BIOTECHNOLOGY IN ACTION: UNVEILING NATURE'S POTENTIAL 2

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EFE ACADEMY PUBLISHING

BOOK TITLE: Biotechnology in Action: Unveiling Nature's Potential 2

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ISBN 978-625-392-311-2
E-ISBN 978-625-392-312-9
DOI https://doi.org/10.59617/efepub20242259
First Edition October 2024

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Library Card

Biotechnology in Action: Unveiling Nature's Potential 2
ÖZDAL, Murat – GÜRKÖK, Sümeyra
First Edition , 462 s., 160 x 235 mm
Type : Original Scientific Book

Dizgi / Design **Kapak Tasarım /** Cover Design	İsa Burak GÜNGÖR (burakgungor@efeakademi.com) Gamze DUMLU (gamzedumlu.2710@gmail.com)
Sertifika No / Certificate No	49168
Matbaa Sertifika No / Printing Certificate No	49168
Efe Akademik Yayıncılık / Efe Akademik Publishing	Cağaloğlu Yokuşu Cemal Nadir Sokak Büyük Milas Han No: 24/125 Fatih/ İSTANBUL 0212 520 52 00 - www.efeakademi.com
Efe Akademik Yayıncılık Matbaa Adres: / Efe Akademik Publishing Printing Adress:	Cağaloğlu Yokuşu Cemal Nadir Sokak Büyük Milas Han No: 24/125 Fatih/ İSTANBUL 0212 520 52 00 - www.efeakademi.com

PREFACE

Biotechnology is basically the deciphering and use of biological information. It is a multidisciplinary branch of science that has relations in many disciplines such as biology, microbiology, biochemistry, molecular biology, genetics, chemistry, and environmental engineering. Biotechnology is a ancient discipline, but it is evolving rapidly and has numerous applications in our daily life, ranging from pharmaceutical discovery to food production and waste disposal.

Biotechnology is the controlled and deliberate manipulation of biological systems (live cells or cell components) in order to effectively produce or process useful products. Because living organisms possess such a diverse range of biological capabilities, selecting appropriate species allows for the production of a large range of compounds, many of which are beneficial to humans as food, fuel, and medicines. Biotechnology, which is heavily dependent on knowledge and technology, plays an important role in promoting innovation, productivity, and economic growth in traditional sectors, as well as contributing to the improvement of the population's quality of life and environmental protection on various levels.

This new book has been enriched with new chapters to complement our first edited book, "Biotechnology in Action: Unveiling Nature's Potential". Therefore, the motivation to compile a comprehensive book on advances in biotechnology stemmed from the increasing awareness of recent developments in cell biology, microbiology, biochemistry, and bioengineering that have accelerated the growth of biotechnology. This book covers diverse aspects of biotechnology, including microbial biopesticides, biological insect control, microbial fermentation, the role of probiotics in biotechnology, biofuel production, plastic biodegradation, phytoremediation, biosorption, antioxidant, thermophilic, and industrial enzyme applications, drug delivery systems, biomaterials, and the biotechnological applications of chitin, bacteriophages, and bryophytes.

This book will serve as a valuable resource for microbial, animal, and plant biotechnologists, as well as biochemists, molecular biologists, pharmacologists, agricultural and environmental engineers. It is also intended

for university students and researchers in the biological sciences at both undergraduate and postgraduate levels. We are confident that this book will not only contribute to scientific knowledge but also engage the interest of the general public.

This book represents the collective efforts of numerous researchers and scientists, each advancing biotechnology within their respective fields. We extend our sincere gratitude to all contributing authors for their valuable contributions.

ERZURUM, 2024

Editors

Assoc. Prof. Dr. Murat OZDAL

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MICROBIAL BIOPESTICIDES AND USAGE AREAS

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Introduction

Biological Control

Biological Control (BM) is a reliable, economical, and successful method in which agricultural pests are neutralized by natural enemies. For this purpose, living organisms such as microorganisms, predators, parasitoid insects, vertebrates, invertebrates, plant substances, and pheromones are used (Özkan et al., 2020). Microbial control is a part of BM and is the application in which plant pests are suppressed using microorganisms or the metabolites they produce. In these applications, microorganisms that are pathogenic to insects are generally used. These bioagents are generally obtained from insects or diseased soils (Allahverdiyev and Şahin, 2011).

Some researchers have stated that the term biopesticides should only be used for living organisms (Glare et al., 2012). Some researchers have limited biopesticides to organisms called direct microbial pest control agents (bacteria, viruses, fungi, protozoa and nematodes) and/or bioactive compounds (metabolites etc.) produced directly from these agents and used to suppress agricultural pest populations. Selected endophytes that have lethal or repellent properties against pests and are introduced to the plant during seed production or propagation have been accepted as biopesticides, although they are not traditionally accepted (Glare et al., 2012). According to this definition, plant extracts and other naturally occurring materials, beneficial macrobiologicals (predators, parasitoids, etc.) and synthetically produced biochemical-like compounds are excluded from the definition of biopesticides. Therefore, in the context of current plant protection, it is thought that a broader definition of biopesticides is needed that covers all molecules of biological origin. Many researchers have defined biopesticides as certain types of pesticides developed from naturally occurring living organisms such as animals, plants and microorganisms (bacteria, fungi and viruses, etc.) and stated that biopesticides and their by-products are used for pest management (Mazid et al., 2011; Senthil-Nathan, 2015). The most thought-provoking difference arising from regions, countries or institutions is that pesticidal substances produced by plants through genetic material transferred to the plant (Bt gene, etc.) are defined as plant-transferred protectors and these are approved as biopesticides (Ndolo et al., 2019). Microbial pesticides consist of a microorganism (bacteria, fungus, virus, protozoa or algae) as the active ingredient (active substance).

Microbial pesticides (MP) are obtained by using microorganisms that exist in the soil, can live a symbiotic life in plant tissues, can convert some nutrient elements such as nitrogen and phosphorus into a form suitable for the use of plants, and produce some enzymes that stimulate the development of plants, or the metabolites produced by these microorganisms. These products do not change the pH of the soil, do not have a toxic effect and do not cause environmental pollution. As an application method; seeds can be coated with these products or they can be applied directly to the soil or plant leaves (Okumuş and Alçınkaya, 2019).

MPs do not have harmful effects on other organisms (including humans) because they target certain organisms, they can be produced in a shorter time and at a lower cost than chemicals, they can be easily obtained from nature and they are biodegradable. They are also widely used in organic agriculture (Liu, 2021). The commercial production of microorganisms that have been tested and shown success in pots and fields is carried out by feeding with pH and temperature control in heated bioreactors. The produced microorganisms are packaged in plastic materials as semi-solid, powder or liquid. The packaged product is stored or offered for sale at 4-12°C (Okumuş and Alçınkaya, 2019). It has been determined that producers cannot easily give up their habits of using chemical pesticides and that they use these chemicals more than necessary due to the development of resistance by pests. This situation poses a threat to human health and the environment. To prevent all of this, it has become an important issue to take steps to research and implement alternative methods such as BM (Topakcı and Keçeci, 2017).

The share of biopesticides in the global plant protection market increased from approximately 0.4% in 1993 to 5.6% in 2016 (McDougall, 2019). The largest share in this market belongs to North America. The reason for this is thought to be the use of plant-transferred preservatives in the USA through gene transfer. The reason for the low rates of biopesticides use in other regions may be that plant-transferred preservatives are not considered biopesticides (Balcı and Durmuşoğlu, 2020).

Microbial Agents Used in Biological Control and Their Mechanisms of Action

Most of the fungi, bacteria, nematodes, protozoa and viruses used against insects or pathogens in plant protection have been mass-produced as MPs. The most successful of these insecticides produced is *Bacillus*

thuringiensis (Özkan et al., 2020). According to data from 2013, most of the MPs licensed in our country contain *B. thuringiensis* bacteria. This is followed by fungal pesticides and *Bacillus subtilis* (Ministry of Food, Agriculture and Livestock, 2015).

a) Microorganisms in Plant Roots

They are more important than all other organisms as a source of organic matter. These roots provide dead tissue to soil microorganisms. While plant roots are alive, they provide a balance by taking in soluble nutrients in the soil and they also directly affect the nutrients becoming useful.

b) Algae

Most algae are organisms that contain chlorophyll. For this reason, they are commonly found in the part close to the surface of the soil if other conditions are suitable. Some types of algae can also be found in lower horizons because they obtain a large part of their necessary energy from organic substances.

c) Fungi

Fungi play a major role in the transformation of soil structure materials. Like bacteria and actinomycetes, fungi do not contain chlorophyll and therefore need organic matter for their energy and carbon requirements. Most of the fungi found in soil are in the form of molds, and a few yeasts are also found.

Most fungal agents that cause insect diseases are spread through asexual spores. *Gliocladium, Trichoderma, Ampelomyces, Candida* and *Coniothyrium* species are among the effective fungi in plant protection. Microorganism spores pass into vegetative form on the insect cuticle and thus the microorganism can reach the body of the insect by passing through the cuticle as seen in the Figure 1. As fungal infections progress, infected insects are killed by the toxins of the microorganism (Işık and Çınar, 2022). The most widely used genus in fungal control is *Trichoderma*. Many species of this genus can improve the soil environment surrounding the plant rhizosphere (the soil environment surrounding plant roots) by degrading hydrocarbons, chlorophenolic compounds, polysaccharides, and xenobiotic pesticides used in agriculture (Liu et al., 2021).

In addition, fungi produce mixtures of different volatile organic compounds belonging to aldehydes, ketones, benzene derivatives, alcohols, hydrocarbons, heterocycles, cyclohexanes, thioalcohols and thioesters. These compounds have a preventive and repellent effect against plant pathogens and insects (Karslı and Şahin, 2021). Mycorrhizal fungi take the carbon and essential organic substances necessary for their development from the plant, while helping the plant to take in water, nutrients, salt and metabolites.

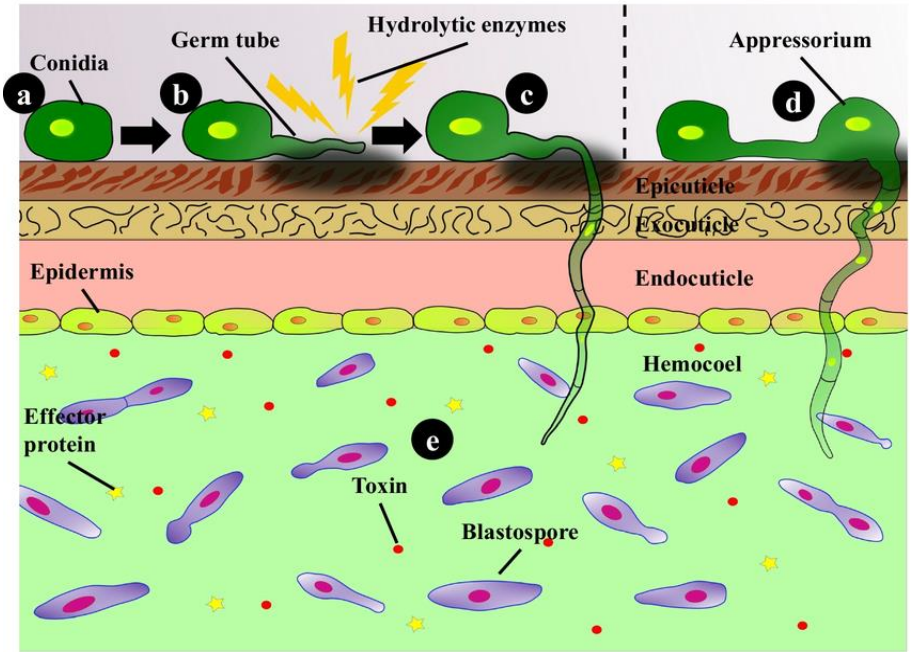

Figure 1. Invasion of the insect's body by fungal spores (Vidhate et al., 2023)

d) Actinomycetes

They are microflora that develop in moist and poorly ventilated soils. They sometimes show more activity than bacteria and fungi in dry times. They are sensitive to acidic soils and their optimum development is seen in soils with a pH between 6.0-7.5. They are the most numerous microorganisms in the soil after bacteria. Actinomycetes play a major role in the dissolution of organic residues and the release of nutrients.

e) *Bacillus thuringiensis*

Bacillus thuringiensis has an insecticidal effect thanks to its Cry and Cyt toxins (Pardo-Lopez et al., 2013). This bacterium is the most commonly used bacterium in MP production because it multiplies rapidly in fermentation tanks, easily forms spores, and is suitable for the production of biopesticides containing endotoxin. Many licensed biopesticides containing *B. thuringiensis* are also used in our country (Kılınçer et al., 2010). The pest to be affected consumes the leaves treated with *B. thuringiensis* (spores and crystal toxin). When cry proteins (crystal toxin) bind to the intestinal wall, the insect's intestinal wall breaks down. As the spores multiply, the insect dies within 1-2 days due to harmful septicemia (Rahul et al., 2020) (Figure 2). In addition, this bacterium can also act by breaking down the chitin layer of insects thanks to the chitinase enzyme. This enzyme is especially beneficial in fungal plant diseases (Mnif et al., 2015).

Bacillus species gain resistance to heat and drying thanks to their spore formation and can therefore be easily formulated as powder (Okumuş and Alçınkaya, 2019). They are highly specific for target insects and kill a limited number of species. *Bacillus* toxins are harmless to humans, vertebrates and plants and are completely biodegradable (Pardo-Lopez et al., 2013). In addition, the gene of this bacterium that produces a crystal protein that is toxic to pests has been transferred to corn plants and it has been reported that it has a preventive effect against corn earworm (Akpınar and Halkman, 2019). Thanks to a broad spectrum of bioactivity, *B. thuringiensis*-based biopesticides have represented approximately 95% of the microorganisms used for pest control (Mnif and Ghribi, 2015).

Because their constituents are widely regarded as harmless and are target-specific, microbial pesticides often have no negative impact on the environment, agricultural producers, or consumers (Guven et al., 2021). Furthermore, as compared to chemical pesticides, their application results in fewer greenhouse gas emissions (Llamas et al., 2021). In addition, a vast range of organisms may be used to create microbial biopesticides, which guarantee sustainability and address the issue of resistance. It may be difficult for vendors, manufacturers, marketers, and final consumers to handle the storage and shipping of various microorganisms employed as biopesticides since they may require varying storage conditions. Therefore, more study is required to guarantee that microbial pesticides have a long and sustainable shelf life.

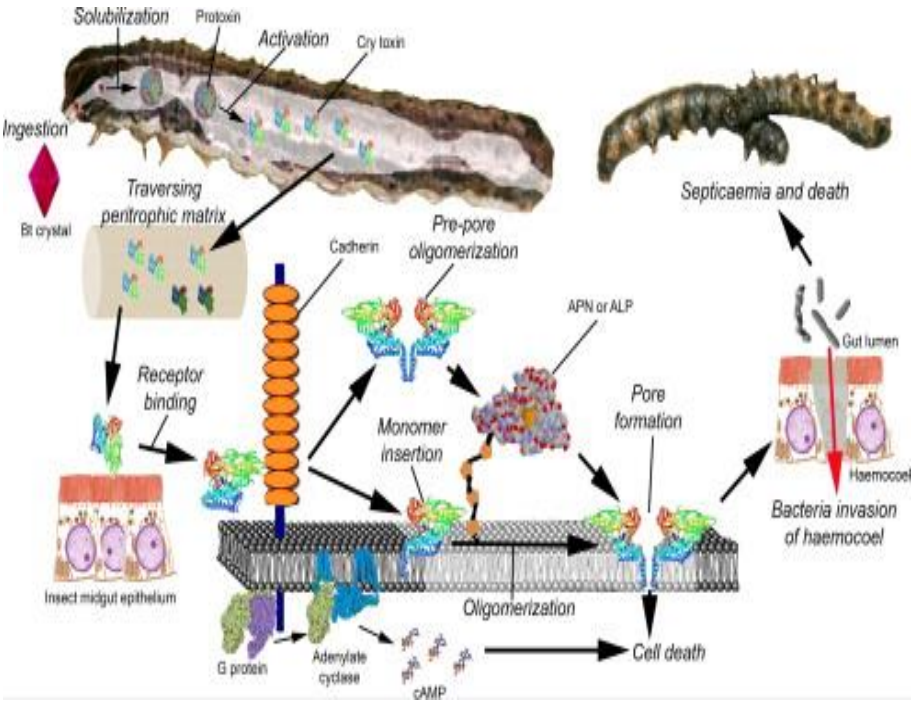

Figure 2. *Bacillus thuringiensis* Crystal Toxins and Mechanism of Action (Adang et al., 2014)

f) *Bacillus subtilis*

Bacillius subtilis is a rhizobacterium and has antibacterial effects against plant pathogens with the bacteriocins it produces (Caulier, et al., 2019). For example, it has antifungal effects against *Aspergillus flavus* and *Colletotrichum gloeosporioides* thanks to surfactin (Mnif et al., 2015). In some studies, it has been stated that *B. subtilis* uses biofilm formation on plant roots to produce these agents (Bais et al., 2004). In a study, the effects of five plant activators used in agriculture (Crop-Set, ISR-2000, KingBo, Sergomil L60, Turf-Set) and a biological plant protection product containing *B. subtilis* (1.34% *B. subtilis* QST 713) against bacterial spot disease in tomatoes were investigated under greenhouse conditions. As a result of the application, it was stated that disease symptoms were reduced by 16-50% in the leaf parts and 25-50% in the stem parts (Aktepe, 2021).

g) *Bacillus sphaericus*

Another bacteria is *Bacillus sphaericus*; it also produces toxins. Its characteristic structure is similar to *B. thuringiensis*, but this bacteria is more effective in dirty water, while *B. thuringiensis* is more effective in clean water. It is ineffective against houseflies or Aedes. *B. sphaericus* is generally more effective for a longer time than *B. thuringiensis*. Research on this bacteria is ongoing, and some products have been introduced. In field trials, it was determined that its pellet and solid formulations provided a protective effect against mosquito larvae for over eight weeks (Rozendaal, 1997).

h) Paenibacillus popilliae

Paenibacillus popilliae is a Gram-positive bacterium. It is rod-shaped, motile and catalase-negative. *P. popilliae* causes a fatal disease called milk disease in *Japanese beetle* larvae. After being ingested by the larvae, *P. popilliae* spores begin to multiply in the intestine and invade the hemolymph (Splittstoesser et al., 1973). A typical milky appearance is observed after approximately 10 days. Within 7-21 days, the larvae die and billions of new spores are released into the soil as the insects decompose (Figure 3). During sporulation *P. popilliae* produces parasporal crystals. During sporulation Cry proteins are synthesized and released as protoxin inclusions. They also noticed that ingestion of even high numbers of vegetative cells of *P. popilliae* by mayfly (*Melolontha melolontha* L.) larvae did not cause milk sickness, but ingestion of spores did. After addition of parasporal crystal protein, loss of viability was observed in primary hemocyte cultures of mayfly (Zhang et al., 1997).



Figure 3. Milky spore disease (*Bacillus popilliae*)

i) Viruses

Viruses that are pathogens of insects are called "baculoviruses". Caterpillars ingest infected leaves. In the midgut, the virus is dissolved and released into the intestinal lumen (Haase et al., 2015). In advanced stages of the disease caused by the virus, the virus spreads to other body tissues, the caterpillar's internal organs liquefy, the cuticle (body covering) changes color, and eventually ruptures. Wet-looking caterpillars that die from viral infection often remain attached to leaves or branches for several days, releasing virus particles. Other larvae feed on these released viruses. In this way, the disease spreads (Liu, 2021). Baculoviruses are fast-moving and pathogenic to some of the most globally important pest species, such as the diamondback moth (*Plutella xylostella*), *Heliothis/Helicoverpa*. These pests can exhibit high resistance to chemical insecticides (Grzywacz, 2017). To increase the insecticidal activity of baculoviruses, recombinant baculoviruses that produce insect-specific toxins, hormones, and enzymes can be developed (Liu, 2021).

j) Phages

Bacteriophages produce new phages by using the bacterial cell as a host. They then break down the bacterial cell and spread to the surroundings (Esmer et al., 2021). It has also been observed that phages can grow on the exopolysaccharide structure produced by the plant pathogen *Erwinia amylovora*. This can help neutralize the pathogen. The ability of bacteria to develop resistance to phages can make this biocontrol method difficult (Svircev et al., 2018). Bacteriophage isolates isolated from soil samples taken from cherry, plum and apricot orchards in different parts of England were found to be *Pseudomonas syringae* pv. In a study examining the effect of 66% of the isolates against bacterial disease caused by syringae, it was stated that 66% of the isolates reduced the disease severity by more than 50% (Akbaba, 2019).

k) Protozoa

Protozoa, which are single-celled eukaryotes, are free-living organisms found mostly in soil and water (Seenivasagan and Babalola, 2021). Protozoan pathogens are generally host-specific and their effects are slow. Spores formed by protozoans are ingested by insects and pass into vegetative form in the midgut. The infection that occurs in this way has a debilitating effect by

slowing down the feeding and fertility of insects (Kachhawa, 2017). A study was conducted to examine whether the effect of beneficial *Pseudomonas* on wheat growth and health was altered by co-inoculation with *Acanthamoeba castellanii.* The effect of this co-inoculation was examined in the presence and absence of the root pathogen *Pythium ultimum*. In the absence of *A. castellanii*, bacterial isolates were found to have little beneficial effect. It has been determined that inoculation with *A. castellanii* promotes plant growth in the absence of pathogens and reduces harmful effects in the presence of pathogens (Weidner et al., 2017).

l) Nematodes

Nematodes are multicellular roundworms, not microbial agents. However, nematodes used in insecticide products are nearly microscopic in size and are used in a manner very similar to the microbial products discussed previously (Liu, 2021). Entomopathogenic nematodes in the genera *Heterorhabditis* and *Steinernema* work in conjunction with insect pathogenic bacteria. These nematodes, which have been widely commercialized for the control of insect pests, are potent microbial control agents (Shapiro-Ilan et al., 2017). They exhibit host-seeking and capturing behavior. They enable the transmission of bacteria to the insect host. In this way, the bacteria penetrate the insect. After the insect dies, the nematodes feed on these insects and spread the bacteria to the surrounding areas (Dillman, et al., 2012).

Molecular Mechanisms of the Application of Biopesticides

Because it is so efficient against a variety of arthropod hosts, *Beauveria bassiana* is one example of an entomopathogenic fungus that is frequently employed as a biopesticide (Boomsma et al., 2014). However, a thorough assessment of its molecular pathogenicity mechanisms beyond the conventional method is required to comprehend its sustainability and efficiency against pests. According to Wojda et al. (2009), the pathogenicity mechanism of *B. bassiana* starts with adherence to the host pest, cuticle penetration, and colonization of the pest's heat emulsion. *B. bassiana*'s hydrophobic contact with insect cuticles is made possible by its hydrophobin-coated aerial conidia (Holder and Keyhani, 2005).

The function that several *B. bassiana*-expressed genes play in maintaining lipid homeostasis may have an impact on the hydrophobicity of

the aerial conidia of the fungus. When mammalian-like perilipin 1 (MPL1) genes are overexpressed, *B. bassiana'*s conidia can transport and store lipids while maintaining lipid homeostasis (Chen et al., 2018). The deletion of the MPL1 gene reduces the turgor pressure of the appressoria, which reduces *B. bassiana'*s adhesiveness, making the gene's function critical (Wang and Leger, 2007). Additionally, *B. bassiana'*s CFEM-domain-containing genes enable surface sensing and signaling for conidia germination and appressoria production (Sabnam and Barman, 2017).

Oosporeins, beauvericin, isarolides, beauverolides, tenellins, and bassianolide are some of these metabolites (Chandler, 2017). The overexpression of the polyketide synthase (PKS) gene mediates the production of oosporein, which takes place in the hemocoel (Lai et al., 2017). It's interesting to notice that when living insect tissues are available, *B. bassiana* tends to release more beauverolides than when dead insect tissues are present (de Bekker et al., 2013). Among the entomopathogenic fungi, *B. bassiana* stands out thanks to these amazing methods of action, which makes it a desirable and popular biopesticide against a variety of arthropod hosts.

Finally, it is important to remember that no single omics method can fully comprehend the complicated pathophysiology displayed by *B. bassiana*; instead, it is necessary to look at the overall expressions of various proteins, secondary metabolites, and their genes at every stage of infection. Therefore, in order to acquire a comprehensive understanding of the mechanisms of action of various biopesticides, researchers from various domains of omics must cooperate and study under the same circumstances.

Conclusion

Considering the damages caused to the environment and living beings by chemical pesticides used for protection against agricultural pests, the research of natural methods that can be an alternative to chemicals has become an important issue. One of these natural methods is microbial struggles with microorganisms that are commonly found in the environment. The strains of the microorganisms to be used that are not toxic to humans and animals are turned into products for this purpose. Their advantages include not being harmful to the environment and not posing a danger to beneficial organisms due to their pest-specific nature. MPs offer effective alternatives for the control of many insect pests. *Bacillus* spp. emerges as an effective bacterial group in this regard. The most promising microorganism is *Bacillus*

thuringiensis because it is resistant to environmental conditions as it forms spores, is easy to mass produce, and has the opportunity to be easily used in transgenic plant production to give resistance to the plant. MPs are likely to become increasingly important tools in pest management. Increasing awareness on this issue and conducting educational activities will be beneficial in protecting the environment and living beings. Since most MPs are only effective against a narrow range of pests, users should properly identify target pests and plan the most effective application. To avoid this disadvantage, several strains or bacteria can be used together. In order for living organisms not to be hindered by their sensitivity to the environment, application can be made at hours appropriate to the optimum working conditions of the microorganism. In addition, the physical conditions of the environment can also be adjusted accordingly. The types of pests and the periods in which they appear can be determined for different plants, and microorganisms that can affect the pests in question and adapt to the weather conditions at that time can be produced and used as microbial pesticides. In order to increase resistance against pests, studies can be carried out to combat pests by transferring some enzymes or different metabolites (chitinase, protease inhibitor, lectins, *Bacillus thuringiensis* gene, etc.) obtained from microorganisms to plants (transgenic plants).

REFERENCES

Adang, M. J., Crickmore, N., Jurat-Fuentes, J. L. (2014). Diversity of *Bacillus thuringiensis* crystal toxins and mechanism of action. In *Advances in Insect Physiology*, 47, 39-87.

Akbaba, M. (2019). Kirazda bakteriyel kansere neden olan etmenlerin moleküler tanısı ve mücadelesine yönelik biyolojik yaklaşımlar. İzmir.

Akpınar, M., Halkman, A. K. (2019). Gıda patojenlerinin biyokontrolünde bakteriyofaj uygulamaları. Gıda, 44(6), 1106-1120.

Aktepe, B.P. (2021). Domateste bakteriyel benek hastalığının biyolojik mücadelesinde farklı bitki aktivatörleri ve biyolojik preparatların etkisi. Mustafa Kemal Üniversitesi Tarım Bilimleri Dergisi, 26(2), 355-364.

Allahverdiyev, A. M., Şahin, F. (2011). Biyoinsektisit özelliği olan mikrobiyal formülasyonların geliştirilmesi, sera ve tarla koşullarında kullanım olanaklarının araştırılması.

Bais, H. P., Fall, R., Vivanco, J. M. (2004). Biocontrol of *Bacillus subtilis* against infection of arabidopsis roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. Plant Physiology, 134(1), 307-319.

Balcı, H., Durmuşoğlu, E. (2020). Bitki koruma ürünü olarak biyopestisitler: tanımları, sınıflandırılmaları, mevzuat ve pazarları üzerine bir değerlendirme. Türkiye Biyolojik Mücadele Dergisi, 11(2), 261-274

Boomsma, J. J., Jensen, A. B., Meyling, N. V., and Eilenberg, J. (2014). Evolutionary interaction networks of insect pathogenic fungi. Annual Review of Entomology 59, 467–485.

Caulier, S., Nannan, C., Gillis, A., Licciardi, F., Bragard, C., & Mahillon, J. (2019). Overview of the antimicrobial compounds produced by members of the *Bacillus subtilis* group. Frontiers in Microbiology, 302.

Chandler, D. (2017). Basic and applied research on entomopathogenic fungi. In Microbial Control of Insect and Mite Pests (pp. 69-89). Amsterdam: Elsevier).

Chen, A., Wang, Y., Shao, Y., Zhou, Q., Chen, S., Wu, Y., et al. (2018). Genes involved in *Beauveria bassiana* infection to galleria mellonella. Archives of Microbiology, 200, 541–552.

de Bekker, C., Smith, P. B., Patterson, A. D., and Hughes, D. P. (2013). Metabolomics reveals the heterogeneous secretome of two entomopathogenic fungi to ex vivo cultured insect tissues. PLoS One 8, e70609.

Dillman, A. R., Chaston, J. M., Adams, B. J., Ciche, T. A., Goodrich-Blair, H., Stock, S. P., Sternberg, P. W. (2012). An entomopathogenic nematode by any other name. PLoS Pathogens, 8(3), e1002527.

Esmer, E., Bayrak, R., Küçükduman, Y., Akocak, P. B. (2021). Gıda teknolojilerinde inovatif bir yaklaşım olarak "Bakteriyofajlar". Avrupa Bilim ve Teknoloji Dergisi, 27, 6- 16.

Federici, B. A. (2009). Pathogens of insects. In *Encyclopedia of insects* (pp. 757-765). Academic Press.

Grzywacz, D. (2017). Basic and applied research: Baculovirus. Microbial Control of İnsect And Mite Pests. Academic Press, 27-46.

Guven, O., Aydin, T., Karaca, I., and Butt, T. (2021). Biopesticides offer an environmentally friendly solution for control of pine processionary moth (*Thaumetopoea wilkinsoni* tams) larvae and pupae in urban areas. Biocontrol Science and Technology, 31, 35–52.

Haase, S., Sciocco-Cap, A., Romanowski, V. (2015). Baculovirus insecticides in Latin America: historical overview, current status and future perspectives. Viruses, 7(5), 2230-2267.

Holder, D. J., and Keyhani, N. O. (2005). Adhesion of the entomopathogenic fungus *Beauveria* (*Cordyceps*) *bassiana* to substrata. Applied and Environmental Microbiology, 71, 5260–5266.

Işık, Ç., Çınar, A. (2022). Bitki Korumada Mikrobiyal Pestisitlerin Kullanım Olanakları ve Etki Mekanizmaları. Avrupa Bilim ve Teknoloji Dergisi, (36), 214-221.

Kachhawa, D. (2017). Microorganisms as a biopesticides. Journal of Entomology and Zoology Studies, 5(3), 468-473.

Karslı, A., Şahin, Y. S. (2021). The role of fungal volatile organic compounds (FVOCs) in biological control. Türkiye Biyolojik Mücadele Dergisi, 12(1), 79-92.

Kılınçer, N., Yiğit, A., Kazak, C., Er, M. K., Kurtuluş, A., Uygun, N. (2010). Teoriden pratiğe zararlılarla biyolojik mücadele. Türkiye Biyolojik Mücadele Dergisi, 1(1), 15-60.

Liu, X., Cao, A., Yan, D., Ouyang, C., Wang, Q., Li, Y. (2021). Overview of mechanisms and uses of biopesticides. International Journal of Pest Management, 67(1), 65-72.

Llamas, B., Suarez-Rodriguez, M. C., Gonzalez-Lopez, C. V., Mora, P., and Acien, F. G. (2021). Techno-economic analysis of microalgae related processes for CO_2 bio-fixation. Algal Research, 57, 102339.

McDougall, P. (2019). Evolution of the crop protection industry since 1960. Pathhead, Scotland.

Mnif, I., Ghribi, D. (2015). Potential of bacterial derived biopesticides in pest management. Crop Protection, 77, 52-64.

Okumuş, A., Alçınkaya, T. (2019). Toprak ve bitki destekleyicileri: biopestisit ve mikrobiyal gübreler. Soil And Plant Promotors: Biopest and Biofertilizers. Samsun.

Özkan, C., Özpınar, A., Karsavuran, R. U. Y., Özaktan, H., Yücel, N. Ö. İ. K. S., Yarpuzlu, F., Demirel14, K. K. E. (2020). Biyolojik Mücadele Uygulamarında Mevcut Durum Ve Gelecek. Türkiye Ziraat Mühendisliği IX. Teknik Kongresi Bildiriler Kitabı-2, 27.

Pardo-Lopez, L., Soberon, M., Bravo, A. (2013). *Bacillus thuringiensis* insecticidal three-domain Cry toxins: mode of action, insect resistance and consequences for crop protection. FEMS Microbiology Reviews, 37(1), 3-22.

Rahul, S., N., S. (2020). Role of microbial insecticides in ınsect pest management. Pop Kheti, 8, 88-92.

Rozendaal, J.A. (1997). Vector control: methods for use by individuals and communities. World Health Organization.

Sabnam, N., and Barman, S. R. (2017). WISH, a novel CFEM GPCR is indispensable for surface sensing, asexual and pathogenic differentiation in rice blast fungus. Fungal Genetics and Biology, 105, 37–51.

Seenivasagan, R., Babalola, O. O. (2021). Utilization of microbial consortia as biofertilizers and biopesticides for the production of feasible agricultural product. Biology, 10(11), 1111.

Shapiro-Ilan, D., Hazir, S., Glazer, I. (2017). Basic and applied research: entomopathogenic nematodes. Microbial Control of İnsect and Mite Pests. Academic Press, 91-105.

Splittstoesser, C. M., Tashiro, H., Lin, S. L., Steinkraus, K. H., Fiori, B. J. (1973). Histopathology of the European chafer, *Amphimallon majalis*, infected with *Bacillus popilliae*. Journal of Invertebrate Pathology, 22(2), 161-167.

Svircev, A., Roach, D., Castle, A. (2018). Framing the future with bacteriophages in agriculture. Viruses, 10(5), 218.

Topakcı, N., Keçeci, M. (2017). Türkiye'de örtüaltında zararlılara karşı biyolojik mücadele uygulamalarının gelişimi: Araştırmadan pratiğe Antalya örneği. Türkiye Biyolojik Mücadele Dergisi, 8(2), 161-174.

Vidhate, R. P., Dawkar, V. V., Punekar, S. A., & Giri, A. P. (2023). Genomic determinants of entomopathogenic fungi and their involvement in pathogenesis. Microbial Ecology, 1-12

Weidner, S., Latz, E., Agaras, B., Valverde, C., Jousset, A. (2017). Protozoa stimulate the plant beneficial activity of rhizospheric pseudomonads. Plant and Soil, 410(1), 509-515.

Wojda, I., Kowalski, P., and Jakubowicz, T. (2009). Humoral immune response of galleria mellonella larvae after infection by *Beauveria bassiana* under optimal and heat-shock conditions. Journal of Insect Physiology, 55, 525–531.

Zhang, J., Hodgman, T. C., Krieger, L., Schnetter, W., Schairer, H. U. (1997). Cloning and analysis of the first cry gene from *Bacillus popilliae*, Journal of Bacteriology, 179(13), 4336-4341.

BIOLOGICAL CONTROL OF APHIDS

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Indroduction

Aphids are one of the most studied insect groups because they cause significant damage to plants. Various species of aphids feed on the leaves, shoots, branches, trunks, fruits and roots of plants, causing serious damage and deformations, and they also pollute plants due to the sweet substances they secrete, and the fumagine formed by the dust and fungi that develop therein, reduces the photosynthesis and respiration capacity of plants. For example; Apples infected with the apple grey aphid, *Dysaphis plantaginea* (Passerini), have been recorded to have a diameter reduction of approximately 22-28%, weight reduction of 30-53% and seed yield reduction of 35-48% compared to uninfected apples. As a result of their nutrition, changes are also determined in the sugar, protein and vitamin contents of apples. At the same time, aphids are vectors for viruses and virus-like organisms, and their damage in this way is often much more significant than their other damage. (Kennedy et al. 1962; Conti, 1985; Lodos, 1986; Blackman and Eastop, 1994; Ölmez and Ulusoy, 2002; Görür, 2008). Aphids are recorded to carry 66% of the 370 viruses that cause disease in plants (Matheus, 1993).

Due to their reproductive characteristics, aphids can produce large numbers of individuals in a short time and form large colonies. In general, a viviparous aphid can produce 100 individuals within 30 days. In their life cycle, both parthenogenetic and sexual reproduction occur. This situation is called cyclic parthenogenesis. In this way, it can multiply very quickly even in changing environmental conditions (Şahin, 2007).

Aphids, which feed on plant sap and can easily adapt to the environmental conditions in which they live, are represented by approximately 4500 species in the world and 480 species in Turkey, and have an important place among insects. They release toxic substances while sucking plant sap, and these toxic substances cause quality and yield losses such as gall formation, leaf curling, drying, and color change. The ecological changes occurring globally are causing them to spread farther and cause more damage (Görür, 2021).

Aphids are a group of economic importance because they directly damage plants and are carriers of many plant diseases. Today, aphids are rapidly expanding both the damage they cause and their distribution areas around the world (Çıraklı et al. 2018). Their rapid reproduction and dense population formation necessitate constant control of aphids. For this purpose,

the use of chemicals not only increases costs but also causes significant damage to the environment (Bulut and Kedici, 1992).

It has been reported that 60% of all herbicides, 90% of all fungal disease drugs and 30% of all insecticides currently used contain carcinogenic substances. In addition to cancer, pesticides cause congenital disabilities, nervous system disorders and genetic changes (Aksoy, 2005).

Studies have shown that beneficial organisms such as parasites and predators are more affected by pesticides than harmful insects (Akkuzu et al. 2022).

Due to the threat to human and animal health, drug residues in foodstuffs and high drug prices, it has become necessary to choose environmentally friendly and cheaper alternative control methods to chemical control. (Uygun, 2022).

The most promising, most environmentally friendly, cheapest and most sustainable of these methods is "biological control." Biological Control can be defined as “the use of beneficial organisms found in nature in combating harmful organisms that cause economic losses in plant production”. The highest success in biological control is seen in predators, parasitoids and entomopathogens (Uygun et al. 2010; Birişik, 2018.).

One of the fundamental approaches in biological control is the mass rearing and release of natural enemies under laboratory conditions. (Külbaş and Uğur, 2015).

Organisms used in Biological Control of Aphids

Predators

The most common predators of aphids are ladybugs (Coleoptera: Coccinellidae). Species belonging to the orders Hemiptera, Diptera, Neuroptera and Dermaptera also feed on aphids (Sunderland, 1988; Solomon et al. 2000). When aphid density is already high, these predatory species are prevalent (Solomon et al. 2000; Dixon, 1998).

Predators are highly variable in their success in suppressing aphid populations. It has been observed that some species can reduce pest

populations by an average of 73.9% (Bouvet et al. 2021). Predators are generally polyphagous. There are very few that specialize in a particular pest. Both their pre-adult and adult stages are generally hunters. These are called specialized aphid hunters. Specialist aphid predators depend on aphids for reproduction. These predators attack when aphid populations reach sufficient levels to lay their eggs. In such cases, they can multiply very quickly and destroy the aphid epidemic. Although not very common, adults of some predators feed not on their prey but on substances such as honeydew, nectar, pollen, water, etc. These predators are called generalist hunters. While generalist hunters can prevent an outbreak, specialist hunters can suppress an ongoing outbreak. Therefore, the effectiveness of generalist predators is lower than that of specialized predators (Uygun, 2022; Toft, 2005).

Aphids show defensive and escape behaviors when attacked by predators (Dill et al. 1990). When it detects a disturbance, it may kick or adjust its body posture defensively without taking its mouthparts (stylets) out of the plant. Consequently, hunting attempts are prevented (Ibbotson and Kennedy, 1951; Dixon, 1958; Brown, 1974; Hartbauer, 2010). If an aphid judges the level of danger to be high, it will stop feeding, retract its needles and prepare for the impending attack by throwing themselves off the plant (Dixon, 1958; Klingauf, 1967; Roitberg and Myers, 1978; Clegg and Barlow, 1982; Dill et al. 1990). When it does so, it risks being preyed upon by ground predators or not being able to find a suitable host plant and eventually dies (Dill et al. 1990; Losey and Denno, 1998; Nelson et al. 2004; Nelson, 2007).

Parasitoids

Braconids (Aphidiinae) and chalcidoids (Aphelinidae) of the order Hymenoptera are the primary parasitoids of aphids.

Parasitoids lay their eggs directly on the aphid, and the aphid develops through the egg and larval stages. The young larva hatches from the egg and begins eating the aphid tissues in about 48 hours. Aphids die as a result of the larvae consuming all of the host's tissues in the later stages of development, including their muscles and digestive tracts. The larva attaches the aphid to the leaf and makes a small hole in the aphid. The larvae pupate while still inside the dead aphid. At this time, the aphid disappears to just its skin, forming a "mummy.’’As the parasitoid kills its host, it begins to cover the aphid with silk, and the aphid's skin dries and becomes a mummy in which the parasitoid pupates. After the development period, The little wasp will pierce

the mummy to break free from the aphid's skin and emerge as an adult who can live on its own. It can take nine to twenty-three days for an egg to mature into an adult. The temperature affects the rate of development; Development happens more quickly at high temperatures and more slowly at low ones. Adults have a seven-to 15-day lifespan. Rain, humidity, wind, and other elements can also have an impact on the growth and lifespan of parasitoids. Although they are wandering, adults often require nectar, honeydew and pollen to thrive. However, some species continue to feed on their hosts during their adult stages. Parasitoids are generally smaller than their hosts and parasitize their hosts for a certain period of time.

Parasitoids usually attack only one host species or several related species. This situation has made parasitoids the most suitable agent to be used in biological control (Godfray, 1994; Bernal and González, 1995; Müller and Godfray, 1999; Sampaio et al. 2001; Capinera, 2004; Soglia et al. 2006; Sidney et al. 2010; Uygun et al. 2010).

Entomopathogens

Entomopathogens used in biological control against harmful insects are bacteria, fungi, viruses, and nematodes. Entomopathogens found naturally in nature attack insects, make them sick and sometimes kill them. Many entomopathogens have been commercially produced and marketed as “biological insecticides” (Uygun et al. 2010).

Entomopathogenic bacteria

Recently, entomopathogenic bacteria have attracted significant attention as biological control agents. More than 100 bacterial species with entomopathogenic effects have been identified as exo- and endopathogens of insects (Hafsa and Benfekih, 2024). *Brevibacillus*, *Bacillus*, *Pseudomonas*, *Photorhabdus*, *Peanibacillus*, *Serratia* and *Xenorhabdus* are the most prevalent entomopathogenic bacteria in the environment. They can successfully fight against various harmful insects (Oulebsir-Mohand kaci et al. 2015; (Ciche et al. 2006; Koppenhofer and Gaugler, 2009; Ruiu et al. 2022).

These bacteria are facultative or obligate insect pathogens and therefore have multiple host spectrums and routes of infection (Boemare and Tailliez, 2009).

The most widely used environmentally friendly microbial pathogen in pest control is *Bacillus thuringiensis* (*Bt*). *Bt* products cause damage to the midgut of insects in their larval stages or lead to septicaemia (Raymond et al. 2010). The crystal proteins they form during sporulation are commercially formulated and used for biological control purposes (Tamez et al. 2004). Insecticidal crystal proteins exhibit toxicity against a wide range of pests and disease vectors (Bravo et al. 2007). *Bt* strains are the most studied entomopathogens because they are environmentally safe and species specific (Han et al. 2006).

Researchers are interested in the bacterial symbionts of EPNs because of their use in biological control. This symbiotic situation has a high degree of specificity (Boemare et al. 1997; Ciche et al. 2006).

The gram-negative bacterium *Xenorhabdus* spp. is symbiotically connected to Steinernematidae family entomopathogenic nematodes (Boemare et al. 1993; Forst et al. 1997). Likewise, entomopathogenic bacteria such as *Photorhabdus* sp. and *Xenorhabdus* sp. live in symbiotic relationships with entomopathogenic nematodes such as *Heterorhabditis* and *Steinernema* (Boemare et al. 1993; Sajnaga and Kazimierczak, 2020). Their association has a significant toxic effect against many insect species (Akhurst, 1983; Herbert and Goodrich-Blair, 2007; Herbert et al. 2009). When nematodes infect their insect hosts, they release symbiotic bacteria from their gut into the insect's hemocel, where in 48 hours, the bacteria proliferate and kill the insect. After this, the bacteria convert the insect into a food source the nematode can eat (Akhurst and Bedding, 1986). It has been demonstrated that Xenorhabdus bacteria may grow successfully in a lab. It has been claimed that this bacterium kills and negatively affects a variety of insect pests (Fuchs et al. 2014).

Entomopathogenic fungi

Entomopathogenic fungi have a wider host range than other biological control agents (Azizoğlu et al. 2012).

It is estimated that there are more than 1000 entomopathogenic fungi (EPF) in the world and there are over 200 fungal entomopathogens with significant potential against insect pests (Shang et al. 2015; Gouli et al. 2020; Ravindran et al. 2018).

Entomopathogenic fungi (EPF) play a role in controlling numerous pest populations (Noman et al. 2021).

Given that the EPF spores can penetrate the host's cuticle, they are effective against phloem-feeding pests such as aphids. Only surface inoculation is sufficient for the insects to become infected (Im et al. 2022., Singh and Kaur, 2020).

When an entomopathogenic fungus infects a host insect, it breaks down the body wall of the host insect by cooperating with enzymes such as chitinase and protease (Askary et al. 1999).

Fungi have high effects under field and laboratory conditions. They are specialized for their hosts and are harmless to beneficials and non-target organisms. They constitute the most suitable natural enemy group to be used in biological control (Kütük, 2018).

Some of the entomopathogenic fungi used against aphids are *Beauveria bassiana*, *Lecanicillium lecanii*, *Metarhizium anisopliae* and *Nomuraea rileyi* (Devi et al. 2003; Shi and Feng 2004; Kılıç and Yıldırım, 2008; İnanlı et al. 2012; Ujjan and Shahzad, 2012; Jandricic et al. 2014;).

It has been recorded that fungi are effective in the pre-adult and adult stages of insects (Herlinda, 2010).

Entomopathogenic Nematodes

Considering their success in biological control, 10 nematode families have come to the fore (Mermithidae, Allantonematidae, Aphelenchoididae, Neotylenchidae, Sphaerularidae, Tetratonematidae, Phaenopsitylenchidae, Rhabditidae, Steinernematidae, Heterorhabditidae). Today, Heterorhabditidae and Steinernematidae have become the most studied families due to their success in biological control (Grewal et al. 2005; Nguyen and Hunt, 2010).

They do not pose a threat to the environment, non-target species, or people (Koppenhofer et al. 2020). Thanks to the symbiotic relationship they establish with bacteria, toxins are secreted, which usually leads to the death of the insect within 48 hours (Tarasco et al. 2023).

The cost-effectiveness of their manufacture has significantly decreased the usage of chemical pesticides in many North American and European

countries (Askary and Abd-Elgawad, 2021; Dillman et al. 2012; Lacey et al. 2015).

The families Heterorhabditidae and Steinernematidae are obligate parasites of insects. Larvae in their third stage, called "infectious larvae", initiate infection and can survive in the external environment (Addis et al. 2016). Bacteria called Photorhabdus and Xenorhabdus are present in the guts of infectious larvae (Fuenmayor et al. 2021; Karthik et al. 2021; Pratissoli et al. 2021).

Entomopathogenic Viruses

Entomopathogenic viruses are important agents of biological control because of their environmental friendliness (Clem and Passarelli, 2013). There are more than 1100 species in 16 families of entomopathogenic viruses (Adams, 1991). Viruses can be produced and applied as bioinsecticide after being obtained from infected insects. *Baculoviruses* (Bv), *nuclear polyhedrosis viruses* (NPv), and *Granulosis viruses* (Gv) are typically employed to get rid of insect pests (Suman and Dikshit, 2010; Arthurs and Lacey, 2004; Arthurs et al. 2005). *Baculoviruses* in particular are very important due to their entomopathogenicity (Hails, 2001; Karabörklü et al. 2018).

A protein coat known as the capsid surrounds the nucleic acid of viruses and is essential to the process of infecting host cells. Once a virus gets into a cell, its nucleic acid takes over the metabolism of the host and multiplies until the cell dies (van Regenmortel et al. 2000). The first symptoms in insects are the cessation of feeding and growth and a slowdown in their activities. The infection is usually transmitted through ingestion. The infection progresses first to the hemocoel and then to the vital organs, resulting in death of the host within 5-14 days (Granados and Williams, 1986).

REFERENCES

Adams, J.R. (1991). Introduction and classifcation of viruses of invertebrates. In: Adams JR, Bonami JR (eds) Atlas of invertebrate viruses. CRC Press Inc., Boca Raton, pp 1–8.

Addis, T., Teshome, A., Strauch, O., & Ehlers, R. U. (2016). Life history trait analysis of the entomopathogenic nematode Steinernema feltiae provides the basis for prediction of dauer juvenile yields in monoxenic liquid culture. *Applied Microbiology and Biotechnology, 100,* 4357-4366.

Akhurst, R. J. (1983). Neoaplectana species: specificity of association with bacteria of the genus *Xenorhabdus*. *Experimental Parasitology, 55*(2), 258-263.

Akhurst, R. J., & Bedding, R. A. (1986). Natural occurrence of insect pathogenic nematodes (Steinernematidae and Heterorhabditidae) in soil in Australia. *Australian Journal of Entomology, 25*(3), 241-244.

Akkuzu, E., Ayberk, H., Mol, T. (2022). Pestisit kullanımı ve faydalı arthropodlar üzerindeki etkileri. *İstanbul Üniversitesi Orman Fakültesi Dergisi, 51*(2), 85-90.

Aksoy, U. (2005). Bahçe bitkileri tarımında çevre dostu üretim teknikleri. Ege Üniversitesi, Ziraat Fakültesi, 140s, İzmir.

Arthurs, S. P., & Lacey, L. A. (2004). Field evaluation of commercial formulations of the codling moth granulovirus: persistence of activity and success of seasonal applications against natural infestations of codling moth in Pacific Northwest apple orchards. *Biological Control, 31*(3), 388-397.

Arthurs, S. P., Lacey, L. A., & Fritts Jr, R. (2005). Optimizing use of codling moth granulovirus: effects of application rate and spraying frequency on control of codling moth larvae in Pacific Northwest apple orchards. *Journal of Economic Entomology, 98*(5), 1459-1468.

Askary, H., Benhamou, N., & Brodeur, J. (1999). Ultrastructural and cytochemical characterization of aphid invasion by the hyphomycete *Verticillium lecanii*. *Journal of Invertebrate Pathology, 74*(1), 1-13.

Askary, T. H., & Abd-Elgawad, M. M. (2021). Opportunities and challenges of entomopathogenic nematodes as biocontrol agents in their tripartite interactions. *Egyptian Journal of Biological Pest Control, 31*, 1-10.

Azizoğlu, U., Bulut, S., Yılmaz, S. (2012). Organik tarımda biyolojik mücadele; entomopatojen biyoinsektisitler. *Erciyes Üniversitesi Fen Bilimleri Enstitüsü Dergisi*, 28(5), 367-373.

Bernal, J., González, D. (1995). Thermal Requirements of Diaeretiella rapae (M'Intosh) on Russian wheat aphid (Diuraphis noxia Mordwilko, Hom., Aphididae) hosts. *Journal of Applied Entomology, 119*, 273-277.

Birişik, N. (2018). Teoriden Pratiğe Biyolojik Mücadele ve Gelecek Stratejisi. *Teoriden Pratiğe Biyolojik Mücadele, Gıda Tarım ve Hayvancılık Bakanlığı Yayınları*.

Blackman, R.L., Eastop, V.F. (1994). Aphids on the World's Trees. Cab International. 987s, UK.

Boemare, N. E., Akhurst, R. J., & Mourant, R. G. (1993). DNA relatedness between Xenorhabdus spp. (Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes, and a proposal to transfer *Xenorhabdus luminescens* to a new genus, Photorhabdus gen. nov. *International Journal of Systematic Bacteriology*, *43*(2), 249-255.

Boemare, N., Givaudan, A., Brehelin, M., Laumond, C. (1997). Symbiosis and pathogenicity of nematode-bacterium complexes. *Symbiosis*, *22*(1–2).

Boemare, N., Tailliez, P. (2009). Molecular approaches and techniques for the study of entomopathogenic bacteria. In: Stock P, Vandenberg J, Glazer I, Boemare N (eds) Insect pathogens: molecular approaches and technique. CABI Publishing, pp 32–49.

Bouvet, J. P. R., Urbaneja, A., Monzó, C. (2021). Aphid Predators in Citrus Crops: the Least Voracious Predators are the Most Efective. *Journal of Pest Science, 94*, 321–333.

Bravo, A., Gill, S.S., Soberon, M. (2007). Mode of Action of Bacillus thuringiensis Cry and Cyt Toxins and Their Potential for Insect Control. *Toxicon, 49*, 423-435.

Brown, H. D. (1974). Defensive behaviour of the wheat aphid, Schizaphis graminum (Rondani) (Hemiptera: Aphididae), against Coccinellidae. *Journal of Entomology Series A, General Entomology, 48*(2), 157-165.

Bulut, H., & Kedici, R. (1992). Elma yeşil yaprakbiti (Aphis pomi De Geer) (Homoptera: Aphididae)'ne tavsiye edilen bazı ilaçların etkili en düşük dozlarının saptanması ve bunun ekonomik açıdan değerlendirilmesi. Türkiye II. Entomoloji Kongresi (28-31 Ocak 1992, Adana), 297-305.

Capinera, J.L. (2004). Encyclopedia of Entomology. Vols. 3. Kluwer Academic Press, Dordrecht, The Netherlands. 2580 pp.

Ciche, T. A., Darby, C., Ehlers, R. U., Forst, S., & Goodrich-Blair, H. (2006). Dangerous liaisons: the symbiosis of entomopathogenic nematodes and bacteria. *Biological Control*, *38*(1), 22-46.

Clegg, J. M., & Barlow, C. A. (1982). Escape behaviour of the pea aphid Acyrthosiphon pisum (Harris) in response to alarm pheromone and vibration. *Canadian Journal of Zoology*, *60*(10), 2245-2252.

Clem, R. J., & Passarelli, A. L. (2013). Baculoviruses: sophisticated pathogens of insects. *PLoS Pathogens*, *9*(11), e1003729.

Conti, M. (1985). Transmission of Plant Viruses by Leafhoppers and Planthoppers. *A Wiley Interscience Publication*, 289-307 s, New York.

Çıraklı, A., Görür, G., Işık, M. (2018). Denizli il merkezinde belirlenen afit (Hemiptera: Aphididae) Türleri. *Selçuk Üniversitesi Ziraat Fakültesi Dergisi*, 22 (44), 12-18.

Devi, P. S., Prasad, Y. G., Anitha Chowdary, D., Mallikarjuna Rao, L., & Balakrishnan, K. (2003). Identification of virulent isolates of the entomopathogenic fungus *Nomuraea rileyi* (F) Samson for the management of Helicoverpa armigera and Spodoptera litura. *Mycopathologia, 156*, 365-373.

Dill, L. M., Fraser, A. H., & Roitberg, B. D. (1990). The economics of escape behaviour in the pea aphid, *Acyrthosiphon pisum*. *Oecologia, 83*, 473-478.

Dillman, A. R., Chaston, J. M., Adams, B. J., Ciche, T. A., Goodrich-Blair, H., Stock, S. P., & Sternberg, P. W. (2012). An entomopathogenic nematode by any other name. *PLoS Pathogens*, *8*(3), e1002527.

Dixon, A.F.G. (1998). Aphid Ecology. An Optimization Approach. Chapman & Hall, London, UK, 300 pp.

Dixon, A. F. G. (1958). The escape responses shown by certain aphids to the presence of the coccinellid *Adalia decempunctata* (L.). *Transactions of the Royal Entomological Society of London*, *110*(11), 319-334.

Forst, S., Dowds, B., Boemare, N., & Stackebrandt, E. (1997). Xenorhabdus and Photorhabdus spp.: bugs that kill bugs. *Annual Review of Microbiology*, *51*(1), 47-72.

Fuchs, S. W., Grundmann, F., Kurz, M., Kaiser, M., & Bode, H. B. (2014). Fabclavines: Bioactive Peptide–Polyketide-Polyamino Hybrids from Xenorhabdus. *ChemBioChem*, *15*(4), 512-516.

Fuenmayor, Y., Portillo, E., Bastidas, B., Guerra, M., & San-Blas, E. (2020). Infection parameters of (Nematoda: Heterorhabditidae) in different stages of *Hibiscus pink* mealybug. *Journal of Nematology*, *52*(1), 1-7.

Godfray, H. C. J. (1994). Parasitoids: behavioral and evolutionary ecology (Vol. 12). Princeton University Press.

Gouli, V., Marcelino, J.A.P., Gouli, S.Y. (2020). The basic biological resources for the production of microbial pesticides, in: *Microbial Pesticides*, Academic Press, New York, pp. 1–134.

Granados, R.R., Williams, K.A. (1986). In vivo infection and replication of baculoviruses. In: Granados RR, Federici BA (eds) *The biology of baculoviruses*, vol I. CRC Press, Boca Raton, pp 89–108.

Görür, G. (2021). Türkiye Afit Faunasının Son Durumu ve Afitlerin Ekonomik Önemi. *Türk Bilimsel Derlemeler Dergisi*, 1(1), 17-22.

Grewal, P. S., Ehlers, R. U., & Shapiro-Ilan, D. I. (Eds.). (2005). Nematodes as biocontrol agents. Cabi Publishing.

Hafsa, M., & Benfekih, L. A. (2024). New insights on entomopathogenic bacteria isolated from soil of citrus crops to combat the polyphagus aphid pest *Hyalopterus pruni* (Geoffroy 1762) (Hemiptera, Aphididae). *Egyptian Journal of Biological Pest Control*, *34*(1), 51.

Hails, R. (2001). Natural and genetically modified baculoviruses: environmentally friendly pest control or an ecological threat?. *Outlook on Agriculture*, *30*(3), 171-178.

Han, C. S., Xie, G., Challacombe, J. F., Altherr, M. R., Bhotika, S. S., Bruce, D., ... & Gilna, P. (2006). Pathogenomic sequence analysis of *Bacillus cereus* and *Bacillus thuringiensis* isolates closely related to *Bacillus anthracis. Journal of bacteriology*, *188*(9), 3382-3390.

Hartbauer, M. (2010). Collective defense of *Aphis nerii* and *Uroleucon hypochoeridis* (Homoptera, Aphididae) against natural enemies. *PLoS One*, *5*(4), e10417.

Herbert Tran, E. E., Andersen, A. W., & Goodrich-Blair, H. (2009). CpxRA influences *Xenorhabdus nematophila* colonization initiation and outgrowth in *Steinernema carpocapsae* nematodes through regulation of the nil locus. *Applied and Environmental Microbiology*, *75*(12), 4007-4014.

Herbert, E. E., & Goodrich-Blair, H. (2007). Friend and foe: the two faces of *Xenorhabdus nematophila*. *Nature Reviews Microbiology*, *5*(8), 634-646.

Herlinda, S. (2010). Spore density and viability of entomopathogenic fungal isolates from Indonesia, and their virulence against *Aphis gossypii* Glover (Homoptera: Aphididae). *Tropical Life Sciences Research*, *21*(1), 11.

Ibbotson, A., & Kennedy, J. S. (1951). Aggregation in *Aphis fabae* Scop. I. aggregation on plants. *Annals of Applied Biology*, *38*(1), 65-78.

Im, Y., Park, S. E., Lee, S. Y., Kim, J. C., & Kim, J. S. (2022). Early-stage defense mechanism of the cotton aphid *Aphis gossypii* against infection with the insect-

killing fungus *Beauveria bassiana* JEF-544. *Frontiers in Immunology, 13*, 907088.

İnanlı, C., Yoldaş, Z., Birgücü, A.K. (2012). Effects of entomopathogenic fungi, Beauveria bassiana (Bals.) and Metarhizium anisopliae (Metsch.) on larvae and egg stages of Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae). *Ege Üniversitesi Ziraat Fakültesi Dergisi, 49*(3), 239–242.

Jandricic, S. E., Filotas, M., Sanderson, J. P., & Wraight, S. P. (2014). Pathogenicity of conidia-based preparations of entomopathogenic fungi against the greenhouse pest aphids *Myzus persicae*, *Aphis gossypii*, and *Aulacorthum solani* (Hemiptera: Aphididae). *Journal of Invertebrate Pathology, 118*, 34-46.

Karabörklü, S., Azizoglu, U., & Azizoglu, Z. B. (2018). Recombinant entomopathogenic agents: a review of biotechnological approaches to pest insect control. *World Journal of Microbiology and Biotechnology*, *34*, 1-12.

Raja, R. K., Arun, A., Touray, M., Gulsen, S. H., Cimen, H., Gulcu, B., ... & Hazir, S. (2021). Antagonists and defense mechanisms of entomopathogenic nematodes and their mutualistic bacteria. *Biological Control, 152*, 104452.

Kennedy, J. S., Day, M. F., & Eastop, V. F. (1962). A conspectus of aphids as vectors of plant viruses. Commonwealth Institute of Entomology, London, 114 pp, London.

Kılıç, E., & Yıldırım, E. (2008). The use of entomopathogen fungi in control of whiteflies (Homoptera: Aleyrodidae). *Journal of the Faculty of Agriculture of Atatürk University* (Turkey).

Klingauf, F. (1967). Abwehr-und Meidereaktionen von Blattläusen (Aphididae) bei Bedrohung durch Räuber und Parasiten. *Zeitschrift für Angewandte Entomologie, 60*(1-4), 269-317.

Koppenhöfer, H. S., & Gaugler, R. (2009). Entomopathogenic nematode and bacteria mutualism. In *Defensive mutualism in microbial symbiosis* (pp. 117-134). CRC Press.

Koppenhöfer, A. M., Shapiro-Ilan, D. I., & Hiltpold, I. (2020). Entomopathogenic nematodes in sustainable food production. *Frontiers in Sustainable Food Systems*, *4*, 125.

Külbaş, M. D., & Uğur, A. Bazı doğal düşmanların kitle üretiminde kalite kontrolüne yönelik biyolojik parametreler. *Türkiye Entomoloji Bülteni, 5*(1), 35-45.

Kütük, H. (2018). Örtüaltı Sebze Yetiştiriciliğinde Biyolojik Mücadele. Teoriden Pratiğe Biyolojik Mücadele, *Gıda Tarım ve Hayvancılık Bakanlığı Yayınları*.

Lacey, L. A., Grzywacz, D., Shapiro-Ilan, D. I., Frutos, R., Brownbridge, M., & Goettel, M. S. (2015). Insect pathogens as biological control agents: Back to

the future. *Journal of Invertebrate Pathology, 132*, 1-41.

Lodos, N. (1986). Türkiye Entomolojisi II. *Ege Üniversitesi Ziraat Fakültesi Yayınları*, 580s, No 429, İzmir.

Losey, J. E., & Denno, R. F. (1998). Positive predator–predator interactions: enhanced predation rates and synergistic suppression of aphid populations. *Ecology, 79*(6), 2143-2152.

Matheus, R.E.F. (1993). Diagnosis of plant virus diseases. CRS Press Inc, Boca Raton, 374 pp, Florida.

Müller, C. B., & Godfray, H. C. J. (1999). Indirect interactions in aphid–parasitoid communities. *Population Ecology*, *41*(1), 93-106.

Nelson, E. H. (2007). Predator avoidance behavior in the pea aphid: costs, frequency, and population consequences. *Oecologia*, *151*(1), 22-32.

Nelson, E. H., Matthews, C. E., & Rosenheim, J. A. (2004). Predators reduce prey population growth by inducing changes in prey behavior. *Ecology*, *85*(7), 1853-1858.

Nguyen, K., & Hunt, D. (2007). Entomopathogenic nematodes: systematics, phylogeny and bacterial symbionts. *Nematology Monographs and Perspectives* (Vol. 5). Brill.

Noman, A., Aqeel, M., Islam, W., Khalid, N., Akhtar, N., Qasim, M., ... & Al-Sadi, A. (2021). Insects–plants-pathogens: Toxicity, dependence and defense dynamics. *Toxicon*, *197*, 87-98.

Oulebsir-Mohandkaci, H., Doumandji-Mitiche, B., & Behidj, N. (2015). Evaluation de la toxicité de *Pseudomonas fluorescens* et *Bacillus sphaericus* à l'égard du criquet migrateur *Locusta migratoria* (Linné, 1758). *Nature & Technology*, (12), 98.

Ölmez Bayhan, S., & Ulusoy, M. R. (2002). Diyarbakır ilinde Aphidoidea üst familyasına bağlı türlerin predatörlerinin belirlenmesi, 237-246 s. Türkiye V. Biyolojik Mücadele Kongresi Bildirileri (4-7 Eylül 2002, Erzurum).

Pratissoli, D., Damascena, A. P., Fragoso, D. M. F., de Carvalho, J. R., Túler, A. C., Faria, L. V., & de Araujo Junior, L. M. (2021). Performance of entomopathogenic nematodes on neoleucinodes elegantalis (Guenée) (Lepidoptera: Crambidae). *Revista Acta Ambiental Catarinense*, *18*(1), 205-215.

Ravindran, K., Sivaramakrishnan, S., Hussain, M., Dash, C. K., Bamisile, B. S., Qasim, M., & Liande, W. (2018). Investigation and molecular docking studies of Bassianolide from *Lecanicillium lecanii* against *Plutella xylostella* (Lepidoptera: Plutellidae). *Comparative Biochemistry and Physiology Part*

C: Toxicology & Pharmacology, 206, 65-72.

Raymond, B., Johnston, P. R., Nielsen-LeRoux, C., Lereclus, D., & Crickmore, N. (2010). *Bacillus thuringiensis*: an impotent pathogen?. *Trends in Microbiology*, *18*(5), 189-194.

Roitberg, B. D., & Myers, J. H. (1978). Adaptation of alarm pheromone responses of the pea aphid *Acyrthosiphon pisum* (Harris). *Canadian Journal of Zoology*, *56*(1), 103-108.

Ruiu, L., Marche, M. G., Mura, M. E., & Tarasco, E. (2022). Involvement of a novel *Pseudomonas protegens* strain associated with entomopathogenic nematode infective juveniles in insect pathogenesis. *Pest Management Science*, *78*(12), 5437-5443.

Sajnaga, E., & Kazimierczak, W. (2020). Evolution and taxonomy of nematode-associated entomopathogenic bacteria of the genera *Xenorhabdus* and *Photorhabdus*: an overview. *Symbiosis*, *80*(1), 1-13.

Sampaio, M. V., Bueno, V. H., & Pérez-Maluf, R. (2001). Parasitismo de *Aphidius colemani* viereck (Hymenoptera: aphidiidae) em diferentes densidades de *Myzus persicae* (sulzer) (Hemiptera: aphididae). *Neotropical Entomology*, *30*, 81-87.

Shang, Y., Feng, P., & Wang, C. (2015). Fungi that infect insects: altering host behavior and beyond. *PLoS Pathogens*, *11*(8), e1005037.

Shi, W. B., & Feng, M. G. (2004). Lethal effect of *Beauveria bassiana*, *Metarhizium anisopliae*, and *Paecilomyces fumosoroseus* on the eggs of *Tetranychus cinnabarinus* (Acari: Tetranychidae) with a description of a mite egg bioassay system. *Biological Control*, *30*(2), 165-173.

Sidney, L. A., Bueno, V. H., Lins Jr, J. C., Silva, D. B., & Sampaio, M. V. (2010). Quality of different aphids species as hosts for the parasitoid *Aphidius ervi* Haliday (Hymenoptera: Braconidae: Aphidiinae). *Neotropical Entomology*, *39*, 709-713.

Singh, H., & Kaur, T. (2020). Pathogenicity of entomopathogenic fungi against the aphid and the whitefly species on crops grown under greenhouse conditions in India. *Egyptian Journal of Biological Pest Control*, *30*(1), 84.

Soglia, M., Bueno, V. H., Sampaio, M. V., Rodrigues, S. M., & Ledo, C. A. (2006). Desenvolvimento e parasitismo de *Lysiphlebus testaceipes* (Cresson) e *Aphidius colemani* Viereck (Hymenoptera: Braconidae) em Aphis gossypii Glover (Hemiptera: Aphididae) em duas cultivares de crisântemo. *Neotropical Entomology*, *35*, 364-370.

Solomon, M.G., Cross, J.V., Fitzgerald, J.D., Campbell, C.A.M., Jolly, R.L., Olszak, R.W., Niemczyk, E., Vogt, H. (2000). Biocontrol of pests of apples and pears

in northern and central europe. Biocontrol Science Technology, 10, 91–128.

Suman, G., Dikshit, A.K. (2010). Biopesticides: An ecofriendly approach for pest control. *Journal of Biopesticide 3*(1S):186–188.

Sunderland, K.D. (1988). Predators. In: Minks, A.K., Harrewijn, P. (Eds.), *Aphids, Their Biology, Natural Enemies and Control*, vol. 2B. Elsevier, Amsterdam, pp., 293–304.

Şahin, M. (2007). Kayseri Merkez Afit (Homoptera: Aphididae) faunasının belirlenmesi. Yüksek Lisans Tezi, Fen Bilimleri Enstitüsü, Niğde Üniversitesi.

Tamez-Guerra, P., Iracheta, M. M., Pereyra-Alférez, B., Galán-Wong, L. J., Gomez-Flores, R., Tamez-Guerra, R. S., & Rodrıguez-Padilla, C. (2004). Characterization of Mexican *Bacillus thuringiensis* strains toxic for lepidopteran and coleopteran larvae. *Journal of Invertebrate Pathology, 86*(1-2), 7-18.

Tarasco, E., Fanelli, E., Salvemini, C., El-Khoury, Y., Troccoli, A., Vovlas, A., & De Luca, F. (2023). Entomopathogenic nematodes and their symbiotic bacteria: from genes to field uses. *Frontiers in Insect Science, 3*, 1195254.

Toft, S. (2005). The quality of aphids as food for generalist predators: implications for natural control of aphids. *European Journal of Entomology*, *102*(3), 371.

Ujjan, A. A., & Shahzad, S. (2012). Use of entomopathogenic fungi for the control of mustard aphid (*Lipaphis erysimi*) on canola (*Brassica napus* L.). *Pakistan Journal of Botany*, *44*(6), 2081-2086.

Uygun, N. (2022). Zararlılara Karşı Biyolojik Mücadelede Gelişmeler. Türkiye Biyolojik Mücadele Kongresi, Erzurum.

Uygun, N., Ulusoy, M.R., Satar, S. (2010). Biyolojik Mücadele. *Türkiye Biyolojik Mücadele Dergisi, 1*, 1-14.

van Regenmortel, M. H. (2000). Virus taxonomy: classification and nomenclature of viruses. Seventh report of the International Committee on Taxonomy of Viruses. Family Adenoviridae, 227-237.

THE BENEFICIAL ROLES OF MICROBIAL BIOFILMS ON THE ALLEVIATION OF PLANT ABIOTIC STRESSES

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Abstract

Plants encounter several stress factors in their natural habitats, limiting crop growth and productivity, and seriously threating global food security. Although a great variety of plants have developed various specific or non-specific mechanisms to environmental stresses, there are still restrictions in plants' responses at physiological and molecular level, remaining a challenging problem in agricultural production. Inoculation of plant growth-promoting (PGP) microorganisms favors plant growth even under extreme conditions but they are also adversely affected by stressful environments. Therefore, such plant-associated microbes should adapt to changing environmental conditions to exert their beneficial roles in plant stress alleviation and growth promotion. Soil microorganisms alleviate the impacts of plant abiotic stresses through some strategies, but among them, biofilm formation together with exopolysaccharide (EPS) production seems to be an effective approach in plant growth by increasing plant stress tolerance. In previous studies, the role of EPS is well-established but the effects of biofilm formation are still under exploration. Biofilm development by growth-promoting bacteria offers great advantages to not only plant growth but also their effective survival and successful colonization on plant surfaces and/or in the rhizosphere soils. Considering the benefits of plant-associated biofilms on plants and their microbial inhabitants, this chapter aimed to discuss the roles of biofilm and EPS production in the alleviation of plant abiotic stress factors, including drought, salt, heavy metals, and nutrient limitation.

Introduction

The majority of cultivable and almost half of the irrigated areas in the globe are seriously affected by habitat-imposed abiotic stressors (Choudhary, 2012), therefore plants encounter a set of environmental stresses in the natural environment (Cramer et al., 2011; Gong et al., 2020). Ecological effects of global climate change and human activities deteriorate the impacts of such stress conditions on crop quality and productivity (Pereira, 2016; Thakur et al., 2021). These stress factors significantly reduce plant growth and development by changing metabolic activities, consequently causing a significant reduction below optimum levels in agricultural production (Morcillo and Manzanera, 2021; Thakur et al., 2021). It is estimated that abiotic stresses cause a 50% reduction, on average, in the yield of major crop

plants in the world, resulting in unbearable economic losses (Thakur et al., 2021). It is also predicted that this current situation will be more severe, and may increase the yield loss by up to 70% (Thakur et al., 2021). Plants have evolved several complex genetic and molecular mechanisms to make appropriate adjustments for their normal development (Gong et al., 2020) but there are still physiological and molecular limitations in plant responses to the changing environment (Cramer et al., 2011). In many agricultural systems, plants are barely reaching their yield potential because of the stressors (Thakur et al., 2021). According to Pereira (2016), the effects of abiotic stresses will remain a problem for agriculture in the future. The increasing human population also increases the demands for more agricultural production by about 70% (Zörb et al., 2019). Therefore, plant productivity under stress conditions needs to be improved to guarantee global food production.

Plant growth-promoting (PGP) bacteria seem to be a feasible alternative strategy to improve plant fitness under stress conditions (Timmusk et al., 2014; Banerjee et al., 2019). They can colonize the surfaces of plant roots and leaves, plant internal tissues, and rhizosphere soil (Vocciante et al., 2022; Zhang et al., 2022). Following colonization, such bacteria promote plant growth or mitigate the impacts of plant abiotic stresses through various mechanisms such as the synthesis of plant hormones, nitrogen fixation, phosphate solubilization, exopolysaccharide (EPS) production, biofilm formation, the production of siderophore, hydrolytic enzymes, hydrogen cyanide, and antibiotics (Morcillo and Manzanera, 2021; Çam et al., 2023). However, the presence of planktonic microorganisms on plant surfaces or in the rhizosphere soil can be negatively affected by changing environmental factors (Çam et al., 2022; Çam, 2022a). Under natural conditions, interactions between plants and their associated-microorganisms are essential for the growth of plants in a fluctuating environment (Kumar et al., 2021). A successful plant-microbe interaction has a pivotal role in the maintenance of beneficial microbes, soil characteristic, and crop productivity under stress conditions (Kumar et al., 2021). For plant-microbe interactions, microbial adaptation to changing conditions is crucial (Choudhary, 2012). A low potential of a beneficial bacterium is generally due to the lack of adaptation ability (Legein et al., 2020). That is, plant-associated microbes need to adapt to habitat-imposed environmental factors for their survival and long-term persistence (Çam, 2022a). Therefore, PGP microorganisms must have

adaptation mechanisms to colonize and persist on plants or in the rhizosphere soils to exert their beneficial effects (Çam et al., 2022).

The Importance of Biofilms for Microorganisms

Biofilms are the microbial assemblages or communities encased in a self-formed matrix, which attach to a living/non-living macrosurface (Prakash et al., 2003; Rinaudi and Giordano, 2010). Biofilm mode of life provides a reproductive fitness advantage to biofilm cells (Velmourougane et al., 2017). This lifestyle allows microorganisms to survive under unfavorable environmental conditions (Çam and Badıllı, 2024), therefore has been considered a selective survival strategy for biofilm-forming cells (Prakash et al., 2003; Çam and Brinkmeyer, 2020a). The biofilm mode of growth protects the microorganisms from UV radiation, nutrient limitation, desiccation, oxygen radicals, predation, metallic cations, solvents, antimicrobial agents, extreme pH changes, osmotic stress, toxic pollutants, and other hostile environmental factors (Flemming and Wingender, 2010; Rinaudi and Giordano, 2010; Sharma et al., 2020; Çam and Badıllı, 2024). According to Flemming and Wingender (2010), the most successful lifestyle in the natural world is biofilm formation. It is claimed that the majority of cells (up to 80%) on earth live in biofilms (Flemming and Wuertz, 2019). Protection against such detrimental factors may drive microorganisms to reside within a biofilm under natural conditions.

The formation of the three-dimensional biofilm architecture offers an entirely different lifestyle to the cells within a biofilm (Flemming and Wingender, 2010). Biofilm-producing cells are highly organized-complex communities compared to planktonically living-single cells (Flemming and Wuertz, 2019). Various environmental parameters affect biofilm formation (Çam and Brinkmeyer, 2020a; Çam and Bicek, 2023). The process of biofilm development is controlled by multiple genes (Lu, et al., 2018). The gene regulation in biofilms can be different from the planktonic state, depending on environmental parameters (Çam and Brinkmeyer, 2020b), which causes phenotypic and metabolic changes in the biofilm cells over their planktonic counterparts (Velmourougane et al., 2017). Even different groups of cells in a biofilm may reflect distinct behaviors (Stewart and Franklin, 2008). Such organisms change their growth rate and resistance to antimicrobials (Prakash

et al., 2003), which may bring some advantages to biofilm-forming microorganisms. For example, changes in growth rates may increase exopolysaccharide production and the expression of some novel proteins (Prakash et al., 2003) and also offer some cells a better position to more readily reach nutrients (Davey and O'Toole, 2000).

The dry mass of biofilms consists of microbial cells (less than 10%) and the extracellular matrix (more than 90%) that keeps biofilm-forming cells together (Satpathy et al., 2016). Biofilms may be formed by either an individual species or often multiple microbial species that exhibit coordinated collective behaviors (Davey and O'Toole, 2000). The biofilm matrix generally consists of different kinds of exopolysaccharides, proteins, lipids, and DNA (Flemming and Wingender, 2010). Such components in the biofilm matrix, alone or together, offer great physiological and ecological advantages to the cells within a biofilm (Flemming and Wingender, 2010). According to Flemming and Wingender (2010), the advantages of extracellular polymeric substances (EPS) to biofilm organisms can be summarized as follows: EPS; mediate the mechanical stability of a mature biofilm; facilitate the aggregation and colonization of planktonic microorganisms on abiotic/biotic surfaces as well as the long-term persistence; maintain a well-hydrated environment around the cells, thus increasing their resistance against desiccation under drought conditions; act as a protective barrier against various anti-microbial compounds; increase the nutrient availability by accumulating inorganic and organic compounds from the environments and enabling the digestion of the exogenous molecules for energy source; immobilize the cells by keeping them in close proximity, therefore facilitating horizontal gene transfer, cell-cell communications, and the synergistic interactions between biofilm-forming cells; and contribute to environmental detoxification by sequestering heavy metals and xenobiotics from the surroundings (Flemming and Wingender, 2010). Such benefits of biofilm formation extend the survival and existence of biofilm-forming PGP bacteria on plant surfaces under natural conditions; hence, they can exert their beneficial roles on plant growth and productivity.

The Roles of Biofilms on Stress Tolerance

Microbial biofilms are very common among plant-associated microorganisms (Çam, 2021) and considered an important trait for a survival strategy in soils and on plants (Ansari et al., 2019) because biofilm mode of

growth offers several competitive advantages over planktonically-living cells (Wu et al., 2019). This sessile lifestyle helps plant microorganisms resist the hostile environmental conditions and is required for a long-term plant-microbe interaction (Lu et al., 2018). Soil biofilms increase microbial diversity and metabolic activity in soils, driving soil bio-geochemical processes (Wu et al., 2019). They also observed that biofilm formation in soils enhances the response to nutrient provision and respiration rates of soil microbes. Also, cells within soil biofilms are found to be much more active than planktonic cells (Wu et al., 2019). Interestingly, biofilm formation increases the values of microbial growth-promoting activities as compared to free-living individual cells (Triveni et al., 2013).

The beneficial effects of EPS production on abiotic stress tolerance are well-documented in a recent study (Morcillo and Manzanera, 2021) but the ecological roles of biofilm formation in soil are partly underestimated. It is clear that EPS is an indispensable component of bacterial biofilms (Flemming and Wingender, 2010). Exopolysaccharide-defective mutants cannot produce a well-structured robust biofilm (Flemming and Wingender, 2010). Mutational analysis showed that EPS-deficient strain of *Bacillus amyloliquefaciens* is less capable of forming biofilms (Lu et al., 2018). EPS production pattern was also found to be highly correlated with biofilm formation under the stress (Çam et al., 2022), indicating a strong correlation between biofilm formation and EPS production (Çam, 2023). We cannot imagine a well-structured biofilm without EPS production. Therefore, the impacts of biofilm formation along with EPS production on plant abiotic stress tolerance will be explained in this chapter.

Drought

Drought is one of the most detrimental abiotic stresses worldwide and is expected to adversely affect more than half of the arable lands by the mid-21st century (Vurukonda et al., 2016). A great number of studies have worked on the enhancement of plant tolerance to drought stress via genetic modifications in recent years, but the process has not met the expectations (Timmusk et al., 2014). Water stress causes various physiological and morphological problems in plant development (Çam, 2023). It interferes with plant functions by changing plant turgor potential; causes growth reduction; reduces nutrient availability and diffusion; decreases chlorophyll content; and

increases the levels of free radicals, resulting in oxidative stress in plants, consequently membrane deterioration and protein, lipid, and nucleic acid degradation (Vurukonda et al., 2016). Therefore, such drought-induced disorders restrict plant growth and development, subsequently causing significant losses in crop production (Lesk et al., 2016).

Water stress not only affect crop growth but also soil health and stability by changing soil properties, which plays a critical role in soil microbial activity (Morcillo and Manzanera, 2021). The presence of beneficial rhizosphere microorganisms is important to alleviate the effect of plant water stress and maintain a successful plant-microbe interaction (Çam, 2023). PGP bacteria contribute to plant drought stress tolerance through some mechanisms such as hormonal regulation, accumulation of osmolytes and antioxidants, root morphology modification, secretion of volatile organic compounds, EPS production, and biofilm formation (Morcillo and Manzanera, 2021; Çam, 2023). Among those mechanisms, biofilm formation together with EPS production may be a promising strategy in plant tolerance to drought stress.

Only a few recent studies showed the potential of biofilm-forming PGP microbes on drought stress alleviation in plants. For example, biofilm formation by the soil bacterium *Paenibacillus polymyxa* significantly increased plant survival and biomass under water scarcity (Timmusk et al., 2015). Similarly, in a recent study, a more biofilm-forming mutant *Bacillus amyloliquefaciens* strain considerably enhanced root colonization and drought tolerance in tomato seedlings as compared to either biofilm defective mutants or wild-type strain (Wang et al., 2019). In that study, they hypothesized that the respective bacteria sensed and responded to the chemical signals secreted by plant roots, then induced biofilm formation. Their findings also demonstrated that biofilm-producing ability of bacterial strains is positively correlated with their root-colonization ability and water stress tolerance efficacy in tomatoes. Moreover, biofilm-forming PGP rhizobacterial strains favored wheat growth under water stress by affecting root traits and antioxidant defense system (Karimi et al., 2022). It was suggested that bacterial inoculation causes some structural variation in plant roots such as reduced root tissue density, which improves the ability of nutrient uptake under drought conditions (Karimi et al., 2022). Likewise, a novel biofilm-producing strain of *Pseudomonas azotoformans* was found to be very effective in the amelioration of water stress in wheat through several biochemical

mechanisms, including increased root colonization, PGP activities, photosynthetic pigment efficiency, and antioxidant enzymatic activities (Ansari et al., 2021). Co-inoculation of biofilm-forming bacterial strains increased wheat growth under drought conditions by regulating physiological traits and stress indicators (Ansari et al., 2023). Synergistic roles of such biofilm-forming strains also improved soil fertility by maintaining soil physico-chemical properties and enzyme activities in plant rhizosphere even under water-limited conditions (Ansari et al., 2023).

The presence of EPS appears to play an important role in plant drought stress alleviation. First of all, EPS production increases the colonization capacity of biofilm cells on plant root surface (Janczarek, 2011). Lu et al. (2018) observed that EPS-defective bacterial mutant strains reduced their colonization ability on plant roots. In addition to increased microbial colonization, EPS production facilitates long-term persistence of plant-associated microbes on plant surfaces, which enables microorganisms to adapt to a specific environment (Çam, 2023). According to Karimi et al. (2022), rhizosphere microbes in arid regions adapt to the stress conditions of their host plants so they are commonly drought-resistant; their application may be an alternative method to cope with water stress. Enhanced bacterial colonization increases soil aggregate formation around root surfaces and soil macropore volume in the rhizosphere under a drought-stressed environment (Morcillo and Manzanera, 2021). It was shown that inoculation of *B. polymyxa* increased the formation of soil aggregates and porous soil structure in wheat plants (Gouzou et al., 1993), which maintains soil stability by modifying its physical and mechanical properties (Kaci et al., 2005). Soil aggregation around roots enhances the moisture level and water-holding capacity of rhizosphere soils, consequently water and nutrient uptake (Morcillo and Manzanera, 2021). EPS production not only favors plant growth but also microbial survival under drought stress (Çam, 2023). Hydrophilic nature of exopolysaccharides exhibits a high affinity for water so the biofilm matrix creates a highly hydrated environment containing 97% water, which protects biofilm-producing cells from dehydration under water-limiting conditions (Flemming and Wingender, 2010; Naseem and Bano, 2014). Briefly, a high water content-containing EPS layer offers a better resistance for biofilm-forming cells against drying under water restriction and enhances the water availability of rhizosphere soil, which is thought to provide enough time for the host plant to adjust their metabolic functions to withstand the water stress (Morcillo and

Manzanera, 2021). In conclusion, microbial biofilm formation and EPS production together with other PGP attributes contribute to plant growth and productivity under water-stressed conditions by increasing microbial survival and colonization on root surfaces, forming stable soil aggregates around plant roots, and increasing soil water and nutrient availability.

Salinity

Soil salinity is among the most destructive abiotic factors having adverse effects on soil and plant productivity (Banerjee et al., 2019). About 831 million hectares of global land area are under salinization and this situation increases day by day due to prolonged waterlogging, inadequate drainage systems, imposition of seawater to coastal areas, use of agrochemicals, overuse of groundwater, poor irrigation practices, and anthropogenic activities (Srivastava et al., 2019). The ratio of salt-affected areas is expected to increase in the future (Butcher, et al., 2016). Soil salinization leads to land degradation, therefore threatening soil health and food security (Butcher et al., 2016). A higher salt concentration in soil substantially decreases the productivity of many crop plants, subsequently affecting the livelihood of people worldwide (Butcher et al., 2016; Srivastava et al., 2019). Annual loss in global crop production costs over 27 billion US$ due to increasing soil salinization (Qadir et al., 2014).

Excessive amounts of salt ions in soil cause several disorders in plants (Çam, 2022b). Osmotic stress and specific ion toxicity are the two main factors that have direct/indirect effects on plant biological functions and soil physico-chemical conditions, respectively; consequently reducing crop productivity and soil fertility (Butcher et al., 2016). Osmotic stress leads to interruption of cell membrane stability and nutrient imbalance, and reduces photosynthetic activity, transpiration rate, carbon assimilation, and stomatal aperture (Gupta and Huang, 2014). Under salinity stress, accumulation of salt ions in plant tissues causes severe ion toxicity which in turn interferes with the water and nutrient uptake, decreasing plant growth rate, and subsequently may lead to death (Munns, 2002; Gupta and Huang, 2014). When salt concentration exceeds the threshold level, it interrupts plant metabolic and enzymatic activities, eventually causing tissue necrosis (Kasotia et al., 2016). Higher salinity deteriorates not only plant functions and soil structure but also microbial activity and diversity in rhizosphere soil (Morcillo and Manzanera,

2021; Çam, 2022b). Soil microorganisms have a crucial role in plant growth through nutrient cycling so their presence in soil is of utmost importance for plants (Yan et al., 2015).

Plants have various mechanisms to combat salinity stress (Gupta and Huang, 2014). Among such mechanisms, inoculation of salt-tolerant PGP microorganisms seems to play a better role in promoting the growth of agricultural crops by alleviating salinity stress (Banerjee, et al., 2019; Morcillo and Manzanera, 2021). Rhizosphere microorganisms ameliorate the effects of salinity stress in plants through the modulation of stress responsive genes, regulation of plant-associated hormones and antioxidant enzymes, production of secondary metabolites, EPS production, and biofilm formation (Çam, 2022b). Among those PGP bacteria-mediated protective mechanisms, recent investigations have paid more attention to the importance of EPS production in the mitigation of salinity stress (Morcillo and Manzanera, 2021; Çam, 2022b), thus the role of biofilm formation and EPS production on salt stress alleviation will be discussed in the following paragraphs.

Microbial EPS contribute to salt stress alleviation in plants in three ways such as stimulating biofilm formation on plant roots, forming soil aggregates around the roots, and restricting the uptake of salt ions into plants (Çam, 2022b). Increasing salinity triggers bacterial EPS production (Thakur and Yadav, 2024). Increased EPS production in response to a higher salt concentration induces biofilm production (Qurashi and Sabri, 2012). EPS play an important role in microbial adhesion and colonization to the biotic or abiotic surfaces (Flemming and Wingender, 2010), therefore enhanced biofilm formation is attributed to the binding potential of EPS (Morcillo and Manzanera, 2021). In a recent study, salinity stress enhanced the microbial attachment to the root surfaces and subsequently biofilm formation (Ansari, et al., 2019). A strong correlation existed between biofilm and EPS production under salt stress (Çam et al., 2022). Likewise, some *Bacillus* strains enhanced biofilm production in a pattern similar to exopolysaccharide production with increasing salinity (Çam and Badıllı, 2024). However, biofilm and EPS production are differently affected by varying salinity levels in different bacterial strains (Qurashi and Sabri, 2012), therefore it was concluded that EPS production is not considered an indication of biofilm formation under salt stress (Morcillo and Manzanera, 2021).

In recent decades, inoculation studies have focused on biofilm-forming ability of rhizobacteria for salt stress alleviation in plants. For instance, biofilm-forming PGP rhizobacteria increased barley growth by reducing the deleterious effect of salt stress (Kasim et al., 2016). Inoculation of wheat seedlings with a strong biofilm producer and halotolerant strain of *Bacillus pumilus* demonstrated an important improvement in plant growth attributes through its survival stability, salt resistance, root colonization ability, and multiple PGP properties under salinity stress (Ansari et al., 2019). A novel biofilm producer *Pantoea aglomerans* strain improved wheat growth and soil resilience under salt stress by modulating photosynthetic attributes, antioxidant systems, and sulfur metabolism (Ansari et al., 2024). It was suggested that such biofilm-producing strain with multifarious PGP traits successfully colonized plant rhizoplane and rhizosphere, protecting the inoculated plants against salt stress, sustaining physicochemical characteristics of soil, and improving soil hydrolytic enzymes (Ansari et al., 2024). Inoculation of biofilm-forming and salt-tolerant bacterial consortium alleviates salt stress in wheat by increasing membrane stability index and decreasing electrolyte leakage (Thakur and Yadav, 2024). Biofilm-producing PGP bacteria improved plant growth and salt tolerance by increasing photosynthetic pigments, antioxidant enzyme activities, and nutrient uptake in sunflower (Yasmeen et al., 2020). Chickpea plants inoculated with a halotolerant bacterial strain exhibiting biofilm formation and flocculation potential improved their growth attributes under salinity stress by showing a higher level of antioxidant enzymatic activity, relative water contents, and photosynthetic pigment, and a lower level of hydrogen peroxide production (Haroon et al., 2023). Application of biofilm-forming and halotolerant PGP rhizobacteria enhanced plant biomass production, triggered antioxidant defenses, maintained a higher water content, demonstrated a lesser membrane injury, decreased the amount of sodium ions in the roots, and increased the ratio of K^+ to Na^+ in tomato plants under saline conditions (Haque et al., 2022). More biofilm- and EPS-producing salt-resistant *Azotobacter* strains increased maize growth attributes under varying levels of salt concentrations compared to those forming less amount of biofilms (Çam et al., 2022). Biofilm-forming capability of *Bacillus* species was correlated with their salt stress amelioration impact in maize under salinity stress, suggesting that among several PGP traits, increased biofilm production plays a better role in plant growth parameters (Çam et al., 2023).

EPS production greatly contributes to plant salt stress alleviation through soil aggregate formation and cation chelation (Morcillo and Manzanera, 2021). Biofilm production with the help of binding-properties of EPS production sticks soil particles together and with plant roots, intensifying the aggregation of root-adhering soils around plant roots, and stimulating plant growth under salinity stress (Morcillo and Manzanera, 2021). For example, application of EPS formulation enhanced sunflower growth under salt stress, suggesting that EPS production increases soil porosity and quality, water-holding capacity of rhizosphere soils, and plant water and nutrient uptake; thus favors plant growth through the alleviation of salinity stress (Tewari and Arora, 2014). It is believed that the formation of soil aggregates with increasing porosity facilitates the uptake of water and nutrients, which in turn stimulates plant growth under salt-stressed conditions (Banerjee et al., 2019; Morcillo and Manzanera, 2021). In addition, EPS have a high potential to chelate free cautions (like Na^{+}, Ca^{2+}, K^{+}, and Mg^{2+}) from saline soils, making such ions unavailable to the plants by keeping them in the exopolysaccharide network, therefore restricting their uptake by plant roots, maintaining ionic balance in plants, and consequently improving plant growth and salt stress alleviation (Banerjee et al., 2019; Morcillo and Manzanera, 2021).

A higher salt concentration adversely affects bacterial adhesion to root surfaces due to changes in ionic strength (Jofre et al., 1998). Microbial adaptation is crucial for an effective plant-microbe interaction (Choudhary, 2012). Therefore, salt-induced osmotic stress conditions may stimulate EPS production, which in turn drives soil microorganisms to form biofilms under natural conditions (Qurashi and Sabri, 2012; Çam, 2022b) because hydrophilic nature of EPS enhances microbial survival under drought conditions by providing more water around biofilm cells, thus protecting them against desiccation (Flemming and Wingender, 2010). In conclusion, biofilm formation together with EPS production by PGP microorganisms mitigates salt stress in plants by increasing their colonization and survival ability, triggering soil aggregate formation around plant roots, and limiting the uptake of excessive salt ions into plant tissues; therefore improving plant growth under salinity stress.

Heavy Metals

Heavy metal pollution is among the most serious global problems, posing a major threat to the natural ecosystem and living-organisms (Dalvi and Bhalerao, 2013; Ojuederie and Babalola, 2017). The accumulation of heavy metals in agricultural soils adversely affects soil health, rhizosphere microbial activities, and consequently plant growth and productivity (Çam, 2022c). Some certain metals are necessary for the normal functions of plants but a higher quantity of essential/non-essential heavy metals causes various toxic symptoms in plants, even leading to death under extreme conditions (Dalvi and Bhalerao, 2013). The concentration of heavy metals exceeds the optimum level within a plant; they interfere with various physiological, biochemical, and metabolic functions such as, in general, cellular enzyme activities, cell structures, photosynthetic pathways, chlorophyll biosynthesis, uptake of water and nutrients, mitochondrial activity, cell division, seed germination, leaf expansion, membrane permeability, water potential, transpiration rate, ionic balance, membrane ATPase activity, stomatal conductance, and antioxidant systems; subsequently, causing various symptoms in plants (Hassan et al., 2017; Çam, 2022c). A higher level of heavy metals may also indirectly reduce plant growth by interfering with the presence and activity of beneficial soil organisms (Chibuike and Obiora, 2014; Syed et al., 2021). It appears that the clean-up of excessive amounts of toxic metal ions from agricultural soils is essential for plant development.

Plants can escape from the inhibitory effects of metal stress through a number of specific defense strategies, including immobilization by mycorrhizal association, secretion of root exudates, inactivation and detoxification of toxic metals, active efflux pumping, activation of oxidative defense mechanisms, metal-binding properties of cell wall, and modification of rhizosphere pH, and synthesis of stress-related proteins (Dalvi and Bhalerao, 2013). Although plants have developed such mechanisms for the avoidance and tolerance of metal toxicity, heavy metals still lead to significant disorders in plants as summarized by Çam (2022c). Various techniques have been developed for the removal of toxic metals from soils, but among them, the utilization of biological methods is considered the most useful because they are cheap and harmless to the environments (Hassan et al., 2017; Ojuederie and Babalola, 2017).

The use of metal-resistant bacteria is an effective approach to the remediation of toxic metals (Ojuederie and Babalola, 2017; Sharma et al., 2020). Metal-tolerant microbes can remediate and tolerate toxic metals through some mechanisms such as conversion of metals to less-toxic forms by enzymes without noxious end-products, changes of biochemical pathways to restrict metal acquisition, decreasing intracellular metal concentrations using efflux pumps, and sequestration of metal ions by metal-binding proteins/peptides, siderophore production, cell wall components, and EPS production (Ojuederie and Babalola, 2017). EPS production is considered the most notable action in the removal of heavy metals from environments because of its metal-binding potential (Yadav and Chandra, 2020; Çam, 2022c). EPS act as a trap for positively-charged heavy metals such as cadmium, magnesium, copper, zinc, nickel, iron, lead, and manganese (Sharma et al., 2020). Electrostatic interactions between metal cations and negatively-charged EPS residues lead to the formation of stable complexes (Pal and Paul, 2008), which increases metal-sequestration capability of EPS through biosorption (Morcillo and Manzanera, 2021). The anionic nature of EPS is due to the presence of different functional groups and substituents in the composition of microbial EPS (Morcillo and Manzanera, 2021). The anionic moieties in microbial EPS capture positively charged metal ions from their surrounding environments, thus increasing the removal of toxic metal cations (Yadav and Chandra, 2020; Morcillo and Manzanera, 2021). Microorganisms limit bioavailability of toxic metals in plants through one or more mechanisms above, thus indirectly promoting plant growth; but, first of all, microbes must be able to survive under fluctuating environmental conditions to exert their potential in bioremediation (Çam, 2022c).

Soil bioremediation is a challenging process due to soil heterogeneity, thus well-adapted microorganisms are required for the clean-up of toxic pollutants (Hassan et al., 2017; Çam, 2022c). Heavy metal toxicity has an adverse impact on microbial survival so microorganisms in the presence of environmental pollutants prefer a biofilm lifestyle for a better protection against the stress (Yadav and Chandra, 2020). Biofilm-forming organisms are less vulnerable to extreme environmental conditions than their planktonic single cells, so soil microorganisms may switch from planktonic form to biofilm mode of life in the natural environment (Çam, 2022c). For example, biofilm formation ability of *Thiomonas* spp. was strongly modified when exposed to the metal arsenic, resulting increased arsenic resistance, which was

attributed to the protective roles of biofilm (Marchal et al., 2011). Heavy metals also reduce the effect of PGP traits of plant-associated microorganisms (Karthik et al., 2017; Syed et al., 2021). For instance, the production of auxin hormone in *Streptomyces* decreased upon exposure to heavy metal ions (Dimkpa et al., 2008). Moreover, the metal-binding potential of biofilm-forming *Pseudomonas aeruginosa* cells was much higher as compared to planktonically-grown counterparts (Langley and Beveridge, 1999), indicating that biofilm formation contributes to the metal sequestration efficiency of free-living microbial cells. Therefore, the use of microbial biofilms is considered an effective approach in bioremediation because biofilms; 1) can alleviate heavy metal toxicity by absorbing and degrading a great number of different types of toxic pollutants, including heavy metals, and 2) better survive and persist under hostile conditions (Sharma et al., 2020).

Biofilm-mediated bioremediation can be an effective alternative for the eradication of contaminant toxic metals from the environments (Sharma et al., 2020; Jasu et al., 2021). Potential of microbial biofilms in the sequestration of heavy metals from the contaminated solutions has been well-studied (White and Gadd, 2000; Costley and Wallis, 2001; Von Canstein et al., 2002; Templeton et al., 2003; Chang et al., 2006; Kang et al., 2006; Sharma et al., 2020) but their application is limited in the removal of toxic metals from agricultural soils (Çam, 2022c). Only very few studies highlighted the importance of soil biofilms for plant growth under metal-contaminated soils. For example, biofilm formation by *Paenibacillus lentimorbus* protected chickpea in chromium-stressed rhizosphere soil, suggesting that the bacterial cells in biofilms are sheltered from chromium stress due to the physical barrier of the biofilm matrix; as a result, the protected cells exhibited their PGP potential, which in turn promoted chickpea growth and yield (Khan et al., 2012). Inoculation of arsenic-resistant bacterial strains improved rice growth and reduced the metal accumulation in the rice seedlings through the adsorption and bioremediation effect of biofilm and EPS production together with other PGP properties (Mallick et al., 2018). Interestingly, heavy metals decreased the PGP potential of rhizospheric strains of *Pseudomonas* and *Brevundomonas* species but induced biofilm and EPS production; an increase in biofilm formation effectively improved the removal of four common heavy metals (Cu^{2+}, Pb^{2+}, Cd^{2+}, and Zn^{2+}) (Xing et al., 2022). In summary, biofilm-based bioremediation in soils appears to be a promising approach for the removal of heavy metals from the surroundings due to the advantages of

biofilm and EPS production on microbial survival, their PGP potential and metal sequestration efficiency, therefore microbial biofilms produced by metal-resistant PGP rhizobacteria can improve plant growth and productivity under metal-induced stress conditions.

Nutrient Deficiency

Climate change increases the rate of global land degradation day by day, representing a major threat to fertile agricultural lands, therefore causing nutrient-limiting soils around the world, and consequently threatening food security (Arora, 2019). To meet the increasing demands for crop production, modern agriculture is still dependent on the utilization of agricultural chemicals but such hazardous chemical treatments pose an important risk to human and soil health (Hossain et al., 2022). Application of PGP bacteria-based fertilizers can be an alternative to the utilization of synthetic chemicals because of their eco-friendly advantages to the environment and living organisms, so their use has been increasing in agriculture to enhance crop growth in the last decades (Basu et al., 2021). PGP bacteria promote plant growth through a set of beneficial activities, including the solubilization and immobilization of essential minerals/nutrients for soil fertility and production of various growth-promoting substances (Ma et al., 2016; 2021; Vocciante et al., 2022). However, biotic and/or abiotic stress factors affect microbial survival and colonization ability so the performance of microbe-based inoculants may not be consistent under field conditions (Ansari and Ahmad, 2018). Their studies concluded that biofilm-producing ability of the bacteria with the desired growth-promoting traits appears to be a clear advantage for their survival and colonization in the rhizosphere.

Soil is generally a nutritionally poor environment because its organic matter concentrations vary from 0.8 to 2.0%, therefore indigenous plant-associated microorganisms must cope with nutrient deprivation under natural conditions (Davey and O'Toole, 2000). Plant microbes can form biofilms to combat the stressful conditions, including nutrient limitation (Velmourougane, et al., 2017). The nutrient-deprived soil microorganisms may prefer biofilm lifestyle, especially in barren soils, due to the benefits of biofilm formation as also stated by Rinaudi and Giordano (2010). The biofilm matrix allows the absorption of organic compounds from environments, enabling the digestion of the obtained macromolecules through enzymatic

activity, and providing the essential nutrients for biofilm communities (Flemming and Wingender, 2010). The matrix can act as a recycling center by keeping the residual materials of dead cells available within a biofilm (Flemming and Wingender, 2010). It generates an external digestive system thanks to the retention of extracellular enzymes, obtaining dissolved nutrients to be used as nutrient and energy source (Flemming and Wingender, 2010). The anionic character of EPS also helps to trap minerals and nutrients from the environment (Donot et al., 2012). Interestingly, it was proposed that biofilm cells create pores and channels in the matrix, which facilitates nutrient access and mass transport into biofilm architecture (Flemming and Wingender, 2010). These channels allow the biofilm cells to exchange enzymes, nutrients, water, and signals by acting as a regulatory system within the biofilm (Rinaudi and Giordano, 2010). All the benefits mentioned above offer an ecological advantage to biofilm-producing organisms, so may drive rhizobacteria to develop biofilms under nutrient depletion in rhizosphere soils.

Biofilm formation not only increases the survival rate of PGP bacteria under nutrient deficiency, but also enhances the availability of nutrients in soils, resulting in plant growth. For instance, cyanobacterial-biofilmed formulations improved soil fertility, consequently plant productivity (Prasanna et al., 2015). Similarly, biofilm formation by a cyanobacterium species was effective in the nutrient uptake, enzymatic activities, and crop yield in rice plants (Adak et al., 2016). A recent study observed that two EPS-producing rhizobacterial strains with other PGP traits increased the nutrient uptake by plant roots through soil aggregation and enhanced plant growth, which was attributed to biofilm formation (Zainab et al., 2020). EPS production stimulates soil aggregate formation around plant roots, creating an immediate environment in which plant roots take up water and nutrients (Morcillo and Manzanera, 2021). The biofilm matrix is composed of mainly polysaccharides that increase soil physical properties, including soil aggregate formation, reduction in bulk density, and better water and nutrient retention capacity (Velmourougane et al., 2017). According to Morcillo and Manzanera (2021), enhanced soil aggregation and porous soil structure retain more water in the rhizosphere soils, consequently increasing nutrient uptake and plant growth as also suggested by Tewari and Arora (2014). For example, inoculation of EPS-producing PGP rhizobacteria with salicylic acid augmented plant macro- and micro-nutrient availability by increasing soil moisture level and leaf water content (Khan and Bano, 2019). Likewise, the

contribution of biofilm formation to plant nutrient uptake was demonstrated in sunflower plants inoculated with biofilm forming rhizobacterial strains (Yasmeen et al., 2020). Additionally, Karimi et al. (2022) observed that biofilm-forming PGP bacterial strains caused structural changes in root morphology and supported plant nutrition by improving the ability of plant roots for nutrient uptake. In a most recent study, single or co-inoculation of more biofilm-producing rhizobacterial strains with macroalgae extract significantly enhanced barley growth and yield in an organic matter-limited barren soil, suggesting that biofilm and EPS production ability of the bacterial strain appears to be a determining factor in increased plant productivity (Çam et al., 2024). They concluded that bacterial strains residing in biofilms most probably degrade algal extracts into absorbable form that is easily used by not only biofilm-forming bacteria but also barley plants as a source of nutrient in the unproductive soil. From the results of those studies, it can be concluded that biofilm-forming ability of plant-associated microorganisms contributes to plants' nutritional status and their growth by improving soil physical structures through biofilm and EPS production.

Conclusion

The most part of global land areas is under the detrimental effects of habitat-imposed abiotic stresses. Plants encounter various stress factors in the natural environment, causing significant economic losses in agricultural production, threatening food security, therefore the inhibitory impacts of environmental stresses on plants must be reduced to ensure food production. PGP bacteria appear to be a promising effective alternative for plant growth under abiotic stress through biofilm and EPS production. Under stressful conditions, biofilm formation together with EPS production offers several advantages to not only biofilm-forming cells but also plant growth and productivity, in general, such as increased microbial survivability, colonization ability, long-term persistence, soil aggregate formation, and nutrition and water status. Biofilm-and EPS-producing microorganisms with the desired PGP traits can be used to improve crop growth by ameliorating the inhibitory effects of some abiotic stress factors stated above.

REFERENCES

Adak, A., Prasanna, R., Babu, S., Bidyarani, N., Verma, S., Pal, M., Shivay, Y. S., & Nain, L. (2016). Micronutrient enrichment mediated by plant-microbe interactions and rice cultivation practices. *Journal of Plant Nutrition, 39*, 1216-1232.

Ansari, F. A. & Ahmad, I. (2018). Biofilm development, plant growth promoting traits and rhizosphere colonization by *Pseudomonas entomophila* FAP1: A promising PGPR. *Advances in Microbiology, 8*, 235-251.

Ansari, F. A., Ahmad, I., & Pichtel, J. (2019). Growth stimulation and alleviation of salinity stress to wheat by the biofilm forming *Bacillus pumilus* strain FAB10. *Applied Soil Ecology, 143*, 45-54.

Ansari, F. A., Ahmad, I., & Pichtel, J. (2023). Synergistic effects of biofilm-producing PGPR strains on wheat plant colonization, growth and soil resilience under drought stress. *Saudi Journal of Biological Sciences, 30*, 103664.

Ansari, F. A., Ahmad, I., Pichtel, J., & Husain, F. M. (2024). *Pantoea agglomerans* FAP10: A novel biofilm-producing pgpr strain improves wheat growth and soil resilience under salinity stress. *Environmental and Experimental Botany, 222*, 105759.

Ansari, F. A., Jabeen, M., & Ahmad, I. (2021). *Pseudomonas azotoformans* FAB5, a novel biofilm-forming PGPR strain, alleviates drought stress in wheat plant. *International Journal of Environmental Science and Technology, 18*, 3855-3870.

Arora, N. K. (2019). Impact of climate change on agriculture production and its sustainable solutions. *Environmental Sustainability, 2*, 95-96.

Banerjee, A., Sarkar, S., Cuadros-Orellana, S., & Bandopadhyay, R. (2019). Exopolysaccharides and biofilms in mitigating salinity stress: The biotechnological potential of halophilic and soil-inhabiting PGPR microorganisms. *Microorganisms in Saline Environments: Strategies and Functions.* 133-153.

Basu, A., Prasad, P., Das, S. N., Kalam, S., Sayyed, R. Z., Reddy, M. S., & El Enshasy, H. (2021). Plant growth promoting rhizobacteria (PGPR) as green bioinoculants: Recent developments, constraints, and prospects. *Sustainability, 13*.

Butcher, K., Wick, A. F., DeSutter, T., Chatterjee, A., & Harmon, J. (2016). Soil salinity: A threat to global food security. *Agronomy Journal, 108*, 2189-2200.

Chang, W. C., Hsu, G. S., Chiang, S. M., & Su, M. C. (2006). Heavy metal removal from aqueous solution by wasted biomass from a combined as-biofilm process. *Bioresource Technology, 97*, 1503-1508.

Chibuike, G. U. & Obiora, S. C. (2014). Heavy metal polluted soils: Effect on plants and bioremediation methods. *Applied and Environmental Soil Science, 2014*,

752708.

Choudhary, D. K. (2012). Microbial rescue to plant under habitat-imposed abiotic and biotic stresses. *Applied Microbiology and Biotechnology, 96*, 1137-1155.

Costley, S. C. & Wallis, F. M. (2001). Bioremediation of heavy metals in a synthetic wastewater using a rotating biological contactor. *Water Research, 35*, 3715-3723.

Cramer, G. R., Urano, K., Delrot, S., Pezzotti, M., & Shinozaki, K. (2011). Effects of abiotic stress on plants: A systems biology perspective. *BMC Plant Biology, 11*, 163.

Çam, S. (2021). Plant-associated microbial biofilms: From ecology to molecular genetics. In: Aydın B, editor. Current approaches in science of life. Ankara: Iksad Publishing House, 99-121.

Çam, S. (2022a). The potential of bacterial biofilms in biocontrol of microbial plant diseases. In: Çolak M, editor. International research in agriculture sciences. Konya: Eğitim Yayınevi, 37-66.

Çam, S. (2022b). The role of bacterial exopolysaccharides on the amelioration of salt stress in plants. In: Kokten K, Ozdemir S, editors. Integrated alternative farming models. Ankara: Iksad Publishing House, 133-154.

Çam, S. (2022c). Plant-associated microbial biofilms-mediated bioremediation of heavy metals. In: Toncer O, Gunes Z, editors. A look into some recent advances in biology, ecology and agricultural practices. Ankara: Iksad Publishing House, 3-30.

Çam, S. (2023). Potential of plant-associated microbial biofilms in plant drought stress tolerance. In: Çokkızgın A, editor. International research in agriculture sciences II. İstanbul: Eğitim Yayınevi, 23-50.

Çam, S. & Badıllı, İ. (2024). The effect of NaCl, pH, and phosphate on biofilm formation and exopolysaccharide production by high biofilm producers of *Bacillus* strains. *Folia Microbiologica, 69*, 613-624.

Çam, S. & Bicek, S. (2023). The effects of temperature, salt, and phosphate on biofilm and exopolysaccharide production by *Azotobacter* spp. *Archives of Microbiology*, *205*, 87.

Çam, S. & Brinkmeyer, R. (2020a). The effects of temperature, pH, and iron on biofilm formation by clinical versus environmental strains of *Vibrio vulnificus*. *Folia Microbiologica, 65*, 557-566.

Çam, S. & Brinkmeyer, R. (2020b). Differential expression of *vvhA* and CPS operon allele 1 genes in *Vibrio vulnificus* under biofilm and planktonic conditions. *Antonie van Leeuwenhoek, 113*, 1437-1446.

Çam, S., Küçük, Ç., & Almaca, A. (2023). *Bacillus* strains exhibit various plant growth promoting traits and their biofilm-forming capability correlates to their salt stress alleviation effect on maize seedlings. *Journal of Biotechnology*, *369*, 35-42.

Çam, S., Küçük, Ç., & Cevheri, C. (2022). The effect of salinity-resistant biofilm-forming *Azotobacter* spp. on salt tolerance in maize growth. *Zemdirbyste-Agriculture, 109*.

Çam, S., Küçük, Ç., Dikilitaş, S. K., Sezen, G., Almaca, A., & Cevheri, C. (2024). Single and co-inoculation of biofilm-forming rhizobacteria with macroalgae extract increase barley productivity in organic matter-limited soil. *Crop and Pasture Science. 75*.

Dalvi, A. A. & Bhalerao, S. A. (2013). Response of plants towards heavy metal toxicity: An overview of avoidance, tolerance and uptake mechanism. *Annals of Plant Sciences, 2*, 362-368.

Davey, M. E. & O'Toole, G. A. (2000). Microbial biofilms: From ecology to molecular genetics. *Microbiology and Molecular Biology Reviews, 64*, 847-867.

Dimkpa, C. O., Svatoš, A., Dabrowska, P., Schmidt, A., Boland, W., & Kothe, E. (2008). Involvement of siderophores in the reduction of metal-induced inhibition of auxin synthesis in *Streptomyces* spp. *Chemosphere, 74*, 19-25.

Donot, F., Fontana, A., Baccou, J. C., & Schorr-Galindo, S. (2012). Microbial exopolysaccharides: Main examples of synthesis, excretion, genetics and extraction. *Carbohydrate Polymers, 87*, 951-962.

Flemming, H.-C. & Wingender, J. (2010). The biofilm matrix. *Nature Reviews Microbiology, 8*, 623-633.

Flemming, H.-C. & Wuertz, S. (2019). Bacteria and archaea on earth and their abundance in biofilms. *Nature Reviews Microbiology, 17*, 247-260.

Gong, Z., Xiong, L., Shi, H., Yang, S., Herrera-Estrella, L. R., Xu, G., Chao, D.-Y., Li, J., Wang, P.-Y., & Qin, F. (2020). Plant abiotic stress response and nutrient use efficiency. *Science China Life Sciences, 63*, 635-674.

Gouzou, L., Burtin, G., Philippy, R., Bartoli, F., & Heulin, T. (1993). Effect of inoculation with *Bacillus polymyxa* on soil aggregation in the wheat rhizosphere: Preliminary examination. Soil structure/soil biota interrelationships. Elsevier, 479-491

Gupta, B. & Huang, B. (2014). Mechanism of salinity tolerance in plants: Physiological, biochemical, and molecular characterization. *International Journal of Genomics, 2014*.

Haque, M. M., Biswas, M. S., Mosharaf, M. K., Haque, M. A., Islam, M. S., Nahar, K., Islam, M. M., Shozib, H. B., Islam, M. M., & Ferdous, E. E. (2022). Halotolerant biofilm-producing rhizobacteria mitigate seawater-induced salt stress and promote growth of tomato. *Scientific Reports, 12*, 5599.

Haroon, U., Munis, M. F. H., Liaquat, F., Khizar, M., Elahi, M., & Chaudhary, H. J. (2023). Biofilm formation and flocculation potential analysis of halotolerant *Bacillus tequilensis* and its inoculation in soil to mitigate salinity stress of chickpea. *Physiology and Molecular Biology of Plants, 29*, 277-288.

Hassan, Z. U., Ali, S., Rizwan, M., Ibrahim, M., Nafees, M., & Waseem, M. (2017). Role of bioremediation agents (bacteria, fungi, and algae) in alleviating heavy metal toxicity. In: Kumar V et al., editors. Probiotics in Agroecosystem. Singapore: Springer, 517-537.

Hossain, M. E., Shahrukh, S., & Hossain, S. A. (2022). Chemical fertilizers and pesticides: Impacts on soil degradation, groundwater, and human health in Bangladesh. Environmental degradation: Challenges and strategies for mitigation. Springer, 63-92.

Janczarek, M. (2011). Environmental signals and regulatory pathways that influence exopolysaccharide production in rhizobia. *International Journal of Molecular Sciences, 12*, 7898-7933

Jasu, A., Lahiri, D., Nag, M., & Ray, R. R. (2021). Biofilm-associated metal bioremediation. Biotechnology for sustainable environment. Springer, 201-221.

Jofre, E., Fischer, S., Rivarola, V., Balegno, H., & Mori, G. (1998). Saline stress affects the attachment of *Azospirillum brasilense* Cd to maize and wheat roots. *Canadian Journal of Microbiology, 44*, 416-422.

Kaci, Y., Heyraud, A., Barakat, M., & Heulin, T. (2005). Isolation and identification of an EPS-producing rhizobium strain from arid soil (Algeria): Characterization of its EPS and the effect of inoculation on wheat rhizosphere soil structure. *Research in Microbiology, 156*, 522-531.

Kang, S.-Y., Bremer, P. J., Kim, K.-W., & McQuillan, A. J. (2006). Monitoring metal ion binding in single-layer *Pseudomonas aeruginosa* biofilms using ATR-IR spectroscopy. *Langmuir, 22*, 286-291.

Karimi, E., Aliasgharzad, N., Esfandiari, E., Hassanpouraghdam, M. B., Neu, T. R., Buscot, F., Reitz, T., Breitkreuz, C., & Tarkka, M. T. (2022). Biofilm forming rhizobacteria affect the physiological and biochemical responses of wheat to drought. *AMB Express, 12*, 93.

Karthik, C., Elangovan, N., Kumar, T. S., Govindharaju, S., Barathi, S., Oves, M., & Arulselvi, P. I. (2017). Characterization of multifarious plant growth promoting traits of rhizobacterial strain AR6 under chromium (VI) stress. *Microbiological Research, 204*, 65-71.

Kasim, W. A., Gaafar, R. M., Abou-Ali, R. M., Omar, M. N., & Hewait, H. M. (2016). Effect of biofilm forming plant growth promoting rhizobacteria on salinity tolerance in barley. *Annals of Agricultural Sciences, 61*, 217-227.

Kasotia, A., Varma, A., Tuteja, N., & Choudhary, D. K. (2016). Amelioration of soybean plant from saline-induced condition by exopolysaccharide producing *Pseudomonas*-mediated expression of high affinity K^+-transporter (HKT1) gene. *Current Science, 111*, 1961-1967.

Khan, N. & Bano, A. (2019). Exopolysaccharide producing rhizobacteria and their impact on growth and drought tolerance of wheat grown under rainfed conditions. *PLoS One, 14*, e0222302.

Khan, N., Mishra, A., Chauhan, P. S., Sharma, Y. K., & Nautiyal, C. S. (2012). *Paenibacillus lentimorbus* enhances growth of chickpea (*Cicer arietinum* L.) in chromium-amended soil. *Antonie Van Leeuwenhoek, 101*, 453-459.

Kumar, A., Singh, S., Mukherjee, A., Rastogi, R. P., & Verma, J. P. (2021). Salt-tolerant plant growth-promoting *Bacillus pumilus* strain JPVS11 to enhance plant growth attributes of rice and improve soil health under salinity stress. *Microbiological Research, 242*, 126616.

Langley, S. & Beveridge, T. J. (1999). Metal binding by *Pseudomonas aeruginosa* PAO1 is influenced by growth of the cells as a biofilm. *Canadian Journal of Microbiology, 45*, 616-622.

Legein, M., Smets, W., Vandenheuvel, D., Eilers, T., Muyshondt, B., Prinsen, E., Samson, R., & Lebeer, S. (2020). Modes of action of microbial biocontrol in the phyllosphere. *Frontiers in Microbiology, 1619*.

Lesk, C., Rowhani, P., & Ramankutty, N. (2016). Influence of extreme weather disasters on global crop production. *Nature, 529*, 84-87.

Lu, X., Liu, S.-F., Yue, L., Zhao, X., Zhang, Y.-B., Xie, Z.-K., & Wang, R.-Y. (2018). Epsc involved in the encoding of exopolysaccharides produced by *Bacillus amyloliquefaciens* FZB42 act to boost the drought tolerance of *Arabidopsis thaliana*. *International Journal of Molecular Sciences, 19*, 3795.

Ma, Y., Oliveira, R. S., Freitas, H., & Zhang, C. (2016). Biochemical and molecular mechanisms of plant-microbe-metal interactions: Relevance for phytoremediation. *Frontiers in Plant Science, 7*, 918.

Mallick, I., Bhattacharyya, C., Mukherji, S., Dey, D., Sarkar, S. C., Mukhopadhyay, U. K., & Ghosh, A. (2018). Effective rhizoinoculation and biofilm formation by arsenic immobilizing halophilic plant growth promoting bacteria (PGPB) isolated from mangrove rhizosphere: A step towards arsenic rhizoremediation. *Science of The Total Environment, 610*, 1239-1250.

Marchal, M., Briandet, R., Halter, D., Koechler, S., DuBow, M. S., Lett, M.-C., & Bertin, P. N. (2011). Subinhibitory arsenite concentrations lead to population dispersal in *Thiomonas* sp. *PLoS One, 6*, e23181.

Morcillo, R. J. L. & Manzanera, M. (2021). The effects of plant-associated bacterial exopolysaccharides on plant abiotic stress tolerance. *Metabolites, 11*, 337.

Munns, R. (2002). Comparative physiology of salt and water stress. *Plant, Cell & Environment, 25*, 239-250.

Naseem, H. & Bano, A. 2014. Role of plant growth-promoting rhizobacteria and their exopolysaccharide in drought tolerance of maize. *Journal of Plant Interactions, 9*, 689-701.

Ojuederie, O. B. & Babalola, O. O. (2017). Microbial and plant-assisted bioremediation of heavy metal polluted environments: A review. *International Journal of Environmental Research and Public Health, 14*, 1504.

Pal, A. & Paul, A. K. (2008). Microbial extracellular polymeric substances: Central elements in heavy metal bioremediation. *Indian Journal of Microbiology, 48*, 49-64.

Pereira, A. (2016). Plant abiotic stress challenges from the changing environment. *Frontiers Media SA, 7*, 1123.

Prakash, B., Veeregowda, B. M., & Krishnappa, G. (2003). Biofilms: A survival strategy of bacteria. *Current Science,* 1299-1307.

Prasanna, R., Adak, A., Verma, S., Bidyarani, N., Babu, S., Pal, M., Shivay, Y. S., & Nain, L. (2015). Cyanobacterial inoculation in rice grown under flooded and sri modes of cultivation elicits differential effects on plant growth and nutrient dynamics. *Ecological Engineering, 84*, 532-541.

Qadir, M., Quillérou, E., Nangia, V., Murtaza, G., Singh, M., Thomas, R. J., Drechsel, P., & Noble, A. D. (2014). Economics of salt-induced land degradation and restoration. *Natural Resources Forum, 38*, 282-295.

Qurashi, A. W. & Sabri, A. N. (2012). Bacterial exopolysaccharide and biofilm formation stimulate chickpea growth and soil aggregation under salt stress. *Brazilian Journal of Microbiology, 43*, 1183-1191.

Qurashi, A. W. & Sabri, A. N. (2012). Biofilm formation in moderately halophilic bacteria is influenced by varying salinity levels. *Journal of Basic Microbiology, 52*, 566-572.

Rinaudi, L. V. & Giordano, W. (2010). An integrated view of biofilm formation in rhizobia. *FEMS Microbiology Letters, 304*, 1-11.

Satpathy, S., Sen, S. K., Pattanaik, S., & Raut, S. (2016). Review on bacterial biofilm: An universal cause of contamination. *Biocatalysis and Agricultural Biotechnology, 7*, 56-66.

Sharma, A., Jamali, H., Vaishnav, A., Giri, B. S., & Srivastava, A. K. (2020). Microbial biofilm: An advanced eco-friendly approach for bioremediation. New and future developments in microbial biotechnology and bioengineering: Microbial biofilms. Elsevier, 205-219.

Srivastava, P., Wu, Q.-S., & Giri, B. (2019). Salinity: An overview. *Microorganisms in saline environments: Strategies and Functions,* 3-18.

Stewart, P. S. & Franklin, M. J. (2008). Physiological heterogeneity in biofilms. *Nature Reviews Microbiology, 6*, 199-210.

Syed, A., Zeyad, M. T., Shahid, M., Elgorban, A. M., Alkhulaifi, M. M., & Ansari, I. A. (2021). Heavy metals induced modulations in growth, physiology, cellular viability, and biofilm formation of an identified bacterial isolate. *ACS Omega, 6*, 25076-25088.

Templeton, A. S., Spormann, A. M., & Brown, G. E. (2003). Speciation of pb (II) sorbed by *Burkholderia cepacia*/goethite composites. *Environmental Science & Technology, 37*, 2166-2172.

Tewari, S. & Arora, K. (2014). Talc based exopolysaccharides formulation enhancing growth and production of *Hellianthus annuus* under saline conditions. *Cellular and Molecular Biology, 60,* 73-81.

Thakur, R. & Yadav, S. (2024). Biofilm forming, exopolysaccharide producing and halotolerant, bacterial consortium mitigates salinity stress in *Triticum aestivum*. *International Journal of Biological Macromolecules, 262,* 130049.

Thakur, S., Singh, R., Kaur, J., Sharma, M., Pandit, K., Kaur, S., & Kaur, S. (2021). Abiotic stress in plants: An overview. *Environmental Stress Physiology Plants and Crop Product.* 1-19.

Timmusk, S., Abd El-Daim, I. A., Copolovici, L., Tanilas, T., Kännaste, A., Behers, L., Nevo, E., Seisenbaeva, G., Stenström, E., & Niinemets, Ü. (2014). Drought-tolerance of wheat improved by rhizosphere bacteria from harsh environments: Enhanced biomass production and reduced emissions of stress volatiles. *PloS One, 9,* e96086.

Timmusk, S., Kim, S.-B., Nevo, E., Abd El Daim, I., Ek, B., Bergquist, J., & Behers, L. (2015). Sfp-type PPTase inactivation promotes bacterial biofilm formation and ability to enhance wheat drought tolerance. *Frontiers in Microbiology, 6*.

Triveni, S., Prasanna, R., Shukla, L., & Saxena, A. K. (2013). Evaluating the biochemical traits of novel *Trichoderma*-based biofilms for use as plant growth-promoting inoculants. *Annals of Microbiology, 63,* 1147-1156.

Velmourougane, K., Prasanna, R., & Saxena, A. K. (2017). Agriculturally important microbial biofilms: Present status and future prospects. *Journal of Basic Microbiology, 57,* 548-573.

Vocciante, M., Grifoni, M., Fusini, D., Petruzzelli, G., & Franchi, E. (2022). The role of plant growth-promoting rhizobacteria (PGPR) in mitigating plant's environmental stresses. *Applied Sciences, 12,* 1231.

Von Canstein, H., Li, Y., Leonhäuser, J., Haase, E., Felske, A., Deckwer, W.-D., & Wagner-Döbler, I. (2002). Spatially oscillating activity and microbial succession of mercury-reducing biofilms in a technical-scale bioremediation system. *Applied and Environmental Microbiology, 68,* 1938-1946.

Vurukonda, S. S. K. P., Vardharajula, S., Shrivastava, M., & SkZ, A. (2016). Enhancement of drought stress tolerance in crops by plant growth promoting rhizobacteria. *Microbiological Research, 184,* 13-24.

Wang, D.-C., Jiang, C.-H., Zhang, L.-N., Chen, L., Zhang, X.-Y., & Guo, J.-H. (2019). Biofilms positively contribute to *Bacillus amyloliquefaciens* 54-induced drought tolerance in tomato plants. *International Journal of Molecular Sciences, 20*.

White, C. & Gadd, G. M. (2000). Copper accumulation by sulfate-reducing bacterial biofilms. *FEMS Microbiology Letters, 183,* 313-318.

Wu, Y., Cai, P., Jing, X., Niu, X., Ji, D., Ashry, N. M., Gao, C., & Huang, Q. (2019). Soil biofilm formation enhances microbial community diversity and metabolic activity. *Environment International, 132*, 105116.

Xing, Y., Tan, S., Liu, S., Xu, S., Wan, W., Huang, Q., & Chen, W. (2022). Effective immobilization of heavy metals via reactive barrier by rhizosphere bacteria and their biofilms. *Environmental Research, 207*, 112080.

Yadav, S. & Chandra, R. (2020). Biofilm-mediated bioremediation of pollutants from the environment for sustainable development. New and future developments in microbial biotechnology and bioengineering: Microbial biofilms. Elsevier, 177-203

Yan, N., Marschner, P., Cao, W., Zuo, C., & Qin, W. (2015). Influence of salinity and water content on soil microorganisms. *International Soil and Water Conservation Research, 3*, 316-323.

Yasmeen, T., Ahmad, A., Arif, M. S., Mubin, M., Rehman, K., Shahzad, S. M., Iqbal, S., Rizwan, M., Ali, S., Alyemeni, M. N., & Wijaya, L. (2020). Biofilm forming rhizobacteria enhance growth and salt tolerance in sunflower plants by stimulating antioxidant enzymes activity. *Plant Physiology and Biochemistry, 156*, 242-256.

Zainab, N., Din, B. U., Javed, M. T., Afridi, M. S., Mukhtar, T., Kamran, M. A., Khan, A. A., Ali, J., Jatoi, W. N., & Munis, M. F. H. (2020). Deciphering metal toxicity responses of flax (*Linum usitatissimum* L.) with exopolysaccharide and ACC-deaminase producing bacteria in industrially contaminated soils. *Plant Physiology and Biochemistry, 152*, 90-99.

Zhang, X., Hu, Z., Pan, H., Bai, Y., Hu, Y., & Jin, S. (2022). Effects of rare earth elements on bacteria in rhizosphere, root, phyllosphere and leaf of soil–rice ecosystem. *Scientific Reports, 12*, 1-17.

Zörb, C., Geilfus, C. M., & Dietz, K. J. (2019). Salinity and crop yield. *Plant Biology, 21*, 31-38.

OVERVIEW OF PROBIOTICS FROM A BIOTECHNOLOGICAL PERSPECTIVE

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Abstract

Microorganisms are located everywhere. Especially in the intestinal systems of living things, they perform many useful functions for the host. These beneficial bacteria are important in the treatment of cancer, cardiovascular disorders, obesity and diabetes, cholesterol and blood pressure, central nervous system disorders, immune system disorders and viral diseases. Especially with the use of metagenomic techniques, the importance of intestinal flora and probiotic microorganisms was understood, and probiotic microorganisms were used in many treatments. At certain stages of life, the proportion of beneficial microorganism may vary, especially due to nutrition and aging. It is possible to meet these useful microorganisms from fermented nutrients and probiotic products sold commercially. Adequate consumption of live probiotics proves beneficial to human health and well-being because they are useful in the treatment or prevention of many kinds of health problems. In recent years, the use of commercial probiotics has increased for staying healthy and treating diseases due to public awareness. It is possible to access probiotic products from local markets and pharmacies. Therefore, the variety and market of commercial probiotic products continues to grow. In this chapter, general information about the isolation, sources, health effects, and market of probiotics is presented.

Introduction

The concept that microorganisms are beneficial can be difficult to understand because some microorganisms are pathogenic. Currently, some are referred to as friendly, beneficial, or healthy microorganism and they are commonly employed in meals, beverages, and supplements to maintain the health of the gut and immune system. Nowadays, research is supporting these findings and suggesting further benefits (Peter et al., 2022; Singh et al., 2022). Beneficial microbes known as probiotics appear here.

The consumption of living microorganisms known as "probiotics" for health and well-being is expanding over the world (Singh et al., 2022). *Lactobacillus* and *Bifidobacterium* are the most commonly known species in probiotic study and production. *Bacillus* spp., *Enterococci*, *Escherichia coli*, *Weissella* spp., and *Saccharomyces* are among the additional species (El Hage et al., 2017).

The complexity of the human microbiota and its important effects on human health have increased interest in probiotics. Numerous studies have associated the composition of the gut microbiota with diseases and clinical conditions (Flemer et al., 2018; Gilbert et al., 2016; Kåhrström et al., 2016; Douillard and Vos, 2019). It is crucial to understand the structure and functional capabilities of the microbiome in health and disease, as well as the factors that regulate them (Shreiner et al., 2015). The microbiome-microbiota is a highly variable ecological systems that can be influenced by a variety of modifiable and non-modifiable factors, including genetics, exercise, age, geography, environment, nutrition, mode of birth, and medication treatments (Cuffaro et al. 2021). Nutrition is one of the most important modifiable factors influencing microbial diversity, microbiome and microbiota composition, stability, and, depending on certain parameters, therapeutic potential (Leeming et al., 2019). At this stage, probiotics, probiotic metabolites, and probiotic regulators all play crucial roles (Ağagündüz et al., 2022).

The market for functional foods has shown a rapid development in the "probiotic food" category in recent years. It has living colonies of intestinal bacteria that can colonize the digestive tract and influence several bodily metabolic processes, hence enhancing health or curing illnesses (Sionek et al., 2023). In order to develop novel probiotic products and enhance consumer health, it is necessary to isolate probiotic microbes from food and other sources.

Probiotic selection criteria

To improve human health, probiotics are avaliable as lyophilized capsules and food products. The majority of probiotic strains from the Lactobacilli and Bifidobacterium genera have been granted Generally Regarded as Safe (GRAS) status in the United States and European Food Safety Authority (EFSA) safety certification. Probiotics utilized in various food sources are frequently classified into three categories based on their multifactorial selection criteria: having acidic stomach resistance, the ability to bind to the gastrointestinal mucosa, and the ability to activate the immune system (Collins et al., 1998; Gueimonde and Sanchez, 2012; Peivasteh-Roudsari et al., 2019; Cristofori et al., 2023).

Probiotic microorganisms ought to preferably be derived from humans, be acid and bile tolerant, adhere to GIT membranes, prevent the growth of pathogenic bacteria, and have a safe for human consumption. The selection

criteria for probiotics are: 1) To provide benefit to the host; 2) To be able to pass through the intestines; 3) To adhere to the intestinal epithelial cell membrane; 4) To produce antibiotic compounds against pathogens; 5) To be able to stabilize the intestinal microbiota; 6) Not to break down bile salts in the small intestine; 7) Not to transfer antibiotic resistance genes; 8) To be isolated from healthy human gastrointestinal systems; and 9) Not to have any history of pathogenicity (de Melo Pereira et al., 2018; Peivasteh-Roudsari et al., 2019; Tegegne and Kebede, 2022). Probiotic bacteria should not be affected by pH, oxygen, and temperature changes throughout the digestive tract and also should be resistant to enzymes and bacteriophages (de Melo Pereira et al., 2018).

The use of probiotics to improve human health is increasing. So far, lactic acid bacteria isolated from the human gastrointestinal tract have been suggested as probiotics for human use. However, probiotics can also be obtained from fermented animal and plant sources. Traditional fermented products contain a variety of bacteria that may have probiotic properties (Table 1).

Probiotics can be isolated from different sources, such as traditional and non-traditional sources (Sornplang and Piyadeatsoontorn, 2016). One of the reasons why alternative sources are gaining popularity in choosing probiotics is that they are beneficial for individuals with lactose intolerance. Non-traditional sources of microorganisms can be used to isolate potential probiotics from a variety of sources, such as extra-intestinal sources and non-dairy fermented food products (Ramirez-Chavarin et al., 2013; Sornplang and Piyadeatsoontorn, 2016). It is a general assumption that consuming fermented products rich in lactic acid bacteria (LAB), such as yogurt, kefir, pickles, tempeh, and kombucha, has health benefits (Jan, 2024). The development of food fermentation and preservation methods has helped domesticate some of these bacterial species (Douglas and Klaenhammer, 2010).

Tablo 1. Sources of probiotic microorganisms in fermented foods

Name -Region	Microorganisms	References
Kefir Caucasus	*Lactobacillus kefiri, L. paracasei, L. parabuchneri, L. casei, L. lactis, Lactococcus lactis, Acetobacter lovaniensis, Kluyveromyces lactis, Saccharomyces cerevisiae*	Walsh et al., 2016
Kombucha China	*Komagataeibacter xylinus, S. cerevisiae, Zygosaccharomyces bailii, Brettanomyces bruxellensis, Acetobacter pasteurianus, A. aceti, A. xylinum, Gluconacetobacter*	Sanwal et al., 2023
Sauerkraut China	*Leuconostoc mesenteroides, L. plantarum, L. brevis, Enterococcus faecalis, Pediococcus cerevisiae, Rhizopus microsporus*	Zabat et al., 2018
Tempeh Indonesia	*Lactobacillus fermentum, L. plantarum, L. paracasei, L. delbrueckii, L. agilis, L. mucosae, L. paralimentarius, L. zeae, Lactococcus sp, Weisella confusa, W. paramesenteroides, Enterococcus faecalis, E. faecium, Pediococcus pentosaceus*	Virgianti and Suhartati, 2023.
Natto Japan	*Bacillus subtilis* natto	Leñini et al., 2023
Miso Japan	*Enterococcus faecium, Bacillus subtilis, L. plantarum, Leuconstoc citreum, Weissella cibaria, Weissella confuse, Pediococcus acidolactici, Pediococcus pentosaceus, Zygosaccharomyces rouxii*	Allwood et al., 2021
Koji Japan	*Wickerhamomyces anomalus, Akkermansia muciniphilia, Bacteroides dorei, L. delbruekii, L. pentosus, Leu. mesenteroides, Mycobacterium massiliense, Obesumbacterium proteus, Ochrobactrum lupini*	Allwood et al., 2021
Kimchi Korea	*Leu. mesenteroides, L. plantarum, Weissella confusa*	Hwang and Lee, 2019
Tarhana Türkiye	*L. casei, L. plantarum, L. brevis*	Abbas Ahmadi et al., 2014
Bozo Balkan and Asian countries	*L. salivarius, L. sakei, L. maltaromicus, L. fermentum, L. parabuchneri, L. paracasei subsp. paracase, Weissella confusa, Leuconostoc lactis, Leu. amelibiosum, Leu. mesenteroides subsp. Dextranicum, Leu. pseuclomesenteroides, Saccharomyces cerevisiae, Pichia membranifaciens, Dekkera bruxellensis, Nadsonici commutata*	Moncheva et al., 2003

Sources of Probiotics

Another source of probiotics in humans is the gastrointestinal system. There are more than a thousand different types of microorganisms in the human GIT. The majority of probiotic strains utilized now really originate from this source (Fontana et al. 2013).

Breast milk and colostrum are among the greatest settings in which probiotics can be isolated. In part, this is because human milk contains good bacteria that contribute to the health benefits of breastfeeding for newborns (Damaceno et al., 2023). Contrary to popular belief, breast milk contains a variety of microorganisms (over 10^4 cfu/mL) that influence the microbiome of the newborn. Breast milk contains a very complex and diversified bacterial community such as *Actinomyces*, *Bifidobacterium*, *Enterococcus*, *Lactobacillus*, *Propionibacterium*, *Staphylococcus* and *Streptococcus* (Ruiz et al. 2019; Liu et al., 2020). However, a number of bacteria present in a mother's intestines can endogenously pass into the mammary gland (Liu et al., 2020). This demonstrates the importance of the mother's gut microbiota for both the mother and the child.

Probiotics are a significant source of bioactive compounds

Probiotics are valuable suppliers of numerous bioactive substances. Two of these bioactive substances that are produced by different bacteria are proteinaceous toxins called enterotoxins and bacteriocins (Yadav and Tiwari, 2023; Wu et al., 2023). Due to their specificity, they are non-toxic, prevent infections from spreading to other cells, and are eventually being developed into antibiotics. LAB bacteriocins have bactericidal and bacteriostatic effects and are extremely selective to their target species, and do not harm other bacteria. Consequently, the concurrent administration of antibiotics and bacteriocins is becoming more and more viable as a therapeutic approach (Meade et al., 2020; Soltani et al., 2021). Species in the LAB group can create several kinds of organic acids and are part of the hetero-fermentative group. Increased death results from the generated organic acids' effect on the outer surface of pathogenic bacteria and inhibition of their ability to synthesize proteins. Several LAB-groups are able to produce H_2O_2, which works as a harmful oxidation mechanism to prevent harmful bacteria (Mitchell et al., 2015).

Probiotic bacteria are able to secrete exopolysaccharides (EPSs) in large quantities. It has been demonstrated that EPS can decrease blood cholesterol, stimulate the immune system, and have anticancer and antioxidant properties (Ates, 2015; Lee et al., 2022). Probiotics and other intestinal-dwelling bacteria convert non-digestible oligosaccharides into monosaccharides through fermentation, hence supplying the necessary resources for the gut microflora to proliferate (Patel and Goyal, 2012). The immunoglobulin-A (IgA) level is raised by dietary oligosaccharides and naturally contributes to the host's defense.

Numerous vital enzymes, including lactase, amylase, esterase, lipases, and proteases, can be found in probiotics. Lactose is hydrolyzed by lactase to produce glucose and galactose, which are then processed to produce short-chain fatty acids (SCFAs) (Hill et al., 2018). LAB decreases lactose intolerance by producing β-galactosidase (Hill et al., 2018). Similarly, enzymes produced by probiotics (amylase and peptidase) profoundly affect the host's biochemical response, altering its metabolism. Probiotic bacteria can convert amino acids into aldehydes, alcohols and acids. A large number of LAB use casein proteolysis to generate short peptides and amino acids (Rowland et al., 2018).

Numerous probiotic bacteria generate B-group vitamins that are soluble in fat and water. While fat-dissolvable vitamins are consumed as micelles in the digestive system, water-soluble vitamins are absorbed in the intestine. They are necessary for the metabolism of fats, carbohydrates, amino acids, and nucleic acids (Ravisankar et al., 2015). Riboflavin production has been demonstrated by numerous strains of *E. coli* and *Bacillus* (Lin et al., 2014). In a similar vein, certain strains of *Bifidobacteria* generate B-complex vitamins, which support the preservation of the gut's general health (Markowiak and Śliżewska, 2017). Vitamin B12, which is necessary for the nervous system and blood but is not produced by plants, is also synthesized by some bacteria. The cobalamin can be produced by the *L. reuteri* LAB probiotic strain. In addition, propionic acid, vitamin B12, and other beneficial metabolites for industrial use are produced by *Propionibacterium shermani*. Also, *Bifidobacteria* is a thiamine producer (Markowiak and Śliżewska, 2017).

Table 2. Probiotic bacteria's bioactive components and their advantages for health

Probiotic	**Bioactive compounds**	**Impact on health**	**References**
Lactic acid bacteria (LAB)	Bacteriosins	The suppression of pathogens, serves as molecules for signaling	Yadav and Tiwari, 2023
Enterococcus faecium, E. faecalis	Enterocins	Bacteriostatic activity	Wu et al., 2023
LAB	EPS	Stimulate an immunostimulatory response, anticancer, antioxidant action, reduce blood cholesterol, gut adhesion capability	Ates, 2015; Lee et al., 2022
L. crispatus	Lactic acid	Inhibition of bacterial vaginosis, herpes simplex virus 2 and HIV-1 inactivation in vajina, immunomodulatory properties	Tachedjian et al., 2017
F. prausnitzii, Roseburia, Butyrici coccus pullicaecorum Clostridium	Butyric acid	Colonocytes' energy source, with anti-inflammatory and anti-tumor characteristics, heart diseases, preventing and treating distal ulcerative colitis, Crohn's disease, increases the synthesis of mucin.	Geirnaert et al., 2017; Gill et al., 2018; Markowiak-Kopeć and Śliżewska, 2020
Propionibacterium	Propionic acid	Contributes to gluconeogenesis, antimicrobial, anti-inflammatory	Ranaei et al., 2020
Bifidobacterium	Acetic acid	Contributes to the production of cholesterol and antimicrobial activity	Vorobjeva et al., 2008; Arora and Sharma, 2011
Bacteroides, Eubacterium hallii, Clostridium barlettii	Amino acids (phenylalanin e, tyrosine, tryptophan, arginine)	Provides the host with essential amino acids, nutrients for growth and supports the reproductive system.	
Bacteroidetes, Fusobacteria, Proteobacteria, Firmicutes, Actinobacteria	Vitamins (K, B1, B2, B3, B5, B6, B7, B9, B12)	Energy, amino acid and steroid metabolism	Hill, 1997; Rowland et al., 2018
Probioic microorganisms	Lactase, β-galactosidase, amylase, peptidase	SCFAs are produced from carbon sources by lactase. The sensitivity to lactose is decreased by β-galactosidase. Amylase and peptidase regulate metabolism.	Hill et al., 2018

Undigested carbohydrates are fermented by intestinal bacteria to produce energy. Bacteria also convert undigested sugars in the gut into SCFAs such as propionate (C3), butyrate (C4), and acetate (C2). These SCFAs influence lipid, glucose, and cholesterol metabolism in various tissues. It shows that SCFAs are transported from the intestines into the host bloodstream and serve as substrates or signaling molecules in organs (Den Besten et al., 2013). SCFAs are directly related to colon cancer, cardiovascular diseases, obesity, type 2 diabetes, and immune system cells. For example, acetate is linked to neuropeptides that regulate appetite control (Qin et al., 2012; Frost et al., 2014). SCFAs are metabolites of certain bacterial taxa of the gut microbiota and their production is particularly influenced by nutrients non-digestible polysaccharides (Fusco et al., 2023). While numerous bacteria have the ability to produce acetate, only a few bacteria are typically able to make propionate and butyrate (Reichardt et al., 2014).

The Use of Probiotics in Health

Probiotics have been reported to improve in a number of illnesses linked to an imbalanced gut microbiota. Because of enhanced beta-galactosidase activity, consuming yogurt containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus* is beneficial in decreasing lactose intolerance (Kechagia et al., 2013). According to Roškar et al. (2017), the symptoms of diarrhea and flatulence were greatly reduced by *Bifidobacterium animalis* and *L. plantarum*. *L. casei* and *L. rhamnosus* are highly effective in preventing diarrhea associated with *Clostridium difficile* overgrowth (Blaabjerg et al., 2017; Alberda et al. 2018). According to Sniffen et al. (2018), *Saccharomyces boulardii* and *L. rhamnosus* are effective against diarrhea. *L. gasseri* isolated from breast milk reduced the symptoms of diarrhea and irritable bowel syndrome (IBS) (Kim et al., 2018). *H. pylori*, the causal agent of peptic ulcers and chronic gastritis, can be inhibited by *S. boulardii*, *L. johnsonii*, and *L. reuteri* (Homan and Orel, 2015).

Respiratory tract infection (RTI) is significantly reduced by *L. rhamnosus* (Laursen and Hojsak (2018). Probiotic yogurt supplemented with *L. paracasei* has been shown to reduce RTI by enhancing T cells, which regulate the body's immunological system (Pu et al., 2017).

High levels of probiotic colonization of the vagina with *L. crispatus* have been linked to a noteworthy decrease in recurrent **urinary tract infections** (UTIs) (Stapleton et al., 2011). It was shown that oral probiotics

(18 months of use) with a probiotic combination of *Lactobacillus* and *Bifidobacterium* reduced the risk of UTI recurrence (Wolff et al., 2019).

According to numerous studies, there is a correlation between increased intestinal prevalence of Enterobacteriaceae and decreased numbers of *Bifidobacterium* and *Lactobacillus* spp. in patients with **chronic kidney disease** (CKD). Over a period of six months, oral administration of *Lactobacillus* and *Bifidobacterium* spp. dramatically decreased serum endotoxins and proinflammatory cytokines (TNF-α, IL-6, and IL-5), while increasing anti-inflammatory cytokine IL-10 (Sircana et al., 2019).

Regular usage of probiotics such as *Lactobacillus* and *Bifidobacterium* spp. successfully prevents the growth of *Streptococcus mutants* (Hasslöf and Stecksén-Blicks, 2020). It has been demonstrated that long-term daily consumption of milk enriched with the probiotic *L. rhamnosus* reduces the development of **dental caries** in preschoolers (Rodríguez et al., 2016).

The gut microbiota plays an important role in the progression of **osteoporosis** because one of the primary causes of osteoporosis is the imbalance in the GIT microbiota, especially due to an increase in the populations of Firmicutes, Proteobacteria and Bacteroidetes (Aponte et al., 2020). Specifically, *L. acidophilus* and *L. casei* have been documented to inhibit inflammatory biomarkers of osteoporosis (TNF-α, IL-17, and nuclear factor kappa-B ligand) (Collins et al. 2018; Montazeri-Najafabadyt al., 2019).

Probiotics also affect **autoimmune diseases** (Davidson and Diamond, 2010). According to Yousefi et al. (2019), the gut microbiota can play a key role in controlling immune responses and autoimmune illnesses through a variety of mechanisms. Oral probiotic capsules (*L. acidophilus*, *L. casei*, and *B. bifidum*) have been shown to significantly reduce insulin levels, C-reactive protein levels, and joint disease (Zamani et al., 2016). Giving probiotic capsules (*L. acidophilus*, *L. casei*, *B. bifidum*, and *L. fermentum*) to multiple sclerosis patients reduced inflammatory indicators like C-reactive protein and plasma nitric oxide metabolites, as well as the levels of insulin in the blood and β-cell activity (Kouchaki et al., 2017).

Probiotics' ability to reduce inflammation may inhibit the growth of **cancer** through a number of different mechanisms, including elevated levels of immunoglobulin A (IgA) secretion, control over cyclooxygenase-2, and adjustments to nuclear factor kappa B (NF-kB) and interferon-gamma (IFN-

γ) (Shamekhi et al., 2020). Using *in vitro* models of liver, ovarian, breast, and colorectal cancer, the growth inhibition, antiproliferative, or antimetastatic effects of *L. rhamnous* on tumor cells have been thoroughly examined (Behzadi et al., 2017; Mendes et al., 2018).

Obesity is recognized as a worldwide problem caused by changes in eating habits and exercise routines, resulting in abnormal or excessive fat storage that may be detrimental to people's health (Liu et al., 2023). Compared to those of normal weight, obese people typically have lower counts of Bacteroidetes and greater levels of the phylum Firmicutes (Gomes et al., 2018). Supplementation with *B. adolescentis* has been found to reduce weight in rodents (Reichold et al., 2014). Dietary intake of *L. rhamnosus* for 8 weeks in obese mice had a reduced body weight and white adipose tissue (Lee et al., 2006). In both humans and mice, the mucin-degrading bacteria *Akkermansia muciniphila* exhibits anti-obesogenic properties (Dao et al., 2016). In a recent article, it was discovered that obese individuals had much higher amounts of *F. prausnitzii*, *P. copri*, and *C. aerofaciens* than non-obese patients did (Duan et al., 2021).

Lowering blood cholesterol levels significantly reduces the risk of cardiovascular disease, high blood pressure, atherosclerosis, heart attack, and stroke. Consequently, probiotic products may be able to help treat these conditions. Probiotics can lower cholesterol levels by deconjugating bile salts, and turning cholesterol into coprostanol (Nazir et al., 2018). Certain species, particularly *Lactobacillus* (Palaniyandi et al., 2020; Palani Kumar et al., 2021; Widodo et al., 2021) and *Bifidobacterium* (Cheng et al., 2021), as well as *Pediococcus pentosaceus* (Bhukya and Bhukya, 2023) and *Bacillus licheniformis* (He et al., 2021) are effective in lowering cholesterol.

Among non-transmissible diseases, **type 2 diabetes mellitus** (T2DM) ranks fourth in frequency. The most prevalent symptoms include frequent loss of weight, increased urination, thirst, and poor healing capacity for foot wounds. These conditions can eventually cause consequences like obesity, retinal degeneration, coronary heart disease, atherosclerosis, sexual impotence, and renal failure (Sun et al., 2020). The three main probiotics utilized for T2DM intervention are Bifidobacterium, Lactobacillus, and Lactococcus. It was discovered that while the amount of class Betaproteobacteria was significantly up and positively correlated with plasma glucose, the abundance of class Clostridia and phylum Firmicutes in T2DM patients had significantly decreased (Zhang et al., 2021). T2DM is frequently

associated with a drop-in butyrate and a decline in butyrate-producing bacteria, such as Faecalibacterium and Roseburia, which may be the main causes of T2DM. Allin et al. (2018) indicated that during the prediabetic period, abnormal changes in the gut microbiota occur, as evidenced by a significant decrease in the levels of *Clostridium* and *A. muciniphila* and an increase in the levels of *Dorea*, *Ruminococcus*, *Sutterella*, and *Streptococcus*. Improving the number of bacteria that produce butyrate or enhancing gut bacteria's capacity to synthesize butyrate may be useful strategies for treating or preventing diabetes (Zhang et al., 2021).

Nervous system diseases (Alzheimer's [AD], autism, depression, epilepsy, dementia, Parkinson's, migraine, and schizophrenia) characterized by a progressive loss of nerve tissue are known as neurological disorders (Aghamohammad et al., 2023). The two main processes that cause neurodegeneration are oxidation and inflammation, which both disrupt regular physiological processes (Westfall et al., 2017). *L. plantarum* has been demonstrated to increase mice's learning and memory functions. In addition, *L. plantarum* may synthesize acetylcholine, a neurotransmitter that is reduced in people with Alzheimer's, and decrease oxidative stress (Tamtaji et al., 2019). Through the metabolism of the amyloid precursor protein, *L. helveticus* can ameliorate memory impairments linked to AD (Chung et al., 2014). It has been also demonstrated that ALS symptoms get worse by *Ruminococcus torcs* and *Parabacteroides distasonis*, whereas motor symptoms are improved by *Akkermansia muciniphila* by the production of nicotinamide (Blacher et al., 2019).

Parkinson's disease (PD), a neurodegenerative illness that impairs motor function, is caused by dopaminergic neuron loss in the substantia nigra. *Helicobacter pylori*, *Enterococcus faecalis*, Desulfovibrio, *Nocardia asteroides, Proteus mirabilis, Porphyromonas gingivalis* and *Peptoniphilus* bacteria have been found in higher rates in people with PD (Fan et al., 2022). When Parkinson's patients were given probiotic capsules containing numerous strains (*E. faecium*, *L. acidophilus*, *L. paracasei*, *L. rhamnosus*, *B. longum*, *B. bifidum*, and *L. reuteri*), their quality of life improved (Tan et al., 2021).

Psychobiotics are a new form of probiotic that describes live organisms with beneficial effects on **mental health**. Probiotics are a significant supportive agent in the treatment of autism spectrum disorders (ASD). Bacteria in the gut can produce gamma-aminobutyric acid (GABA), 5-hydroxytryptamine (5-HT), dopamine, and SCFAs, all of which have an effect

on the neurological system (Fattorusso et al., 2019). A high-fat diet consumed by the mother during pregnancy changes the microbiome in newborns and may be linked to ASD in people (Connolly et al., 2016). If breastfeeding is sustained for six months, there is a decreased incidence of ASD; in contrast, infants who are formula-fed exhibit a higher gut concentration of *Clostridium difficile* (Schultz et al., 2006). De Angelis et al. (2015) reported that whereas butyric acid levels were lower in children with ASD, propionic and acetic acid levels were greater. It has been reported that parobiotics such as *L. plantarum* (Parracho et al., 2010), *L. acidophilus* (Kałużna-Czaplińska et al., 2012), *L. rhamnosus* (Pärtty et al., 2015), *L. reuteri* (Buffington et al., 2016) can be used in the treatment of autism as well as anxiety and hyperactivity disorders.

Probiotics may reduce **migraine** headaches. A number of studies indicate that serotonin plays an important function in the gut-brain axis. But the central nervous system only contains 3% of an individual's total serotonin; the remainder is found in the gastrointestinal system, i.e. produced by probiotics. Therefore, treating sufferers of migraines by modifying gut function with appropriate probiotics may be a helpful strategy (Yu-Jie et al., 2017; Naghibi et al., 2019).

Globally, **viral infections** still result in a considerable amount of illness and mortality. It is becoming more and more clear that the GIS microbiome acts a significant role in the immune system of the host, regulating immunity and host defense mechanisms (Wang et al., 2022). Various probiotic bacteria have direct or indirect antiviral effects. These mechanisms are the protective function of the mucosa, secretion of antiviral peptides and bacteriocins, prevention of attachment of viruses to host cells, and strengthening of both innate and acquired immunity (Jounai et al., 2012; Schroeder, 2019; Harper et al., 2021). In this context, *L. rhamnosus* (Garcia-Castillo et al., 2020) and *L. gasseri* (Eguchi et al., 2019) against respiratory syncytial virus, *B. bifidum* (Mahooti et al., 2019) against H1N1 influenza virus, *L. plantarum* against herpes simplex virus-1 (Matsusaki et al., 2016), *Lacticaseibacillus rhamnosus* against rotavirus (Aoki-Yoshida et al., 2016), *Bifidobacterium adolescentis* against hepatitis B virus (Lee et al. 2013), *L. paracasei* against SARS-CoV-2 (Salaris et al., 2021) and *L. plantarum* against H1N1 influenza (Maeda et al., 2009) were determined to have antiviral properties.

Age-Related Microbiome Changes

Microbiota is quite variable and some groups of microorganisms may become dominant over time. Firmicutes and Bacteroidetes are two of the most significant bacterial groups in the GIS; others are Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia. The Firmicutes/Bacteroidetes (F/B) ratio is recognized to play a critical role in maintaining intestinal homeostasis. A high or low F/B ratio is considered dysbiosis (Stojanov et al., 2020).

It has been proposed that the F/B ratio is a significant indicator of the gut microbiota's health. Recent years have seen a significant increase in interest in Firmicutes and Bacteroidetes. Dysbiosis is defined as an increased or decreased F/B ratio; the former is typically associated with obesity, while the latter is associated with inflammatory bowel disease (IBD). When given to the host in sufficient quantities, probiotics, can improve their health. Probiotics' nutritional and immunosuppressive qualities are well-documented, as evidenced by studies explaining how they relate to obesity, IBD, and the F/B ratio. Probiotics taken orally may help prevent obesity and inflammatory bowel disease (IBD) as well as aid in the restoration of the dysbiotic microbiome (Stojanov et al., 2020).

The physiological and pathological processes of the host organism, including aging, are significantly influenced by the gut microbiota. It has been established that the composition of the microbiota changes dramatically over the course of a lifetime. The F/B ratio tends to rise with age in both sexes, from the 0–9 age group to the 60–69 age group. From childhood to old age, the relative abundance of the phyla Firmicutes and Actinobacteria increased whereas that of Bacteroidetes dropped. As a result, it has been demonstrated that the F/B ratio increases dramatically till old age (Vaiserman et al., 2020). Therefore, commercial probiotics may be useful in maintaining this balance in the gut.

Market

The GIS is the largest immune organ. Most of the body's immune cells—70–80%—are found in the GIT (Wiertsema et al., 2021). Dysbiosis, or imbalances in the gut microbiota, has been linked to a number of illnesses, including IBS, IBD, obesity, diabetes, allergies, and even mental health issues. Probiotics from dairy products may alter intestinal flora and aid in the

treatment or prevention of certain illnesses. SCFAs, vitamins, and other metabolites that support gut health can all be produced more effectively by them. Probiotics can increase immunity, decrease intestinal inflammation, and enhance the function of the gut barrier (Aziz et al., 2022).

Increased use of probiotics provides many health benefits on the host. They promote a healthy balance of intestinal flora, enhance immune system activity, facilitate digestion, and may have other positive effects on overall health. Fermented foods, which inherently contain live friendly microorganisms, are often included in a diet high in probiotics. Probiotic-rich foods include things like kefir, yogurt, sauerkraut, tempeh, kimchi, and some kinds of cheese. These foods go through fermentation methods promoting the development of particular beneficial bacterial strains. Additionally, probiotic capsules are offered, which contain concentrated doses of particular bacterial strains (Aziz et al., 2024).

Living bacteria known as probiotics can help an individual's health when take in sufficient quantities. There is currently an increase in the amount of commercial products containing probiotics on the market (Grumet et al., 2020). Studies have demonstrated that probiotics can both prevent and treat gastrointestinal and non-gastrointestinal illnesses (Mazziotta et al., 2023; Zaib et al., 2024; Liang et al., 2024). Recently, alternative probiotic candidates have been investigated for the therapy of inflammatory and metabolic illnesses. These include *Eubacterium hallii*, *Bacteroides fragilis*, *Faecalibacterium prausnitzii*, and *Akkermansia muciniphila*. Thanks to their production of SCFAs such butyrate, propionate, and acetate, these organisms aid in the maintenance of gut homeostasis (El Hage et al., 2017). One important butyrate-producing microbe in the human gut is *F. prausnitzii* (Lopez-Siles et al., 2017). *F. prausnitzii* produces butyrate, which inhibits the activation of the NF-κB transcription factor, hence reducing mucosal inflammation (Liu et al., 2017). Similar to this, *E. hallii* is a crucial propionate-producing bacterium that is present in the gut and controls important metabolites that are generated during the fermentation of glucose (Engels et al., 2016).

According to the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), probiotics are usually considered safe. Probiotics are recognized as food supplements, dietetics and pharmaceuticals in the Food Products Directive and Regulation in the European Union (Liang etg al., 2024).

Probiotic-based products (Table 3) are offered to the market in the form of fresh dairy products, capsules, tablets, powder and suppositories (Wang et al., 2022). Probiotics are frequently added to functional food as well as beverages in order to maximize their potential health advantages. The use of probiotics has increased sharply after the 2019 coronavirus pandemic, as people have become increasingly interested in them for boosting their immunity; among Chinese consumers, this growth was 108% year over year (Yoha et al., 2022). It is anticipated that the value of the global probiotics market would increase at a compound annual growth rate (CAGR) of about 8.3%, from $61.1 billion in 2021 to $91.1 billion in 2026 (Liang et al., 2023; Xu et al., 2024). As shown in the data above, probiotics have a lot of promise for use in healthcare applications, leading to an increase in the probiotic market.

Breast milk or donor human milk is one of the most important sources of probiotics. Recently, the number of human milk banks has increased in many countries (approximately 800 in the world, 282 in Europe). However, these are pasteurized before use and given to newborns or babies with insufficient breast milk (Fang et al., 2021). It is also recommended to give human milk to the elderly. Fresh or frozen breast (until 2 weeks) and donor milk is rich in probiotics. Therefore, fresh milk rich in probiotics should be used. Milk samples used or sold after pasteurization are also a rich source of prebiotics (Salcedo et al., 2015; Lackey et al., 2017; Lund et al., 2020).

Fecal microbiota transplantation (FMT) is a well-known practice worldwide used in the treatment of a variety of diseases, particularly *Clostridium difficile* infection (Grigoryan et al., 2020; Yang et al., 2023). Before being transplanted into the recipient, stool samples obtained from suitable donors need to be processed up and prepared (Biazzo & Deidda, 2022). Stool banks have been established to provide standardized screened donor stool suspensions or capsules and ensure safe use of FMT for patients (Van Lingen et al., 2020). This market was valued at $2,114.1 million in 2023 and is expected to reach $3,148.0 million by 2031 (https://www.thebrainyinsights.com/report/fecal-transplant-therapy-market-13247).

Table 3. Commercial products containing probiotics

Manufacture company	**Product form**	**Probiotic microorganisms**	**Country**
BioGaia - Gastrus	Chewable	*L. reuteri* ATCC PTA 6475, *L. reuteri* DSM 17938	Sweden
Danone - Dannon® Activia®	Yogurt	*Bifidobacterium animalis lactis* DN-173 010	French
Lifeway	Kefir	12 live / active cultures	USA
i-Health - Culturelle® M etabolism + Weight Management	Capsules	*Lactobacillus rhamnosus* GG, *L. fermentum*	Swiss-Dutch
i-Health - Culturelle® Abdominal Support	Capsules	*B. coagulans* Unique IS-2	Swiss-Dutch
Yakult - BF-1	Fermented Milk Drink	*B. bifidum* YIT 10347	Japan
PepsiCo - KEVITA Lemon Ginger Sparkling Probiotic	Probiotic drink	Lactic Acid, Kefir Culture, *Bacillus coagulans* Gbi-30 6086	USA
PepsiCo - KeVita Kombucha	Probiotic drink	Kombucha Culture, *Ba. coagulans* MTCC 5856	USA
Custom Probiotics - Six Strain Probiotic Powder	Powder	*L. acidophilus* LA-14, *L. salivarius* LS-33, *L. plantarum* LP-115, *L. rhamnosus* LR-32, *B. lactis* BL-04, *B. bifidum* Bb-06	USA
ProbioFerm	Powder, Capsule	Probiotic species (*Lactobacillus*, *Bifidobacterium*, *Enterococcus faecium*, *Pediococcus*)	USA
Bio-Kult	Capsule	*S. boulardii*	UK
Vitakem-Probiotic Blend	Capsule	*Bacillus subtilis*, *Ba. coagelens*, *B. bifidum*, *L. rhamnosus*, *L. acidophilus*, *L. casei*	USA
Tallinna - Hellus	Probiotic drink	*L. fermentum* ME-3	Estonia
Milkify Singles	Frozen	Breast Milk	USA
SER-109	Capsule	Fecal Microbiota	UK

Conclusions

Probiotics have gained popularity since the discovery of the relationship between nutrition and health. Recent studies have demonstrated that probiotics not only treat a variety of diseases, but also improve the immune system, indicating their promise in biotherapy. Probiotics have a variety of benefits, including antagonistic interactions with harmful microorganisms and immune-modulating properties against infections, cancers, and metabolic diseases.

Today, fast-food nutrition, intense pace and environmental degradation in modern society have caused an unhealthy life in society and encouraged the introduction of alternative healthy products such as probiotics to the market. Probiotics have been recommended by experts as health-improving products for various reasons, from strengthening the intestinal barrier function to their demonstrated advantages in establishing the microbiome-brain-gut axis. Probiotics are widely recognized and accepted as a solution with claims to alleviate or treat a wide range of conditions, from diarrhea to obesity and depression. However, many studies have been conducted recently to confirm these ideas. In the future, probiotic supplements and medicines are expected to be seen as real companions for individuals. Studies are planned for the use of well-defined, therapeutic probiotic strains that are suitable for each individual's microbiota structure. If all of these challenges are resolved and evidence-based health benefits are supplied, probiotics will be a genuinely potent tool in the battle against disease.

REFERENCES

Abbas Ahmadi, M., Tajabadi Ebrahimi, M., Mehrabian, S., Tafvizi, F., Bahrami, H., & Dameshghian, M. (2014). Antimutagenic and anticancer effects of lactic acid bacteria isolated from Tarhana through Ames test and phylogenetic analysis by 16S rDNA. *Nutrition and Cancer*, *66*(8), 1406-1413.

Aghamohammad, S., Hafezi, A., & Rohani, M. (2023). Probiotics as functional foods: How probiotics can alleviate the symptoms of neurological disabilities. *Biomedicine & Pharmacotherapy*, *163*, 114816.

Ağagündüz, D., Gençer Bingöl, F., Çelik, E., Cemali, Ö., Özenir, Ç., Özoğul, F., & Capasso, R. (2022). Recent developments in the probiotics as live biotherapeutic products (LBPs) as modulators of gut brain axis related neurological conditions. *Journal of Translational Medicine*, *20*(1), 460.

Allin, K. H., Tremaroli, V., Caesar, R., Jensen, B. A., Damgaard, M. T., Bahl, M. I., ... & Pedersen, O. (2018). Aberrant intestinal microbiota in individuals with prediabetes. *Diabetologia*, *61*, 810-820.

Allwood, J. G., Wakeling, L. T., & Bean, D. C. (2021). Fermentation and the microbial community of Japanese koji and miso: A review. *Journal of Food Science*, *86*(6), 2194-2207.

Aoki-Yoshida, A., Saito, S., Fukiya, S., Aoki, R., Takayama, Y., Suzuki, C., & Sonoyama, K. (2016). *Lactobacillus rhamnosus* GG increases Toll-like receptor 3 gene expression in murine small intestine ex vivo and in vivo. *Beneficial Microbes*, *7*(3), 421-429.

Aponte, M., Murru, N., & Shoukat, M. (2020). Therapeutic, prophylactic, and functional use of probiotics: a current perspective. *Frontiers in Microbiology*, *11*, 562048.

Arora, T., & Sharma, R. (2011). Fermentation potential of the gut microbiome: implications for energy homeostasis and weight management. *Nutrition Reviews*, *69*(2), 99-106.

Ates, O. (2015). Systems biology of microbial exopolysaccharides production. *Frontiers in Bioengineering and Biotechnology*, *3*, 200.

Aziz, T., Hussain, N., Hameed, Z., & Lin, L. (2024). Elucidating the role of diet in maintaining gut health to reduce the risk of obesity, cardiovascular and other age-related inflammatory diseases: Recent challenges and future recommendations. *Gut Microbes*, *16*(1), 2297864.

Aziz, T., Sarwar, A., Naveed, M., Shahzad, M., Shabbir, M. A., Dablool, A. S., ... & Lin, L. (2022). Bio-Molecular analysis of selected food derived Lactiplantibacillus strains for CLA production reveals possibly a complex mechanism. *Food Research International*, *154*, 111031.

Bhukya, K. K., & Bhukya, B. (2023). Exploration of antidiabetic, cholesterol-lowering, and anticancer upshot of probiotic bacterium *Pediococcus pentosaceus* OBK05 Strain of Buttermilk. *Probiotics and Antimicrobial Proteins*, *15*(6), 1484-1500.

Biazzo, M., & Deidda, G. (2022). Fecal microbiota transplantation as new therapeutic avenue for human diseases. *Journal of Clinical Medicine*, *11*(14), 4119.

Blacher, E., Bashiardes, S., Shapiro, H., Rothschild, D., Mor, U., Dori-Bachash, M., ... & Elinav, E. (2019). Potential roles of gut microbiome and metabolites in modulating ALS in mice. *Nature*, *572*(7770), 474-480.

Buffington, S. A., Di Prisco, G. V., Auchtung, T. A., Ajami, N. J., Petrosino, J. F., & Costa-Mattioli, M. (2016). Microbial reconstitution reverses maternal diet-induced social and synaptic deficits in offspring. *Cell*, *165*(7), 1762-1775.

Cheng, J., Laitila, A., & Ouwehand, A. C. (2021). *Bifidobacterium animalis subsp. lactis* HN019 effects on gut health: a review. *Frontiers in Nutrition*, *8*, 790561.

Chung, Y. C., Jin, H. M., Cui, Y., Jung, J. M., Park, J. I., Jung, E. S., ... & Chae, S. W. (2014). Fermented milk of *Lactobacillus helveticus* IDCC3801 improves cognitive functioning during cognitive fatigue tests in healthy older adults. *Journal of Functional Foods*, *10*, 465-474.

Collins, J. K., Thornton, G., & Sullivan, G. O. (1998). Selection of probiotic strains for human applications. *International Dairy Journal*, *8*, 487-490.

Connolly, N., Anixt, J., Manning, P., Ping-I Lin, D., Marsolo, K. A., & Bowers, K. (2016). Maternal metabolic risk factors for autism spectrum disorder—an analysis of electronic medical records and linked birth data. *Autism Research*, *9*(8), 829-837.

Cristofori, F., Dargenio, V. N., Dargenio, C., Miniello, V. L., Barone, M., & Francavilla, R. (2021). Anti-inflammatory and immunomodulatory effects of probiotics in gut inflammation: a door to the body. *Frontiers in Immunology*, *12*, 578386.

Cuffaro, B., Assohoun, A. L., Boutillier, D., Peucelle, V., Desramaut, J., Boudebbouze, S., ... & Maguin, E. (2021). Identification of new potential biotherapeutics from human gut microbiota-derived bacteria. *Microorganisms*, *9*(3), 565.

Damaceno, Q. S., Gallotti, B., Reis, I. M., Totte, Y. C., Assis, G. B., Figueiredo, H. C., ... & Martins, F. S. (2023). Isolation and identification of potential probiotic bacteria from human milk. *Probiotics and Antimicrobial Proteins*, *15*(3), 491-501.

Dao, M. C., Everard, A., Aron-Wisnewsky, J., Sokolovska, N., Prifti, E., Verger, E. O., ... & MICRO-Obes Consortium. (2016). *Akkermansia muciniphila* and improved metabolic health during a dietary intervention in obesity: relationship with gut microbiome richness and ecology. *Gut*, *65*(3), 426-436.

De Angelis, M., Francavilla, R., Piccolo, M., De Giacomo, A., & Gobbetti, M. (2015). Autism spectrum disorders and intestinal microbiota. *Gut Microbes*, *6*(3), 207-213.

de Melo Pereira, G. V., de Oliveira Coelho, B., Júnior, A. I. M., Thomaz-Soccol, V., & Soccol, C. R. (2018). How to select a probiotic? A review and update of methods and criteria. *Biotechnology Advances*, *36*(8), 2060-2076.

Den Besten, G., Van Eunen, K., Groen, A. K., Venema, K., Reijngoud, D. J., & Bakker, B. M. (2013). The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *Journal of Lipid Research*, *54*(9), 2325-2340.

Douillard, F. P., & de Vos, W. M. (2019). Biotechnology of health-promoting bacteria. *Biotechnology Advances*, *37*(6), 107369.

Duan, M., Wang, Y., Zhang, Q., Zou, R., Guo, M., & Zheng, H. (2021). Characteristics of gut microbiota in people with obesity. *Plos One*, *16*(8), e0255446.

Eguchi, K., Fujitani, N., Nakagawa, H., & Miyazaki, T. (2019). Prevention of respiratory syncytial virus infection with probiotic lactic acid bacterium *Lactobacillus gasseri* SBT2055. *Scientific Reports*, *9*(1), 4812.

El Hage, R., Hernandez-Sanabria, E., & Van de Wiele, T. (2017). Emerging trends in "smart probiotics": functional consideration for the development of novel health and industrial applications. *Frontiers in Microbiology*, *8*, 1889.

Fan, H. X., Sheng, S., & Zhang, F. (2022). New hope for Parkinson's disease treatment: Targeting gut microbiota. *CNS Neuroscience & Therapeutics*, *28*(11), 1675-1688.

Fang, M. T., Chatzixiros, E., Grummer-Strawn, L., Engmann, C., Israel-Ballard, K., Mansen, K., ... & Biller-Andorno, N. (2021). Developing global guidance on human milk banking. *Bulletin of the World Health Organization*, *99*(12), 892.

Fattorusso, A., Di Genova, L., Dell'Isola, G. B., Mencaroni, E., & Esposito, S. (2019). Autism spectrum disorders and the gut microbiota. *Nutrients*, *11*(3), 521.

Flemer, B., Warren, R. D., Barrett, M. P., Cisek, K., Das, A., Jeffery, I. B., ... & Paul, W. T. (2018). The oral microbiota in colorectal cancer is distinctive and predictive. *Gut*, *67*(8), 1454-1463.

Fontana, L., Bermudez-Brito, M., Plaza-Diaz, J., Munoz-Quezada, S., & Gil, A. (2013). Sources, isolation, characterisation and evaluation of probiotics. *British Journal of Nutrition*, *109*(S2), S35-S50.

Frost, G., Sleeth, M. L., Sahuri-Arisoylu, M., Lizarbe, B., Cerdan, S., Brody, L., ... & Bell, J. D. (2014). The short-chain fatty acid acetate reduces appetite via a central homeostatic mechanism. *Nature Communications*, *5*(1), 3611.

Fusco, W., Lorenzo, M. B., Cintoni, M., Porcari, S., Rinninella, E., Kaitsas, F., ... & Ianiro, G. (2023). Short-chain fatty-acid-producing bacteria: key components of the human gut microbiota. *Nutrients*, *15*(9), 2211.

Garcia-Castillo, V., Tomokiyo, M., Raya Tonetti, F., Islam, M. A., Takahashi, H., Kitazawa, H., & Villena, J. (2020). Alveolar macrophages are key players in the modulation of the respiratory antiviral immunity induced by orally administered *Lacticaseibacillus rhamnosus* CRL1505. *Frontiers in Immunology*, *11*, 568636.

Geirnaert A, Calatayud M, Grootaert C, Laukens D, Devriese S, Smagghe G, De Vos M, Boon N, Van de Wiele T (2017) Butyrate-producing bacteria supplemented

in vitro to Crohn's disease patient microbiota increased butyrate production and enhanced intestinal epithelial barrier integrity. *Scientific Reports*, *7*, 11450.

Gilbert, J. A., Quinn, R. A., Debelius, J., Xu, Z. Z., Morton, J., Garg, N., ... & Knight, R. (2016). Microbiome-wide association studies link dynamic microbial consortia to disease. *Nature*, *535*(7610), 94-103.

Gill, P. A., Van Zelm, M. C., Muir, J. G., & Gibson, P. R. (2018). Short chain fatty acids as potential therapeutic agents in human gastrointestinal and inflammatory disorders. *Alimentary Pharmacology & Therapeutics*, *48*(1), 15-34.

Gomes, A. C., Hoffmann, C., & Mota, J. F. (2018). The human gut microbiota: Metabolism and perspective in obesity. *Gut Microbes*, *9*(4), 308-325.

Grigoryan, Z., Shen, M. J., Twardus, S. W., Beuttler, M. M., Chen, L. A., & Bateman-House, A. (2020). Fecal microbiota transplantation: Uses, questions, and ethics. *Medicine in Microecology*, *6*, 100027.

Grumet, L., Tromp, Y., & Stiegelbauer, V. (2020). The development of high-quality multispecies probiotic formulations: from bench to market. *Nutrients*, *12*(8), 2453.

Gueimonde, M., & Sanchez, B. (2012). Enhancing probiotic stability in industrial processes. *Microbial Ecology in Health and Disease*, *23*(1), 18562.

Harper, A., Vijayakumar, V., Ouwehand, A. C., Ter Haar, J., Obis, D., Espadaler, J., ... & Day, R. (2021). Viral infections, the microbiome, and probiotics. *Frontiers in Cellular and Infection Microbiology*, *10*, 596166.

He, Q., Li, J., Ma, Y., Chen, Q., & Chen, G. (2021). Probiotic potential and cholesterol-lowering capabilities of bacterial strains isolated from pericarpium citri reticulatae 'chachiensis'. *Microorganisms*, *9*(6), 1224.

Hill, D., Sugrue, I., Tobin, C., Hill, C., Stanton, C., & Ross, R. P. (2018). The Lactobacillus casei group: history and health related applications. *Frontiers in Microbiology*, *9*, 2107.

Hill, M. J. (1997). Intestinal flora and endogenous vitamin synthesis. *European Journal of Cancer Prevention*, *6*(2), S43-S45.

https://www.thebrainyinsights.com/report/fecal-transplant-therapy-market-13247

Hwang, H., & Lee, J. H. (2019). Evaluation of metabolites derived from lactic acid bacteria isolated from kimchi. In *Chemistry of Korean Foods and Beverages* (pp. 3-10). American Chemical Society.

Jan, T., Negi, R., Sharma, B., Kumar, S., Singh, S., Rai, A. K., ... & Ahmed, N. (2024). Next generation probiotics for human health: An emerging perspective. *Heliyon*.

Jounai, K., Ikado, K., Sugimura, T., Ano, Y., Braun, J., & Fujiwara, D. (2012). Spherical lactic acid bacteria activate plasmacytoid dendritic cells immunomodulatory function via TLR9-dependent crosstalk with myeloid dendritic cells. *PloS One*, *7*(4), e32588.

Kåhrström, C. T., Pariente, N., & Weiss, U. (2016). Intestinal microbiota in health and disease. *Nature*, *535*(7610), 47.

Kałużna-Czaplińska, J., & Błaszczyk, S. (2012). The level of arabinitol in autistic children after probiotic therapy. *Nutrition*, *28*(2), 124-126.

Lackey, K. A., Williams, J. E., Price, W. J., Carrothers, J. M., Brooker, S. L., Shafii, B., ... & McGuire, M. K. (2017). Comparison of commercially-available preservatives for maintaining the integrity of bacterial DNA in human milk. *Journal of Microbiological Methods*, *141*, 73-81.

Lee, M. G., Joeng, H., Shin, J., Kim, S., Lee, C., Song, Y., ... & Park, Y. S. (2022). Potential probiotic properties of exopolysaccharide-producing *Lacticaseibacillus paracasei* EPS DA-BACS and prebiotic activity of its exopolysaccharide. *Microorganisms*, *10*(12), 2431.

Lee, D. K., Kang, J. Y., Shin, H. S., Park, I. H., & Ha, N. J. (2013). Antiviral activity of *Bifidobacterium adolescentis* SPM0212 against Hepatitis B virus. *Archives of Pharmacal Research*, *36*, 1525-1532.

Lee, H. Y., Park, J. H., Seok, S. H., Baek, M. W., Kim, D. J., Lee, K. E., ... & Park, J. H. (2006). Human originated bacteria, *Lactobacillus rhamnosus* PL60, produce conjugated linoleic acid and show anti-obesity effects in diet-induced obese mice. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, *1761*(7), 736-744.

Leeming, E. R., Johnson, A. J., Spector, T. D., & Le Roy, C. I. (2019). Effect of diet on the gut microbiota: rethinking intervention duration. *Nutrients*, *11*(12), 2862.

Leñini, C., Rodriguez Ayala, F., Goñi, A. J., Rateni, L., Nakamura, A., & Grau, R. R. (2023). Probiotic properties of *Bacillus subtilis* DG101 isolated from the traditional Japanese fermented food nattō. *Frontiers in Microbiology*, *14*, 1253480.

Li, Y., Liu, T., Qin, L., & Wu, L. (2023). Effects of probiotic administration on overweight or obese children: a meta-analysis and systematic review. *Journal of Translational Medicine*, *21*(1), 525.

Liang, D., Wu, F., Zhou, D., Tan, B., & Chen, T. (2024). Commercial probiotic products in public health: Current status and potential limitations. *Critical Reviews in Food Science and Nutrition*, *64*(19), 6455-6476.

Lin, Z., Xu, Z., Li, Y., Wang, Z., Chen, T., & Zhao, X. (2014). Metabolic engineering of *Escherichia coli* for the production of riboflavin. *Microbial Cell Factories*, *13*, 1-12.

Liu, W., Chen, M., Duo, L., Wang, J., Guo, S., Sun, H., ... & Zhang, H. (2020). Characterization of potentially probiotic lactic acid bacteria and bifidobacteria isolated from human colostrum. *Journal of Dairy Science*, *103*(5), 4013-4025.

Lund, A. M., Löfqvist, C., Pivodic, A., Lundgren, P., Hård, A. L., Hellström, A., & Hansen-Pupp, I. (2020). Unpasteurised maternal breast milk is positively associated with growth outcomes in extremely preterm infants. *Acta Paediatrica*, *109*(6), 1138-1147.

Maeda, N., Nakamura, R., Hirose, Y., Murosaki, S., Yamamoto, Y., Kase, T., & Yoshikai, Y. (2009). Oral administration of heat-killed *Lactobacillus*

plantarum L-137 enhances protection against influenza virus infection by stimulation of type I interferon production in mice. *International Immunopharmacology*, *9*(9), 1122-1125.

Mahooti, M., Abdolalipour, E., Salehzadeh, A., Mohebbi, S. R., Gorji, A., & Ghaemi, A. (2019). Immunomodulatory and prophylactic effects of *Bifidobacterium bifidum* probiotic strain on influenza infection in mice. *World Journal of Microbiology and Biotechnology*, *35*, 1-9.

Markowiak, P., & Śliżewska, K. (2017). Effects of probiotics, prebiotics, and synbiotics on human health. *Nutrients*, *9*(9), 1021.

Markowiak-Kopeć, P., & Śliżewska, K. (2020). The effect of probiotics on the production of short-chain fatty acids by human intestinal microbiome. *Nutrients*, *12*(4), 1107.

Matsusaki, T., Takeda, S., Takeshita, M., Arima, Y., Tsend-Ayush, C., Oyunsuren, T., Kurokawa, M. (2016). Augmentation of T helper type 1 immune response through intestinal immunity in murine cutaneous herpes simplex virus type 1 infection by probiotic *Lactobacillus plantarum* strain 06CC2. *International Immunopharmacology*, *39*, 320-327.

Mazziotta, C., Tognon, M., Martini, F., Torreggiani, E., & Rotondo, J. C. (2023). Probiotics mechanism of action on immune cells and beneficial effects on human health. *Cells*, *12*(1), 184.

Meade, E., Slattery, M. A., & Garvey, M. (2020). Bacteriocins, potent antimicrobial peptides and the fight against multi drug resistant species: resistance is futile? *Antibiotics*, *9*(1), 32.

Mitchell, A., Chang, H. Y., Daugherty, L., Fraser, M., Hunter, S., Lopez, R., ... & Finn, R. D. (2015). The InterPro protein families database: the classification resource after 15 years. *Nucleic Acids Research*, *43*(D1), D213-D221.

Moncheva, P., Chipeva, V., Kujumdzieva, A., Ivanova, I., Dousset, X., & Gocheva, B. (2003). The composition of the microflora of boza, an original Bulgarian beverage. *Biotechnology & Biotechnological Equipment*, *17*(1), 164-168.

Naghibi, M. M., Day, R., Stone, S., & Harper, A. (2019). Probiotics for the prophylaxis of migraine: A systematic review of randomized placebo controlled trials. *Journal of Clinical Medicine*, *8*(9), 1441.

Nazir, Y., Hussain, S. A., Abdul Hamid, A., & Song, Y. (2018). Probiotics and their potential preventive and therapeutic role for cancer, high serum cholesterol, and allergic and HIV diseases. *BioMed Research International*, *2018*(1), 3428437.

Palani Kumar, M. K., Halami, P. M., & Serva Peddha, M. (2021). Effect of *Lactobacillus fermentum* MCC2760-based probiotic curd on hypercholesterolemic C57BL6 mice. *ACS Omega*, *6*(11), 7701-7710.

Palaniyandi, S. A., Damodharan, K., Suh, J. W., & Yang, S. H. (2020). Probiotic characterization of cholesterol-lowering *Lactobacillus fermentum* MJM60397. *Probiotics and Antimicrobial Proteins*, *12*, 1161-1172.

Parracho, H. M., Gibson, G. R., Knott, F., Bosscher, D., Kleerebezem, M., & McCartney, A. L. (2010). A double-blind, placebo-controlled, crossover-designed probiotic feeding study in children diagnosed with autistic spectrum disorders. *International Journal of Probiotics & Prebiotics*, *5*(2), 69.

Pärtty, A., Kalliomäki, M., Wacklin, P., Salminen, S., & Isolauri, E. (2015). A possible link between early probiotic intervention and the risk of neuropsychiatric disorders later in childhood: a randomized trial. *Pediatric Research*, *77*(6), 823-828.

Patel, S., & Goyal, A. (2012). The current trends and future perspectives of prebiotics research: a review. *3 Biotech*, *2*, 115-125.

Peivasteh-Roudsari, L., Pirhadi, M., Karami, H., Tajdar-Oranj, B., Molaee-Aghaee, E., & Sadighara, P. (2019). Probiotics and food safety: an evidence-based review. *Journal of Food Safety and Hygiene*, *5*(1), 1-9.

Peter, S. B., Qiao, Z., Godspower, H. N., Ajeje, S. B., Xu, M., Zhang, X., ... & Rao, Z. (2022). Biotechnological innovations and therapeutic application of *Pediococcus* and lactic acid bacteria: the next-generation microorganism. *Frontiers in Bioengineering and Biotechnology*, *9*, 802031.

Qin, J., Li, Y., Cai, Z., Li, S., Zhu, J., Zhang, F., ... & Wang, J. (2012). A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature*, *490*(7418), 55-60.

Ramirez-Chavarin, M. L., Wacher, C., Eslava-Campos, C. A., & Perez-Chabela, M. L. (2013). Probiotic potential of thermotolerant lactic acid bacteria strains isolated from cooked meat products. *International Food Research Journal*, *20*(2).

Ranaei, V., Pilevar, Z., Khaneghah, A. M., & Hosseini, H. (2020). Propionic acid: method of production, current state and perspectives. *Food Technology and Biotechnology*, *58*(2), 115.

Ravisankar, P., Reddy, A. A., Nagalakshmi, B., Koushik, O. S., Kumar, B. V., & Anvith, P. S. (2015). The comprehensive review on fat soluble vitamins. *IOSR Journal of Pharmacy*, *5*(11), 12-28.

Reichardt, N., Duncan, S. H., Young, P., Belenguer, A., McWilliam Leitch, C., Scott, K. P., ... & Louis, P. (2014). Phylogenetic distribution of three pathways for propionate production within the human gut microbiota. *The ISME Journal*, *8*(6), 1323-1335.

Reichold, A., Brenner, S. A., Spruss, A., Förster-Fromme, K., Bergheim, I., & Bischoff, S. C. (2014). Bifidobacterium adolescentis protects from the development of nonalcoholic steatohepatitis in a mouse model. *The Journal of Nutritional Biochemistry*, *25*(2), 118-125.

Rose, A. T., Miller, E. R., Butler, M., Eden, C., Kim, J. H., Shah, S. I., & Patel, R. M. (2022). US state policies for Medicaid coverage of donor human milk. *Journal of Perinatology*, *42*(6), 829-834.

Rowland, I., Gibson, G., Heinken, A., Scott, K., Swann, J., Thiele, I., & Tuohy, K. (2018). Gut microbiota functions: metabolism of nutrients and other food components. *European Journal of Nutrition*, *57*, 1-24.

Ruiz, L., García-Carral, C., & Rodriguez, J. M. (2019). Unfolding the human milk microbiome landscape in the omics era. *Frontiers in Microbiology*, *10*, 1378.

Salaris, C., Scarpa, M., Elli, M., Bertolini, A., Guglielmetti, S., Pregliasco, F., ... & Castagliuolo, I. (2021). *Lacticaseibacillus paracasei* DG enhances the lactoferrin anti-SARS-CoV-2 response in Caco-2 cells. *Gut Microbes*, *13*(1), 1961970.

Salcedo, J., Gormaz, M., López-Mendoza, M. C., Nogarotto, E., & Silvestre, D. (2015). Human milk bactericidal properties: Effect of lyophilization and relation to maternal factors and milk components. *Journal of Pediatric Gastroenterology and Nutrition*, *60*(4), 527-532.

Sanwal, N., Gupta, A., Bareen, M. A., Sharma, N., & Sahu, J. K. (2023). Kombucha fermentation: Recent trends in process dynamics, functional bioactivities, toxicity management, and potential applications. *Food Chemistry Advances*, *3*, 100421.

Schroeder, B. O. (2019). Fight them or feed them: how the intestinal mucus layer manages the gut microbiota. *Gastroenterology Report*, *7*(1), 3-12.

Schultz, S. T., Klonoff-Cohen, H. S., Wingard, D. L., Akshoomoff, N. A., Macera, C. A., Ji, M., & Bacher, C. (2006). Breastfeeding, infant formula supplementation, and Autistic Disorder: the results of a parent survey. *International Breastfeeding Journal*, *1*, 1-7.

Shreiner, A. B., Kao, J. Y., & Young, V. B. (2015). The gut microbiome in health and in disease. *Current Opinion in Gastroenterology*, *31*(1), 69-75.

Singh, R. P., Shadan, A., & Ma, Y. (2022). Biotechnological applications of probiotics: a multifarious weapon to disease and metabolic abnormality. *Probiotics and Antimicrobial Proteins*, *14*(6), 1184-1210.

Sionek, B., Szydłowska, A., Zielińska, D., Neffe-Skocińska, K., & Kołożyn-Krajewska, D. (2023). Beneficial Bacteria isolated from food in relation to the next generation of probiotics. *Microorganisms*, *11*(7), 1714.

Sionek, B., Szydłowska, A., Zielińska, D., Neffe-Skocińska, K., & Kołożyn-Krajewska, D. (2023). Beneficial Bacteria isolated from food in relation to the next generation of probiotics. *Microorganisms*, *11*(7), 1714.

Soltani, S., Hammami, R., Cotter, P. D., Rebuffat, S., Said, L. B., Gaudreau, H., ... & Fliss, I. (2021). Bacteriocins as a new generation of antimicrobials: toxicity aspects and regulations. *FEMS Microbiology Reviews*, *45*(1), fuaa039.

Sornplang, P., & Piyadeatsoontorn, S. (2016). Probiotic isolates from unconventional sources: a review. *Journal of Animal Science and Technology*, *58*, 1-11.

Stojanov, S., Berlec, A., & Štrukelj, B. (2020). The influence of probiotics on the firmicutes/bacteroidetes ratio in the treatment of obesity and inflammatory bowel disease. *Microorganisms*, *8*(11), 1715.

Sun, J., Xu, J., Yang, B., Chen, K., Kong, Y., Fang, N., & Liu, J. (2020). Effect of Clostridium butyricum against microglia-mediated neuroinflammation in Alzheimer's disease via regulating gut microbiota and metabolites butyrate. *Molecular Nutrition and Food Research*, *64*(2), 1900636.

Tachedjian, G., Aldunate, M., Bradshaw, C. S., & Cone, R. A. (2017). The role of lactic acid production by probiotic *Lactobacillus* species in vaginal health. *Research in Microbiology*, *168*(9-10), 782-792.

Tamtaji, O. R., Heidari-Soureshjani, R., Mirhosseini, N., Kouchaki, E., Bahmani, F., Aghadavod, E., ... & Asemi, Z. (2019). Probiotic and selenium co-supplementation, and the effects on clinical, metabolic and genetic status in Alzheimer's disease: a randomized, double-blind, controlled trial. *Clinical Nutrition*, *38*(6), 2569-2575.

Tamtaji, O. R., Taghizadeh, M., Kakhaki, R. D., Kouchaki, E., Bahmani, F., Borzabadi, S., ... & Asemi, Z. (2019). Clinical and metabolic response to probiotic administration in people with Parkinson's disease: a randomized, double-blind, placebo-controlled trial. *Clinical Nutrition*, *38*(3), 1031-1035.

Tan, A. H., Lim, S. Y., Chong, K. K., A Manap, M. A. A., Hor, J. W., Lim, J. L., ... & Lang, A. E. (2021). Probiotics for constipation in Parkinson disease: a randomized placebo-controlled study. *Neurology*, *96*(5), e772-e782.

Tegegne, B. A., & Kebede, B. (2022). Probiotics, their prophylactic and therapeutic applications in human health development: A review of the literature. *Heliyon*, *8*(6).

Vaiserman, A., Romanenko, M., Piven, L., Moseiko, V., Lushchak, O., Kryzhanovska, N., ... & Koliada, A. (2020). Differences in the gut Firmicutes to Bacteroidetes ratio across age groups in healthy Ukrainian population. *BMC microbiology*, *20*, 1-8.

Van Lingen, E., Terveer, E. M., van der Meulen-De Jong, A. E., Vendrik, K. E. W., Verspaget, H. W., Kuijper, E. J., ... & Keller, J. J. (2020). Advances in stool banking. *Microbiota In Health And Disease*, *2*, e182.

Virgianti, D. P., & Suhartati, R. (2023). Tempeh's Probiotic Bacteria Content can Boost Its Value as a Functional Food in the COVID-19 Era. *Health Notions*, *7*(4), 80-88.

Vorobjeva, L. I., Khodjaev, E. Y., & Vorobjeva, N. V. (2008). Propionic acid bacteria as probiotics. *Microbial Ecology in Health and Disease*, *20*(2), 109-112.

Walsh, A. M., Crispie, F., Kilcawley, K., O'sullivan, O., O'sullivan, M. G., Claesson, M. J., & Cotter, P. D. (2016). Microbial succession and flavor production in the fermented dairy beverage kefir. *mSystems*, *1*, e00052-16.

Wang, G., Chen, Y., Xia, Y., Song, X., & Ai, L. (2022). Characteristics of probiotic preparations and their applications. *Foods*, *11*(16), 2472.

Wang, Y., Moon, A., Huang, J., Sun, Y., & Qiu, H. J. (2022). Antiviral effects and underlying mechanisms of probiotics as promising antivirals. *Frontiers in Cellular and Infection Microbiology*, *12*, 928050.

Westfall, S., Lomis, N., Kahouli, I., Dia, S. Y., Singh, S. P., & Prakash, S. (2017). Microbiome, probiotics and neurodegenerative diseases: deciphering the gut brain axis. *Cellular and Molecular Life Sciences*, *74*, 3769-3787.

Widodo, W., Fanani, T. H., Fahreza, M. I., & Sukarno, A. S. (2021). Cholesterol assimilation of two probiotic strains of *Lactobacillus casei* used as dairy starter cultures. *Applied Food Biotechnology*, *8*(2), 103-112.

Wiertsema, S. P., van Bergenhenegouwen, J., Garssen, J., & Knippels, L. M. (2021). The interplay between the gut microbiome and the immune system in the context of infectious diseases throughout life and the role of nutrition in optimizing treatment strategies. *Nutrients*, *13*(3), 886.

Wu, Y., Pang, X., Wu, Y., Liu, X., & Zhang, X. (2022). Enterocins: classification, synthesis, antibacterial mechanisms and food applications. *Molecules*, *27*(7), 2258.

Yadav, M. K., & Tiwari, S. K. (2023). Methods for determination of antimicrobial activity of bacteriocins of lactic acid bacteria. *Microbiology*, *92*(6), 745-765.

Yang, R., Chen, Z., & Cai, J. (2023). Fecal microbiota transplantation: Emerging applications in autoimmune diseases. *Journal of Autoimmunity*, *141*, 103038.

Yoha, K. S., Nida, S., & Dutta, S. (2022). Targeted delivery of probiotics: perspectives on research and commercialization. *Probiotics Antimicrob Proteins*, *14*, 15-48.

Yu-Jie, D., Hai-Yan, W., Xi-Jian, W., Kaye, A. D., & Yong-Hai, S. (2017). Potential beneficial effects of probiotics on human migraine headache: a literature review. *Pain physician*, *20*(2), E251.

Zabat, M. A., Sano, W. H., Wurster, J. I., Cabral, D. J., & Belenky, P. (2018). Microbial community analysis of sauerkraut fermentation reveals a stable and rapidly established community. *Foods*, *7*(5), 77.

Zaib, S., Hayat, A., & Khan, I. (2024). Probiotics and their beneficial health effects. *Mini Reviews in Medicinal Chemistry*, *24*(1), 110-125.

Zhang, L., Chu, J., Hao, W., Zhang, J., Li, H., Yang, C., ... & Wang, H. (2021). Gut microbiota and type 2 diabetes mellitus: Association, mechanism, and translational applications. *Mediators of Inflammation*, *2021*(1), 5110276.

MICROBIAL FERMENTATION: PRODUCTION AND BIOTECHNOLOGICAL APPLICATIONS

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Introduction

Microbial fermentation is an area of biotechnology that is of vital importance to mankind and was discovered thousands of years ago in nature. The remarkable capabilities of microorganisms have been utilised for centuries in important areas such as food production and the pharmaceutical industry. Technological advancements of the modern era, including disciplines like microbial genetic engineering, have greatly expanded the potential and scope of fermentation. Today, microbial fermentation is not only increasing the efficiency of industrial production but also revolutionising areas such as biological transformation and innovative product development. Fermentation has evolved from a simple process used in ancient times to produce products such as bread and wine to a fundamental element of complex and critical industrial processes such as pharmaceutical synthesis, bioactive compound production, biofuels and waste management. Effective management and optimisation of these processes are crucial for environmental sustainability.

This section will address the industrial applications, future potential, and innovative uses of microbial fermentation. This is an exciting area of progress for those working in biotechnology and industrial process engineering. Topics such as innovative microbial engineering strategies, smart fermentation technologies and industrial waste recycling illustrate the transformative potential of microbial fermentation in the coming years. Microbial fermentation is a marvellous gift of nature and constant study, and development is required to fully exploit this technology. This section is intended as a guide for anyone wishing to understand the scope, significance, and future potential of microbial fermentation.

Fermentation

Fermentation stands as one of the most ancient biotechnological techniques used extensively employed in processing and preservation of food. Defined as the microbial metabolism-driven chemical modification of an organic substance (Erkmen and Bozoglu, 2016), this process enables the degradation of complex organic compounds into less complex forms through microbial activity. In particular, food components that are affected by microbiological or enzymatic processes often ferment, leading to beneficial biochemical alterations that are responsible for the remarkable food

modifications (Sharma et al., 2020). For thousands of years, people have been using microorganisms to ferment food in order to extend its shelf life and improve its function, flavour and consistency (Hill et al., 2017). For instance, the earliest written records of this process date back 6000 years and describe beer brewing by the Sumerians (Bud, 1993). Approximately 3500 years ago, soya fermentation was introduced in China. From the mid-1830s onwards, evidence accumulated that proved the biological nature of fermentation. Schwann in 1837 and Cagniard-Latour in 1838 proved that yeast is a microorganism, and that the fermentation of alcohol requires living yeast. Microbial fermentation methods are used in many different industries, e.g. in waste management, the food industry, the pharmaceutical sector, and in the production of biofuel. These processes' efficiency, control, and optimisation play a significant role in industrial applications and are constantly being improved through research and development efforts.

Industrial Applications of Microbial Fermentation

Food Industry

In the food industry, microbial fermentation is a key method in producing numerous food products. Additionally, it has been used for centuries to increase the nutritional content and shelf life of food. This process is based on the metabolism of, carbohydrates, proteins and fats by microorganisms in a nutrient-rich environment, which gives the products specific flavours, aromas, textures and nutritional values to the products. The well-known fermented products include yoghurt, kefir, kimchi, sauerkraut, kombucha, cheese, beer, tempeh, wine, and natto. Fermentation is usually initiated by introducing starter cultures into the food. For example, in **kombucha** production, acetic acid bacteria from the genera *Komagataeibacter*, *Gluconobacter*, and *Acetobacter*, as well as yeasts such as *Zygosaccharomyces*, *Saccharomyces*, and *Brettanomyces*, are essential factors in fermentation (Mayser et al., 1995; Liu et al., 1996). **Kefir** production requires kefir grains that contains yeasts such as *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, and *S. unisporus*, lactic acid and acetic acid bacteria (Lopitz-Otsoa et al., 2006). *Bacillus subtilis* is required for natto, a traditional Japanese soybean dish (Hosoi and Kiuchi, 2003), *Aspergillus oryzae* for miso, another traditional Japanese fermented food (Kusumoto et al., 2021), and *Rhizopus oligoporus* for tempeh, a traditional Indonesian

fermented soybean product (Hachmeister and Fung, 1993). In the production of **kimchi** or **traditional sauerkraut**, naturally occurring microorganisms in raw foods can also lead to spontaneous fermentation (Augustin et al., 2023).

Fermented products undergo various fermentation processes carried out by microorganisms, each of which uniquely contributes to the final product. In the production of yoghurt, for example, the bacteria *Lactobacillus delbrueckii* and *Streptococcus thermophilus* ferment the milk to produce lactic acid, which gives the yoghurt its characteristic texture and flavour (Lourens-Hattingh and Viljoen, 2001). Lactic acid bacteria, such as *Lactobacillus plantarum* and *L. brevis*, produce lactic acid during pickle-making, creating an acidic environment that inhibits spoiling. (Swain et al., 2014). In the production of fermented **meat products**, starter cultures are crucial. Microorganisms such as *L. sakei* and *L. lactis* play a significant role by producing some organic acids, bacteriocins and acetaldehyde to improve the preservation of the product (Zagorec and Champomier-Vergès, 2017; Franciosa et al., 2018). These microorganisms also improve the probiotic content of food and promote digestive health.

The production of functional foods is a key component of food fermentation. During fermentation, microorganisms can produce health-promoting components such as vitamins, enzymes, biochemically active compounds and antioxidants. For example, fermented fish products like **narezushi**, which is consumed in Japan, and **jeotgal**, which is consumed in Korea are known for their high protein content and unique flavours (Kumar and Nayak, 2015). Fermented soybean products such as **Miso** and **natto** are fermented soybean products. They are rich in proteins and vitamins, which increases their nutritional value (Saeed et al., 2022). During the fermentation of **yoghurt, Bifidus milk** and **kefir**, lactic acid bacteria increase the content of riboflavin, niacin and folic acid (Parvez et al., 2006). Fermented beverages such as **kefir** and **kombucha** also have a high content of phenols and flavonoids, which contribute to human health through their antioxidant, anticancer and antigenotoxic properties (Tefon-Öztürk et al., 2023; da Anunciação et al., 2024; Burgazlı et al., 2024).

Fermentation technology is not only essential for the production of traditional foods but is also a key component in the production of next-generation functional foods. For instance, water kefir, a fermented drink without dairy products, is being developed as a substitute for the supply of probiotics for people who follow a vegan diet or are lactose intolerant

(Cufaoglu and Erdinc, 2023). Additionally, microbial fermentation is used to produce milk proteins such as casein and whey, creating alternatives to animal dairy products (Hilgendorf et al., 2024). Plant-based meat alternatives produced through fermentation are usually made from protein-rich plant-based raw materials and offer similar textures and flavour profiles to traditional meat products (Kumar et al., 2017). Edible and biodegradable packaging materials made from bacterial cellulose and other biopolymers produced by microbial fermentation offer environmentally friendly packaging solutions (Shi et al., 2014). Furthermore, innovative methods are being developed to convert waste into high-quality components through fermentation. For example, fruit and vegetable waste can be processed by fermentation to produce enzymes, organic acids and bioactive compounds. These applications help to reduce food waste and support a circular economy (Louhasakul and Cheirsilp, 2013; Ahuja et al., 2021; Yafetto et al., 2023).

Innovations in genetic engineering and synthetic biology are improving fermentation processes by enabling microorganisms to produce more efficiently and specifically. This development facilitates the production of more diverse and nutritious products in the food industry. Precision fermentation technologies in particular increase the production of proteins, lipids and other vital food components by microorganisms and create new possibilities for the production of sustainably produced food. In summary, the applications of microbial fermentation in the food industry cover a broad spectrum, ranging from traditional production methods to modern technologies. These processes not only enhance the nutritional quality of food but also offer significant opportunities for sustainable and innovative food production.

Biopolymer Production

The industrial use of polymers is often based on materials derived from plants or seaweeds, such as alginate, mannan and cellulose. However, these polymers face issues related to insecure supply and quality variations (Vijayendra and Shamala, 2014). Microorganisms synthesise extracellular or intracellular polymers known as biopolymers, which are crucial for their functions and survival (Angelina and Vijayendra, 2015). These biopolymers fulfil specific tasks such as protective substances, energy reserves, support of cellular processes, osmotic adaptation and establishment of symbioses, and generally help the microorganism adapt to fluctuating environmental

conditions. Recently, much research has been carried out into the use of microbial biopolymers as packaging materials. These polymers will be used in a range of industries, including printing, wastewater treatment, mining, packaging, oil extraction, cosmetics, agriculture, food, medicine and healthcare (Angelina and Vijayendra, 2015).

Many bacterial species have the ability to synthesise biopolymers by converting waste products into cellular products such as polyhydroxyalkanoates (PHA) and extracellular products such as exopolysaccharides (EPS). Specifically, a variety of bacteria, including *Nocardia* spp., *Bacillus* spp., *Alcaligenes eutrophus*, *A. latus*, *Pseudomonas* spp., *Azotobacter vinelandii*, *A. beijerincki*, *A. chroococcum*, recombinant *Escherichia coli* and *Rhizobium* spp., have been used to produce **PHAs** from various organic by-products on an industrial scale (Pagliano et al., 2017). PHAs are biopolymers produced by microorganisms to increase their resistance to environmental stresses and to store energy. They are synthesised as a natural energy store and carbon source and accumulate in the cytoplasm in the form of granules. In most cases, PHA is formed when cells are exposed to too many carbon sources and too little nitrogen, oxygen, sulphur, phosphorus, or other nutrients. PHAs are broken down by intracellular depolymerases and metabolised as a source of carbon and energy when the limiting nutrients are replenished. The biodegradability and biocompatibility of PHAs also favour their use in biomedical and industrial applications. Because of these qualities, they are a valuable and sustainable substitute for plastic and other synthetic materials in the environment (Cavalheiro et al., 2009).

Microbial fermentation can also lead to the accumulation of extracellular molecules like EPS, which can be used to produce biopolymers. These polymers are used in the food, chemical, cosmetics and packaging industries as adhesives, lubricants, absorbents and cosmetics (Pepe et al., 2013). EPS has a diverse structure containing carbohydrate components such as rhamnose, glucose and mannose as well as non-carbohydrate components such as uronic acids, succinate, pyruvate and phosphate (Vijayendra and Shamala, 2014). The production of EPS can vary depending on factors such as temperature, fermentation time, aeration, medium composition, agitation, carbon source and concentration, pH, trace elements, nitrogen, and dissolved oxygen levels. For instance, **pullulan**, a linear and branched EPS, is primarily produced by dimorphic fungi such as *Aureobasidium pullulans* and some

bacterial species. Although too much nitrogen in the fermentation medium promotes the growth of *A. pullulans*, it has a detrimental effect on the yield of the polymers (Jiang and Wu, 2011). Maltose residues are linked together by α-1,4- and α-1,6-glycosidic bonds and form pullulan. It is a crucial biomolecule that has several advantages as a drug delivery mechanism and has high water solubility when used to make polysaccharide-based hydrogels (Patil et al., 2020; Ari et al., 2023). **Bacterial cellulose**, an unbranched polymer, is produced through fermentation by various bacterial genera such as *Achromobacter, Rhizobium, Azotobacter, Acetobacter, Agrobacterium* and *Aerobacter* (Lahiri et al., 2021). Previous studies have reported that the addition of L-methionine (Matsuoka et al., 1996) and ethanol as an additional carbon source (Son et al., 2001) to the fermentation medium increases the growth of *Acetobacter xylinum* and cellulose yield. Additionally, cultivating cultures in shake flasks or fermenters can lead to higher cellulose production compared to static fermentation (Suwannapinunt et al., 2007). Despite various physicochemical and mechanical properties that make it an interesting substitute for plant cellulose, bacterial cellulose is chemically similar to plant cellulose. Higher degrees of polymerisation, purity, tensile strength, increased capacity for absorbing and holding onto water, and improved biocompatibility are some of these properties (Gea et al., 2011; Lin et al., 2020). **Levan,** a branched neutral homopolysaccharide consisting exclusively of fructose units, is mainly biosynthesised by the enzyme sucrose 6-fucosyltransferase, also known as levansucrase (Hernández et al., 1999). Species from genera include *Aerobacter*, *Lactobacillus*, *Zymomonas*, *Erwinia*, *Streptococcus*, *Pseudomonas* and *Rahnella* produce this extracellular enzyme. (Han, 1989; Han, 1990; Bahl et al., 2010; Abdel-Fattah et al., 2012). In addition to its biodegradability, renewability, flexibility and environmental friendliness, levan exhibits significant biomedical properties, including anti-hyperglycemic, antioxidant, anti-cancer and anti-inflammatory effects (Dahech et al., 2011; Abdel-Fattah et al., 2012). Furthermore, it is an amphiphilic polymer with strong adhesive properties, and its applications extend to the manufacture of cosmetic, pharmaceutical and food products (Jakob et al., 2012; Öner et al., 2016; Sezer et al., 2017). **Kefiran**, which consists of equal parts galactose and glucose, is usually produced by lactic acid bacteria, yeasts and acetic acid bacteria found in the flora of the kefir grain (Kooiman, 1968; Zajsek et al., 2011). This polymer is not only highly durable, but also has fertilisable, biodegradable, and edible properties. As a result, the food industry uses it extensively, especially as a stabiliser, emulsifier, gelling agent and fat substitute (Piermaria et al., 2015; Moradi and

Kalanpour, 2019). Another microbiological polymer, **gellan**, is commercially produced by the exopolysaccharide fermentation of *Sphingomonas elodea* and *S. paucimobilis* bacteria (Giavasis et al., 2000; Vijayendra and Shamala, 2014; Feketshane et al., 2022). This polymer consists of a tetrasaccharide repeating unit, which includes two β-d-glucose, one β-d-glucuronate, and one α-l-rhamnose unit (Kuo et al., 1986; Gong et al., 2009), and is used as a gelling agent in foods.

The search for sustainable and eco-friendly solutions today underscores the increasing importance of microbial biopolymers. These biopolymers, which have a broad range of potential applications from food packaging to medical materials, contribute to reducing plastic pollution and minimise the ecological footprint. The optimisation of production processes for microbial biopolymers and the exploration of new application areas will therefore bring significant economic and ecological benefits. In this context, the wider application of microbial biopolymers will be representing an important step towards a sustainable world in the future.

Biofuel Production

Due to the social, environmental and economic impact of biofuel production, many scientists are focussing on this field. The use of biofuels as renewable energy sources is particularly noteworthy as they reduce carbon dioxide emissions and overall pollution (Prasad et al., 2020). Biofuels are recognised as carbon-neutral pathways as they are made from biomass and absorb an enormous amount of the carbon dioxide emitted into the atmosphere (Prasad et al., 2012). Microbial fermentation is pivotal in generating biofuels and is increasingly applied to create various fuels such as bioethanol, biodiesel, biomethane, biohydrogen, and biobutanol (Agrawal, 2012; Adegboye et al., 2021; Paul et al., 2021; Love, 2022).

In order to meet energy demands and lessen reliance on fossil fuels, the production of biofuels has been developed in four generations, using different raw materials and production techniques in each generation. First-generation biofuels, for example, are produced from edible raw materials such as barley, wheat, potatoes, rice, sugar cane and vegetable oils. The fermentation of starchy or sugary plants such as maize or sugar cane usually produces ethanol, while the transesterification of vegetable oils with methanol or ethanol produces biodiesel (Bhargavi et al., 2018). The benefits of this production include the ability to lower carbon emissions compared to fossil fuels and

compatibility with current agricultural infrastructure and equipment. However, it can compete with food resources, leading to an increase in food prices, and the use of agricultural land for biofuel production can lead to deforestation and a reduction in biodiversity (Paschalidou et al., 2016). To overcome the limitations of the first generation, the production of second-generation of biofuels has been proposed. Second-generation biofuels are produced from lignocellulosic biomass such as agricultural residues or wood crisps. There are two production processes: the conversion of biomass into synthetic gas or biofuels through processes like gasification and pyrolysis (thermochemical conversion) or the conversion of cellulose into sugar by enzymatic hydrolysis followed by fermentation to produce bioethanol (biochemical conversion). This production does not compete with food resources and enables the utilisation of agricultural and forestry residues. However, it requires complex and costly production processes and the development of highly efficient enzymes for enzymatic hydrolysis is a challenge (Srivastava et al., 2019; Alalwan et al., 2019). To address the shortcomings of first and second-generation biofuels, the use of microalgae and seaweeds as feedstock has led to the development of third-generation biofuels. In this generation, microalgae are used to produce fatty acids through photosynthesis. The produced oils are converted into biodiesel, and the sugars obtained from microalgae are converted into bioethanol through fermentation. However, subsequent studies have shown that biofuels derived from microalgae are highly volatile and less stable. Additionally, technological advancements are required for large-scale production (Shah et al. 2018). This has led to research into fourth generation biofuels. Fourth generation aims to convert carbon dioxide directly into biofuels using genetically modified microalgae or bacteria, where the microorganisms are manipulated to produce biofuels directly (Ng et al., 2017). However, this application is still in the development phase, and it has been reported that it may not be suitable for all microalgae species due to limited genetic and biological knowledge of the organisms used (Abdullah et al., 2019). To date, biological data from certain microalgae such as *Chlorella variabilis* (Blanc et al., 2010), *Chlamydomonas reinhardtii* (Merchant et al., 2007) and *C. sorokiniana* (Arriola et al., 2018), as well as cyanobacterial species belonging to the genera *Nostoc*, *Synechocystis*, and *Synechococcus*, which are intended for the production of fourth-generation biofuels, have been reported and genetic modifications have been studied (Santos-Merino et al., 2018; Eungrasamee et al., 2019). Additionally, various organisms have been utilised to develop efficient and stable microbial systems for biofuel production due to their ability to ferment

a wide range of feedstocks, their efficiency in the targeted biofuel production and their known genomes (Mi et al., 2014; Xu et al., 2016; Yan et al., 2019; Du et al., 2022).

Biofuel production plays a critical role in environmental sustainability, energy security and reducing dependence on fossil fuels. The development of different generations of biofuels, each with their benefits and challenges, allows for more efficient utilisation of renewable energy sources. In particular, the role of microorganisms in biofuel production helps to reduce the environmental impact and increase the economic benefits of utilising different types of biomasses. Future research is expected to focus on the development of fourth generation biofuels through genetically modified organisms and innovative methods to find more sustainable and effective solutions in the field of bioenergy. In this context, the potential of microorganisms could lead to significant transformations in biofuel production and open new horizons in energy production.

Pharmaceutical Industry

Fermentation technology has long been acknowledged for its contribution to the production of crucial medical products, such as antibiotics, solvents like ethanol, intermediates such as citric acid, and probiotics including yogurt. The latest advances in fermentation provide antiviral agents, therapeutic recombinant proteins, and monoclonal antibodies (Rahman, 2013). Microbial fermentation holds a significant place in the pharmaceutical industry, playing a critical role in the production of various therapeutic components. Additionally, fermented raw pharmaceuticals are also produced. For instance, Shinkiku, which is used in East Asia to treat dyspepsia, nausea and anorexia, is produced by fermenting a mixture of wheat, apricot kernels, red beans, watercress, sweet wormwood and chicory. Solid cultures of filamentous fungi such as *Rhizopus*, *Aspergillus* or *Mucor* are usually used in this fermentation (Wang et al., 2020).

Today, microbial fermentation is utilized to produce a wide range of biological products, including hormones, vitamins, enzymes, vaccines and antineoplastic drugs. Alexander Fleming discovered penicillin in 1928. It was extracted from Penicillium notatum and used extensively to treat infections during the Second World War (Fleming, 1929). Streptomycin, produced through the fermentation of *Streptomyces griseus* bacteria, was discovered in 1944 by Selman Waksman and Albert Schatz and revolutionised the treatment

of tuberculosis (Schatz et al., 1944). These discoveries paved the way to produce antibiotics through microbial fermentation. Recombinant DNA technology advancements have led to the increasing use of microbial fermentation in the production of hormones. In 1982, *E. coli* was engineered to produce human insulin, marking a significant innovation in the treatment of diabetes (Johnson 1983). This method eliminated the need to obtain insulin from animal sources and enabled the production of purer, human-like insulin products. Similar to such, recombinant DNA technology is also used for producing human growth hormone in bacteria (Goeddel et al., 1979), which represents an important therapeutic alternative for people with growth hormone deficiency.

Propionibacterium freudenreichii is effectively used for the production of vitamin B12 (Piao et al., 2004; Piwowarek et al., 2018). Anaemia can result from a lack of vitamin B12, which is necessary for the synthesis of DNA and the production of red blood cells. The fungus *A. niger* is used in the production of lactase enzyme (Corazza, 1992; Mirsalami and Alihosseini, 2021). Lactase helps people with lactose intolerance to digest dairy products and thus reduces digestive problems. The hepatitis B vaccine was produced in *S. cerevisiae* yeast cells (McAleer et al., 1984; Kniskem et al., 1986). This method provides high efficiency and safety in vaccine production. The BCG vaccine (Bacillus Calmette-Guérin) is produced through the fermentation of an attenuated strain of *Mycobacterium bovis* and is effective in the prevention of tuberculosis (Calmette, 1927; Pascoe et al., 2020). The BCG vaccine is widely used globally to prevent the spread of tuberculosis, particularly in children. Actinomycin D, an antineoplastic agent, is produced from *Streptomyces parvulus* and exhibits anticancer properties by causing DNA damage in cancer cells (Williams and Katz, 1977; Cibi and Nair, 2016). Similarly, doxorubicin is produced through the fermentation of *S. peucetius* and is used in the treatment of various types of cancer (Shrestha et al., 2019). These agents play a crucial role in chemotherapy.

Microbial fermentation is an important method for the production as well as the development of new therapeutic agents. For example, teixobactin, which has been discovered in recent years, is a new antibiotic produced by microbial fermentation that is effective against multidrug-resistant bacteria (Ling et al., 2015; Karas et al., 2020). Genome editing techniques and synthetic biology approaches are being used to improve the effectiveness of microbial fermentation. Technologies such as CRISPR-Cas9 enable the

modification of the genomes of microorganisms and facilitate the creation of more efficient bioprocesses. This enables greater efficiency in drug production and the production of specific products (Doudna and Charpentier, 2014). Ensuring high efficiency, low cost, and sustainability in this sector makes it a favoured method in the pharmaceutical industry. This biotechnological process has a direct impact on drug production and offers safer, more effective and more economical treatment options. As microbial fermentation technology advances and new applications are explored, its significance in the pharmaceutical industry will continue to grow.

Conclusion

Microbial fermentation stands out as a crucial technology for the production of food, pharmaceuticals, biofuels and biopolymers. In the food industry, microbial fermentation enables the production of nutrients and environmentally friendly packaging solutions. In the pharmaceutical sector, this technology is used to produce effective and safe therapeutic agents. Additionally, the biopolymers produced with this technology are valuable components that offer renewable alternatives and reduce environmental impact. By reducing dependence on fossil fuels, the development of biofuels also contributes to environmental sustainability and energy security. The further development of these technologies will bring significant environmental and economic benefits in the future and represent an important step towards a sustainable world.

REFERENCES

Abdel-Fattah, A. M., Gamal-Eldeen, A. M., Helmy, W. A., & Esawy, M. A. (2012). Antitumor and antioxidant activities of levan and its derivative from the isolate *Bacillus subtilis* NRC1aza. *Carbohydrate Polymers*, *89(2)*, 314-322.

Abdullah, B., Muhammad, S. A. F. S., Shokravi, Z., Ismail, S., Kassim, K. A., Mahmood, A. N., & Aziz, M. M. A. (2019). Fourth generation biofuel: A review on risks and mitigation strategies. *Renewable and Sustainable Energy Reviews*, *107*, 37-50.

Adegboye, M. F., Ojuederie, O. B., Talia, P. M., & Babalola, O. O. (2021). Bioprospecting of microbial strains for biofuel production: metabolic engineering, applications, and challenges. *Biotechnology for Biofuels*, *14(1)*, 5.

Agrawal, M. (2012). Metabolic engineering of *Zymomonas mobilis* for improved production of ethanol from lignocelluloses. *Georgia Institute of Technology*.

Ahuja, V., Bhatt, A. K., Sharma, V., Rathour, R. K., Rana, N., Bhatia, R. K., et al. (2021). Advances in glucosamine production from waste biomass and microbial fermentation technology and its applications. *Biomass Conversion and Biorefinery*, 1-23.

Alalwan, H. A., Alminshid, A. H., & Aljaafari, H. A. (2019). Promising evolution of biofuel generations. Subject review. *Renewable Energy Focus*, *28*, 127-139.

Angelina, & Vijayendra, S. V. N. (2015). Microbial biopolymers: the exopolysaccharides. In *Microbial Factories: Biodiversity, Biopolymers, Bioactive Molecules: Volume 2* (pp. 113-125). New Delhi: Springer India.

Ari, B., Sahiner, M., Suner, S. S., Demirci, S., & Sahiner, N. (2023). Super-macroporous pulluan cryogels as controlled active delivery systems with controlled degradability. *Micromachines*, *14(7)*, 1323.

Arriola, M. B., Velmurugan, N., Zhang, Y., Plunkett, M. H., Hondzo, H., & Barney, B. M. (2018). Genome sequences of *Chlorella sorokiniana* UTEX 1602 and *Micractinium conductrix* SAG 241.80: implications to maltose excretion by a green alga. *The Plant Journal*, *93(3)*, 566-586.

Augustin, M. A., Hartley, C. J., Maloney, G., & Tyndall, S. (2023). Innovation in precision fermentation for food ingredients. *Critical Reviews in Food Science and Nutrition*, 1-21.

Bahl, M. A., Schultheis, E., Hempel, D. C., Nörtemann, B., & Franco-Lara, E. (2010). Recovery and purification of the exopolysaccharide PS-EDIV from *Sphingomonas pituitosa* DSM 13101. *Carbohydrate Polymers*, *80(4)*, 1037-1041.

Bhargavi, G., Nageswara Rao, P., & Renganathan, S. (2018, March). Review on the Extraction Methods of Crude oil from all Generation Biofuels in last few Decades. In *IOP conference series: materials science and engineering* (Vol. 330, p. 012024). IOP Publishing.

Blanc, G., Duncan, G., Agarkova, I., Borodovsky, M., Gurnon, J., Kuo, A., et al. (2010). The *Chlorella variabilis* NC64A genome reveals adaptation to photosymbiosis, coevolution with viruses, and cryptic sex. *The Plant Cell*, *22(9)*, 2943-2955.

Bud, R. (1993). The uses of life, a history of biotechnology, Cambridge University Press, Cambridge; (1995) German translation: Wie wir das Leben nutzbar machten. Vieweg, Braunschweig (citations follow the 1995 edition).

Burgazlı, A. Y., Tagorti, G., Yalçın, B., Güneş, M., Eroğlu, B., Delik, E., et al. (2024). Antigenotoxic and Life-Prolonging Effects of Flavoured Kombuchas on *Drosophila melanogaster*. *Food Technology and Biotechnology*, *62*(2), 133-139.

Cagniard-Latour, C. (1838). Mémoire sur la fermentation vineuse. *Annales de chimie et de physique*, *68*, 206-222.

Calmette, A., Guérin, C., Boquet, A., & Nègre, L. (1927). *La vaccination préventive contre la tuberculose par le" BCG,"*. Masson et cie.

Cavalheiro, J. M., de Almeida, M. C. M., Grandfils, C., & Da Fonseca, M. M. R. (2009). Poly (3-hydroxybutyrate) production by *Cupriavidus necator* using waste glycerol. *Process Biochemistry*, *44*(5), 509-515.

Cibi, R., & Nair, A. J. (2016). Evaluation of the efficacy of the compound actinomycin d in tumor cell lines. *International Journal of Current Microbiology and Applied Sciences*, *5*, 405-411.

Corazza, G. R., Benati, G., Sorge, M., Strocchi, A., Calza, G., & Gasbarrini, G. (1992). β-Galactosidase from *Aspergillus niger* in adult lactose malabsorption: a double-blind crossover study. *Alimentary Pharmacology & Therapeutics*, *6(1)*, 61-66.

Cufaoglu, G., & Erdinc, A. N. (2023). An alternative source of probiotics: Water kefir. *Food Frontiers*, *4*(1), 21-31.

da Anunciação, T. A., Guedes, J. D. S., Tavares, P. P. L. G., de Melo Borges, F. E., Ferreira, D. D., Costa, J. A. V., et al. (2024). Biological Significance of Probiotic Microorganisms from Kefir and Kombucha: A Review. *Microorganisms*, *12*(6), 1127.

Dahech, I., Belghith, K. S., Hamden, K., Feki, A., Belghith, H., & Mejdoub, H. (2011). Oral administration of levan polysaccharide reduces the alloxan-induced oxidative stress in rats. *International Journal of Biological Macromolecules*, *49(5)*, 942-947.

Doudna, J. A., & Charpentier, E. (2014). The new frontier of genome engineering with CRISPR-Cas9. *Science*, *346(6213)*, 1258096.

Du, G., Wu, Y., Kang, W., Xu, Y., Li, S., & Xue, C. (2022). Enhanced butanol production in *Clostridium acetobutylicum* by manipulating metabolic pathway genes. *Process Biochemistry*, *114*, 134-138.

Erkmen, O., & Bozoglu, T. F. (2016). Basic principles of food fermentation. Food Microbiology: Principles into Practice. Chichester, the United Kingdom, John Wiley and Sons Ltd, 228-252.

Eungrasamee, K., Miao, R., Incharoensakdi, A., Lindblad, P., & Jantaro, S. (2019). Improved lipid production via fatty acid biosynthesis and free fatty acid recycling in engineered *Synechocystis* sp. PCC 6803. *Biotechnology for Biofuels*, *12*, 1-13.

Feketshane, Z., Alven, S., & Aderibigbe, B. A. (2022). Gellan gum in wound dressing scaffolds. *Polymers*, *14*(19), 4098.

Fleming, A. (1929). On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of *B. influenzae. British Journal of Experimental Pathology*, *10(3)*, 226.

Franciosa, I., Alessandria, V., Dolci, P., Rantsiou, K., & Cocolin, L. (2018). Sausage fermentation and starter cultures in the era of molecular biology methods. *International Journal of Food Microbiology*, *279*, 26-32.

Gea, S., Reynolds, C. T., Roohpour, N., Wirjosentono, B., Soykeabkaew, N., Bilotti, E., & Peijs, T. (2011). Investigation into the structural, morphological, mechanical and thermal behaviour of bacterial cellulose after a two-step purification process. *Bioresource Technology*, *102(19)*, 9105-9110.

Giavasis, I., Harvey, L. M., & McNeil, B. (2000). Gellan gum. *Critical Reviews in Biotechnology*, *20*(3), 177-211.

Goeddel, D. V., Heyneker, H. L., Hozumi, T., Arentzen, R., Itakura, K., Yansura, D. G., ... & Seeburg, P. H. (1979). Direct expression in *Escherichia coli* of a DNA sequence coding for human growth hormone. *Nature*, *281(5732)*, 544-548.

Gong, Y., Wang, C., Lai, R. C., Su, K., Zhang, F., & Wang, D. A. (2009). An improved injectable polysaccharide hydrogel: modified gellan gum for long-term cartilage regeneration in vitro. *Journal of Materials Chemistry*, *19(14)*, 1968-1977.

Hachmeister, K. A., & Fung, D. Y. (1993). Tempeh: a mold-modified indigenous fermented food made from soybeans and/or cereal grains. *Critical Reviews in Microbiology*, *19*(3), 137-188.

Han, Y. W. (1989). Levan production by *Bacillus polymyxa*. *Journal of Industrial Microbiology and Biotechnology*, *4*(6), 447-451.

Han, Y. W. (1990). Microbial levan. *Advances in Applied Microbiology*, *35*, 171-194.

Hernández, L., Arrieta, J., Betancourt, L., Falcón, V., Madrazo, J., Coego, A., & Menéndez, C. (1999). Levansucrase from *Acetobacter diazotrophicus* SRT4 is secreted via periplasm by a signal-peptide-dependent pathway. *Current Microbiology*, *39*, 146-152.

Hilgendorf, K., Wang, Y., Miller, M. J., & Jin, Y. S. (2024). Precision fermentation for improving the quality, flavor, safety, and sustainability of foods. *Current Opinion in Biotechnology*, *86*, 103084.

Hill, D., Sugrue, I., Arendt, E., Hill, C., Stanton, C., & Ross, R. P. (2017). Recent advances in microbial fermentation for dairy and health. *F1000 Research*, *6*.

Hosoi, T., & Kiuchi, K. (2003). Natto–a food made by fermenting cooked soybeans with *Bacillus subtilis* (natto). *Handbook of Fermented Functional Foods*, 227-245.

Jakob, F., Meißner, D., & Vogel, R. F. (2012). Comparison of novel GH 68 levansucrases of levan-overproducing *Gluconobacter* species. *Acetic Acid Bacteria*, *1(1)*, 2.

Jiang, L., & Wu, S. (2011). Effect of different nitrogen sources on activities of UDPG-pyrophosphorylase involved in pullulan synthesis and pullulan production by *Aureobasidium pullulans. Carbohydrate polymers*, *86*(2), 1085-1088.

Johnson, I. S. (1983). Human insulin from recombinant DNA technology. *Science*, *219(4585)*, 632-637.

Karas, J. A., Chen, F., Schneider-Futschik, E. K., Kang, Z., Hussein, M., Swarbrick, J., et al. (2020). Synthesis and structure− activity relationships of teixobactin. *Annals of the New York Academy of Sciences*, *1459(1)*, 86-105.

Kniskem, P. J., Hagopian, A., Montgomery, D. L., Burke, P., Dunn, N. R., Hofmann, K. J., et al. (1986). Unusually high-level expression of a foreign gene (hepatitis B virus core antigen) in *Saccharomyces cerevisiae*. *Gene*, *46*(1), 135-141.

Kooiman, P. (1968). The chemical structure of kefiran, the water-soluble polysaccharide of the kefir grain. *Carbohydrate Research*, *7*(2), 200-211.

Kumar, P., Chatli, M. K., Mehta, N., Singh, P., Malav, O. P., & Verma, A. K. (2017). Meat analogues: Health promising sustainable meat substitutes. *Critical Reviews in Food Science and Nutrition*, *57*(5), 923-932.

Kumar, S., & Nayak, B. B. (2015). Health benefits of fermented fish. *Health Benefits of Fermented Foods and Beverages*, 490-503.

Kuo, M. S., Mort, A. J., & Dell, A. (1986). Identification and location of L-glycerate, an unusual acyl substituent in gellan gum. *Carbohydrate Research*, *156*, 173-187.

Kusumoto, K. I., Yamagata, Y., Tazawa, R., Kitagawa, M., Kato, T., Isobe, K., & Kashiwagi, Y. (2021). Japanese traditional Miso and Koji making. *Journal of Fungi*, *7*(7), 579.

Lahiri, D., Nag, M., Dutta, B., Dey, A., Sarkar, T., Pati, S., et al. (2021). Bacterial cellulose: Production, characterization, and application as antimicrobial agent. *International Journal of Molecular Sciences*, *22*(23), 12984.

Lin, D., Liu, Z., Shen, R., Chen, S., & Yang, X. (2020). Bacterial cellulose in food industry: Current research and future prospects. *International Journal of Biological Macromolecules*, 158, 1007-1019.

Ling, L. L., Schneider, T., Peoples, A. J., Spoering, A. L., Engels, I., Conlon, B. P., et al. (2015). A new antibiotic kills pathogens without detectable resistance. *Nature, 517(7535)*, 455-459.

Liu, C. H., Hsu, W. H., Lee, F. L., & Liao, C. C. (1996). The isolation and identification of microbes from a fermented tea beverage, Haipao, and their interactions during Haipao fermentation. *Food Microbiology, 13(6),* 407-415.

Lopitz-Otsoa, F., Rementeria, A., Elguezabal, N., & Garaizar, J. (2006). Kefir: A symbiotic yeast-bacteria community with alleged healthy capabilities. *Revista Iberoamericana de Micologia, 23(2),* 67.

Louhasakul, Y., & Cheirsilp, B. (2013). Industrial waste utilization for low-cost production of raw material oil through microbial fermentation. *Applied Biochemistry and Biotechnology*, *169*, 110-122.

Lourens-Hattingh, A., & Viljoen, B. C. (2001). Yogurt as probiotic carrier food. *International Dairy Journal*, *11*(1-2), 1-17.

Love, J. (2022). Microbial pathways for advanced biofuel production. *Biochemical Society Transactions, 50(2),* 987-1001.

Matsuoka, M., Tsuchida, T., Matsushita, K., Adachi, O., & Yoshinaga, F. (1996). A synthetic medium for bacterial cellulose production by *Acetobacter xylinum* subsp. *sucrofermentans*. *Bioscience, Biotechnology, and Biochemistry*, *60*(4), 575-579.

Mayser, P., Fromme, S., Leitzmann, G., & Gründer, K. (1995). The yeast spectrum of the 'tea fungus Kombucha' Das Hefespektrum des 'Teepilzes Kombucha'. *Mycoses*, *38*(7-8), 289-295.

McAleer, W. J., Buynak, E. B., Maigetter, R. Z., Wampler, D. E., Miller, W. J., & Hilleman, M. R. (1984). Human hepatitis B vaccine from recombinant yeast. *Nature*, *307*(5947), 178-180.

Merchant, S. S., Prochnik, S. E., Vallon, O., Harris, E. H., Karpowicz, S. J., Witman, G. B., et al. (2007). The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. *Science*, *318*(5848), 245-250.

Mi, J., Becher, D., Lubuta, P., Dany, S., Tusch, K., Schewe, H., et al. (2014). *De novo* production of the monoterpenoid geranic acid by metabolically engineered *Pseudomonas putida*. *Microbial Cell Factories*, *13*, 1-11.

Mirsalami, S. M., & Alihosseini, A. (2021). Selection of the most effective kinetic model of lactase hydrolysis by immobilized *Aspergillus niger* and free β-galactosidase. *Journal of Saudi Chemical Society, 25(12),* 101395.

Moradi, Z., & Kalanpour, N. (2019). Kefiran, a branched polysaccharide: Preparation, properties and applications: A review. *Carbohydrate Polymers, 223*, 115100.

Ng, I. S., Tan, S. I., Kao, P. H., Chang, Y. K., & Chang, J. S. (2017). Recent developments on genetic engineering of microalgae for biofuels and bio-based chemicals. *Biotechnology Journal, 12(10),* 1600644.

Öner, E. T., Hernández, L., & Combie, J. (2016). Review of Levan polysaccharide: From a century of past experiences to future prospects. *Biotechnology Advances*, *34*(5), 827-844.

Pagliano, G., Ventorino, V., Panico, A., & Pepe, O. (2017). Integrated systems for biopolymers and bioenergy production from organic waste and by-products: a review of microbial processes. *Biotechnology for Biofuels*, *10*, 1-24.

Parvez, S., Malik, K. A., Ah Kang, S., & Kim, H. Y. (2006). Probiotics and their fermented food products are beneficial for health. *Journal of Applied Microbiology*, *100*(6), 1171-1185.

Paschalidou, A., Tsatiris, M., & Kitikidou, K. (2016). Energy crops for biofuel production or for food? -SWOT analysis (case study: Greece). *Renewable Energy*, *93*, 636-647.

Pascoe, J., Hendon-Dunn, C. L., Birch, C. P., Williams, G. A., Chambers, M. A., & Bacon, J. (2020). Optimisation of *Mycobacterium bovis* bcg fermentation and storage survival. *Pharmaceutics, 12(9),* 900.

Patil, S. B., Inamdar, S. Z., Das, K. K., Akamanchi, K. G., Patil, A. V., Inamadar, A. C., et al. (2020). Tailor-made electrically-responsive poly (acrylamide)-graft-pullulan copolymer based transdermal drug delivery systems: synthesis, characterization, in-vitro and *ex-vivo* evaluation. *Journal of Drug Delivery Science and Technology*, *56*, 101525.

Paul, D., Arora, A., & Verma, M. L. (2021). Advances in microbial biofuel production. *Frontiers in Microbiology*, *12*, 746216.

Pepe, O., Ventorino, V., Cavella, S., Fagnano, M., & Brugno, R. (2013). Prebiotic content of bread prepared with flour from immature wheat grain and selected dextran-producing lactic acid bacteria. *Applied and Environmental Microbiology, 79(12),* 3779-3785.

Piao, Y., Yamashita, M., Kawaraichi, N., Asegawa, R., Ono, H., & Murooka, Y. (2004). Production of vitamin B12 in genetically engineered *Propionibacterium freudenreichii. Journal of Bioscience and Bioengineering, 98(3),* 167-173.

Piermaria, J., Diosma, G., Aquino, C., Garrote, G., & Abraham, A. (2015). Edible kefiran films as vehicle for probiotic microorganisms. *Innovative Food Science & Emerging Technologies*, *32*, 193-199.

Piwowarek, K., Lipińska, E., Hać-Szymańczuk, E., Bzducha-Wróbel, A., & Synowiec, A. (2018). Research on the ability of propionic acid and vitamin B12 biosynthesis by *Propionibacterium freudenreichii* strain T82. *Antonie van leeuwenhoek, 111,* 921-932.

Prasad, S., Dhanya, M. S., Gupta, N., & Kumar, A. (2012). Biofuels from biomass: a sustainable alternative to energy and environment. *Biochemical and Cellular Archives 12(2)*, 255-260.

Prasad, S., Kumar, S., Yadav, K. K., Choudhry, J., Kamyab, H., Bach, Q. V., et al. (2020). Screening and evaluation of cellulytic fungal strains for saccharification and bioethanol production from rice residue. *Energy, 190*, 116422.

Rahman, M. (2013). Medical applications of fermentation technology. *Advanced Materials Research, 810*, 127-157.

Saeed, F., Afzaal, M., Shah, Y. A., Khan, M. H., Hussain, M., Ikram, A., et al. (2022). Miso: A traditional nutritious & health-endorsing fermented product. *Food Science & Nutrition, 10*(12), 4103-4111.

Santos-Merino, M., Garcillán-Barcia, M. P., & De La Cruz, F. (2018). Engineering the fatty acid synthesis pathway in *Synechococcus elongatus* PCC 7942 improves omega-3 fatty acid production. *Biotechnology for Biofuels*, *11*, 1-13.

Schatz, A., Bugle, E., & Waksman, S. A. (1944). Streptomycin, a substance exhibiting antibiotic activity against gram-positive and gram-negative bacteria.∗. *Proceedings of the Society for Experimental Biology and Medicine*, *55*(1), 66-69.

Schwann T (1837) Vorläufige Mittheilung, betreffend Versuche über die Weingärung und Fäulnis. *Annals of Physics 11*(2):184

Sezer, A. D., Kazak Sarılmışer, H., Rayaman, E., Çevikbaş, A., Öner, E. T., & Akbuğa, J. (2017). Development and characterization of vancomycin-loaded levan-based microparticular system for drug delivery. *Pharmaceutical Development and Technology*, *22*(5), 627-634.

Shah, S. H., Raja, I. A., Rizwan, M., Rashid, N., Mahmood, Q., Shah, F. A., & Pervez, A. (2018). Potential of microalgal biodiesel production and its sustainability perspectives in Pakistan. *Renewable and Sustainable Energy Reviews*, *81*, 76-92.

Sharma, R., Garg, P., Kumar, P., Bhatia, S. K., & Kulshrestha, S. (2020). Microbial fermentation and its role in quality improvement of fermented foods. *Fermentation*, *6*(4), 106.

Shi, Z., Zhang, Y., Phillips, G. O., & Yang, G. (2014). Utilization of bacterial cellulose in food. *Food Hydrocolloids*, *35*, 539-545.

Shrestha, B., Pokhrel, A. R., Darsandhari, S., Parajuli, P., Sohng, J. K., & Pandey, R. P. (2019). Engineering *Streptomyces peucetius* for doxorubicin and daunorubicin biosynthesis. Pharmaceuticals from Microbes: *The Bioengineering Perspective*, 191-209.

Son, H. J., Heo, M. S., Kim, Y. G., & Lee, S. J. (2001). Optimization of fermentation conditions for the production of bacterial cellulose by a newly isolated *Acetobacter*. *Biotechnology and applied biochemistry*, *33*(1), 1-5.

Srivastava, N., Kharwar, R. K., & Mishra, P. K. (2019). Cost economy analysis of biomass-based biofuel production. In New and Future Developments in Microbial Biotechnology and Bioengineering (pp. 1-10). Elsevier.

Suwannapinunt, N., Burakorn, J., & Thaenthanee, S. (2007). Effect of culture conditions on bacterial cellulose (BC) production from *Acetobacter xylinum* TISTR976 and physical properties of BC parchment paper. *Suranaree Journal of Science and Technology 14*(4), 357-365.

Swain, M. R., Anandharaj, M., Ray, R. C., & Parveen Rani, R. (2014). Fermented fruits and vegetables of Asia: a potential source of probiotics. *Biotechnology Research International*, *2014*(1), 250424.

Tefon-Öztürk, B. E., Eroğlu, B., Delik, E., Çiçek, M., & Çiçek, E., (2023). Comprehensive Evaluation of Three Important Herbs for Kombucha Fermentation. *Food Technology and Biotechnology*, vol.61, no.1, 1-28.

Vijayendra, S. V. N., & Shamala, T. R. (2014). Film forming microbial biopolymers for commercial applications A review. *Critical Reviews in Biotechnology*, *34*(4), 338-357.

Wang, Z., Okutsu, K., Futagami, T., Yoshizaki, Y., Tamaki, H., Maruyama, T., et al. (2020). Microbial community structure and chemical constituents in Shinkiku, a fermented crude drug used in Kampo medicine. *Frontiers in Nutrition*, *7*, 115.

Williams, W. K., & Katz, E. (1977). Development of a chemically defined medium for the synthesis of actinomycin D by *Streptomyces parvulus*. *Antimicrobial Agents and Chemotherapy*, *11*(2), 281-290.

Xu, P., Qiao, K., Ahn, W. S., & Stephanopoulos, G. (2016). Engineering Yarrowia lipolytica as a platform for synthesis of drop-in transportation fuels and oleochemicals. *Proceedings of the National Academy of Sciences*, *113*(39), 10848-10853.

Yafetto, L., Odamtten, G. T., & Wiafe-Kwagyan, M. (2023). Valorization of agro-industrial wastes into animal feed through microbial fermentation: A review of the global and Ghanaian case. *Heliyon*, *9*(4).

Yan, J., Kuang, Y., Gui, X., Han, X., & Yan, Y. (2019). Engineering a malic enzyme to enhance lipid accumulation in *Chlorella protothecoides* and direct production of biodiesel from the microalgal biomass. *Biomass and Bioenergy*, *122*, 298-304.

Zagorec, M., & Champomier-Vergès, M. C. (2017). *Lactobacillus sakei*: A starter for sausage fermentation, a protective culture for meat products. *Microorganisms*, *5*(3), 56.

Zajšek, K., Kolar, M., & Goršek, A. (2011). Characterisation of the exopolysaccharide kefiran produced by lactic acid bacteria entrapped within natural kefir grains. *International Journal of Dairy Technology*, *64*(4), 544-548.

UNVEILING NATURE'S POTENTIAL IN BIOFUELS – EXPLORING TYPES, GENERATIONS, PRETREATMENT METHODS, CHALLENGES, AND RECENT ADVANCES IN PRODUCTION TECHNOLOGIES

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Abstract

The world's population is growing and, in parallel, people's energy demands are increasing rapidly. Since fossil fuels will eventually run out, researchers have begun the search for new alternative energy sources. The desire to reduce greenhouse gas emissions and the increasing demand for energy emphasize the need to produce sustainable and financially viable biofuels. The present chapter provides an overview of current studies and developments in the production of biofuels. The chapter examines various sustainable and cost-efficient biofuel production methods, such as utilizing non-edible and waste materials, and algae as feedstock. It discusses advancements in catalysis and explores genetic and metabolic engineering, and synthetic biology approaches to increase biofuel production efficiency. Therefore, this chapter provides valuable insights into future directions for affordable biofuel production and explores diverse sustainable and economically feasible pathways to satisfy the increasing demand for renewable energy and improve biofuels' competitiveness with fossil fuels.

Introduction

Fossil fuels are still the main energy source necessary for many businesses. Global population growth and improvements in living standards have led to a sharp increase in the demand for energy. However, using fossil fuels comes with a lot of risks and disadvantages. These fuels are used, which pollutes the environment and endangers the lives of living things. Burning fossil fuels discharge numerous pollutants into the atmosphere, including carbon dioxide (CO_2), nitrogen oxides (NO), sulfur dioxide (SO_2), methane (CH_4), particulate matter, and mercury (Golomb et al., 2020; Dunkle and Winniford, 2020). These emissions, in turn, promote climate change, causing acid rains, biological extinctions, and several health issues. Additionally, governments are concerned about the security of energy supply, as fossil fuel will ultimately run out due to their non-renewable and unsustainable nature. For all these reasons, the search for alternative fuels has increased interest and research in renewable energy sources, especially biofuels (Gurkok, 2024).

Biofuels are a diverse category of renewable, biodegradable, and environmentally friendly fuels produced from organic materials, primarily plants, offering a sustainable alternative to traditional fossil fuels. Based on

the feedstocks used for their production, biofuels are categorized into four generations as given in Table 1.

Table 1. Generations of Biofuels

Generations	Feedstocks	References
First generation	Food Crops: Corn, sugarcane, wheat, sugar cane, sugar beet, canola, maize, sunflower oil, soybean oil, palm oil, rapeseed oil	Singh et al., 2023
Second generation	Agricultural/domestic/industrial residues: Wood, straw, stalks and cobs, manure, food waste. Dedicated energy crops: Miscanthus, switchgrass, Jatropa, Camelina, poplar	Ingale et al., 2014; Han et al., 2020
Third generation	Algae and other microorganisms	Rafa et al., 2021; Wang et al., 2024
Fourth generation	Genetically modified microorganisms/microalgae	Tandon and Jin, 2017; Abdullah et al., 2019; Shokravi et al., 2022

The first-generation biofuels are derived from food crops including corn, soybeans, and sugarcane. The second-generation biofuels utilize non-food biomass like agricultural residues, switchgrass, wood, and dedicated energy crops. The third-generation biofuels come from algae and other microorganisms, which have higher growth rates and can be grown on non-arable land (Rafa et al., 2021). Fourth-generation biofuels employ advanced technologies, including genetically engineered organisms and carbon capture and utilization techniques, to enhance sustainability and reduce environmental impact (Shokravi et al., 2022). It is anticipated that biofuels of fifth-generation, which are now in the research and development phase, will employ synthetic biology to produce entirely synthetic biofuels (Padder et al., 2024).

Biofuels can exist in liquid, solid, and gaseous states and come in various types (Figure 1). Liquid biofuels include biodiesel and bioethanol, which are commonly used as substitutes for diesel and gasoline, respectively. Solid biofuels, such as biomass pellets (compressed sawdust, wood shavings) and briquettes, are used for heating and electricity generation. Gaseous biofuels, like biogas (CH_4, CO_2, H_2S) and syngas (CO, H_2, CH_4, CO_2), can be used for heating, electricity, and as a substitute for natural gas. Each type of

biofuel has specific applications depending on its state and properties, contributing to the versatility and adaptability of biofuels in different energy sectors.

Advantages and Disadvantages of Biofuels

Biofuels offer several advantages as well as disadvantages. Biofuels are derived from renewable sources, making them potentially sustainable compared to fossil fuels. Typically, they emit fewer greenhouse gases during combustion juxtaposed with fossil fuels, contributing to lower carbon footprints. Biofuels can improve energy security by lowering reliance on imported fossil fuels, especially in regions with abundant biomass resources. Many biofuels can be employed with little to no change in currently installed combustion engines and infrastructure.

However, difficulties arise in producing biofuels, like demand for agricultural area use, costs, and availability of feedstocks, need for skilled professionals, technical constraints, sustainability concerns, and limitations in infrastructure and market access. Production of biofuels, especially first-generation biofuels, may put food crops in competition with it for resources and land, potentially impacting food prices and security. Increased demand for biofuel feedstocks may drive land use changes, such as deforestation or displacement of food production, with potential negative environmental consequences. Intensive farming practices for biofuel feedstocks may result in habitat loss, deforestation, and increased usage of fertilizers and pesticides, affecting biodiversity and water quality.

Types of Biofuels

Bioethanol

Bioethanol is a liquid type of alcohol produced through fermentation procedures using glucose obtained from crops including sugarcane, corn, wheat, molasses, potato, maize, sweet sorghum, and fruits. Agriculture waste, wood and trees containing cellulose can also be digested into glucose molecules by different pretreatment methods and then can be used in fermentation to produce bioethanol.

The production of bioethanol involves a number of steps. The pretreatment and digestion of macromolecules (polysaccharides) into

micromolecules (glucose) constitutes the initial stage. The second stage is the fermentation, which utilizes microorganisms and/or enzymatic activity to convert glucose into ethanol. The final stage is the purification of ethanol, which is typically achieved through distillation.

As an alternative to traditional fossil fuels, bioethanol can be used in a variety of applications. It is commonly used as an additive in conventional gasoline. Biofuel can be mixed to create ethanol-blended gasoline fuels such as E10 (90% gasoline, 10% ethanol,) and E85 (15% gasoline, 85% ethanol), which then can be used in flex-fuel vehicles as transportation fuel. Bioethanol can also be used as a solvent for the manufacture of a number of products such as personal care items, paints, pharmaceuticals, and cosmetics. It can play role as a feedstock for producing chemicals like ethylene, which is used to make plastics and other synthetic materials.

Biodiesel

Biodiesel is a kind of biofuel produced from fats, oils, or oil-derivatives through a process called transesterification. The feedstocks for biodiesel production include animal fats, vegetable oils (including sunflower oil, soybean oil, and palm oil), recycled cooking oils, and algae. In transesterification, these feedstocks react with methanol (or ethanol) facilitated by a catalyst, which can be either acidic or basic. This reaction generates fatty acid methyl esters as the main product and glycerol as a byproduct.

Biodiesel can be employed in diesel-powered vehicles in its pure form (B100) and other modified equipment when blended with petroleum diesel. However, in cold weather, especially during winter, issues may arise due to the higher viscosity of biodiesel at low temperatures, which depends on the type of biomass used (Najafi et al., 2009).

Green diesel, also known as hydrotreated vegetable oil, is produced through a process called hydro-processing. In this process, vegetable oils, animal fats, or other renewable lipids are treated with H_2 with the aid of a catalyst. This process removes oxygen and converts the triglycerides and free fatty acids into hydrocarbons, resulting in a fuel that is chemically similar to petroleum diesel. Green diesel consists of hydrocarbons that are very similar to those found in conventional diesel. This similarity ensures that green diesel has properties nearly identical to fossil diesel, making it suitable with diesel

engines that are currently in use and infrastructure without alterations. Therefore, green diesel typically offers superior performance and combustion properties compared to biodiesel. It burns cleaner, leading to fewer emissions of pollutants like CO, nitrogen oxides, and particulate matter.

Biogas

Biogas is another environmentally friendly and renewable energy source generated during break down of organic compounds by microorganisms anaerobically. This gas primarily consists of CH_4 and CO_2, along with small quantities of additional gases like hydrogen sulfide (H_2S), ammonia (NH_3), water vapor, hydrocarbons, siloxanes, CO, N_2, and O_2 (Kougias and Angelidaki, 2018). The byproduct of biogas, in solid form, can serve as fertilizer or biofuel. Once other mixtures and CO_2 are removed from it, biogas is known as CH_4 gas. Biogas can be utilized for heating, electricity generation, and as a vehicle fuel.

Biogas can be generated either from crops or through the biodegradation of waste materials including agricultural waste, food waste, sewage sludge, and manure under anaerobic conditions. Biogas production process includes four key phases: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Rasapoor et al., 2020). Firstly, complicated larger organic substances are separated into simpler soluble molecules, which were then converted into volatile fatty acids, alcohols, and gases. Volatile fatty acids are further transformed into acetic acid, H_2, and CO_2 during acetogenesis. Ultimately, acetic acid, H_2, and CO_2 are transformed into CH_4 and CO_2 by methanogenic archaea.

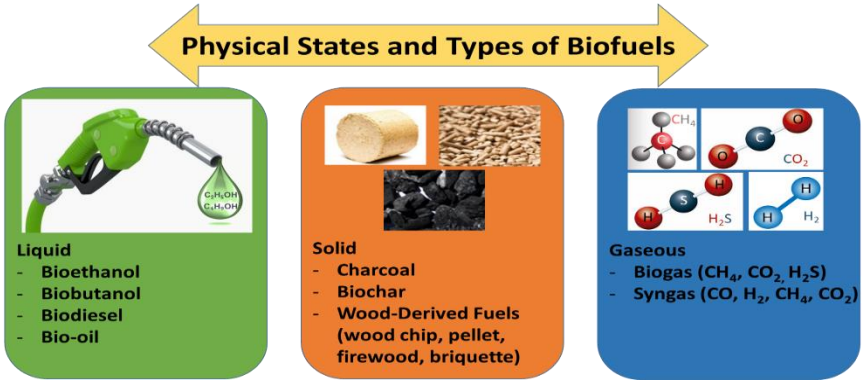

Figure 1. Overview of the physical states and types of biofuels

Generations of Biofuels

First-Generation (Conventional) Biofuel

First-generation biofuels, also known as conventional biofuels, are derived from feedstocks such as corn, cassava, barley, wheat, potatoes, sorghum, rice, and soybeans cultivated on arable land for bioethanol production. Similarly, first-generation biodiesel is created from edible oils such as soybean oil, corn oil, rapeseed oil, rice oil, coconut oil, and mustard oil converted into bioethanol and biodiesel through yeast fermentation and transesterification (Singh et al., 2023).

Second-Generation Biofuels

Second-generation biofuels, called as advanced biofuels, are generated from non-food sources. Second-generation biofuels are derived from lignocellulosic materials (e.g., dedicated energy crops, agricultural and forestry wastes) and other non-food biomass, as opposed to first-generation biofuels, which are manufactured from food crops like corn and sugarcane. These biofuels aim to address the limitations of first-generation biofuels, including competition with food production and land use changes (Ingale et al., 2014).

Cellulosic feedstocks like crop residues, wood chips, grasses, corn stover, wheat straw, switchgrass, and miscanthus are broken down into sugars through pretreatment and enzymatic hydrolysis. These sugars are then fermented to produce ethanol. Lignocellulosic feedstocks like agricultural residues, wood, and energy crops are also fermented using specific microorganisms to produce butanol, a biofuel with properties similar to gasoline.

Forestry, agricultural, and municipal solid residues can be gasified to produce syngas (CO and H_2 mixture) and then, it is transformed into liquid hydrocarbons via the Fischer-Tropsch synthesis or other catalytic processes (dos Santos and Alencar, 2020).

Non-food oil and fat sources including used cooking oil, animal fats, and non-edible plant oils like jatropha and camelina are hydrotreated, converting them into hydrocarbons similar to conventional diesel and jet fuels.

The utilization of non-food feedstocks that does not compete with food crops, addressing food security concerns and reducing pressure on arable land. Additionally, converting agricultural, forestry, and municipal waste into valuable energy resources, second generation biofuels contribute to waste management and circular economy principles. They also offer significant greenhouse gas emission reductions compared to fossil fuels, depending on the feedstock and production process.

Third-Generation (Algae-Based) Biofuels

Third-generation biofuels are algae-based biofuels. In addition to algae, other advanced biological systems are employed as feedstocks in the third generation biofuels. Third-generation biofuel production aim to overcome the limitations of first and second-generation biofuels, such as competition with food crops and the need for large agricultural lands. Algae are accepted as one of the most promising sources for third generation biofuels due to their fast growth rates, high photosynthesis efficiency, ability to grow in non-arable land and water, and lipid-rich content (Peng et al., 2020).

Microalgae and macroalgae can be both utilized for the generation of biofuels (Ismail et al., 2024). Microalgae are unicellular organisms that can be cultivated in freshwater, seawater, or wastewater. They can make a high yield of lipids which can be transformed to biofuels (Wang et al., 2024). Macroalgae on the other hand, are multicellular algae and are harvested for their carbohydrate content. These carbohydrate content can be subjected to fermentation to produce ethanol and other biofuels (Twigg et al., 2024). Algae biomass is heated to high temperatures and pressures in water, breaking down the organic material into bio-crude oil during hydrothermal liquefaction. This bio-crude can be refined into a variety of fuels, such as gasoline, and diesel. In transesterification, lipids extracted from algae are interacted with an alcohol (methanol or ethanol) with the help of a catalyst to generate biodiesel and glycerol. Carbohydrates in algae can also be fermented by microorganisms to produce ethanol, butanol, and other alcohol-based biofuels (Kulikova et al., 2024; Suhartini et al., 2024).

Fourth-Generation Biofuels

Fourth-generation biofuels are still in research and development phase, aiming to overcome limitations/challanges of earlier generations of biofuels. The fourth-generation of biofuel is produced by genetically modified

organisms, such as cyanobacteria and algae. Techniques such as enhancing photosynthetic efficiency, improving light absorption, and minimizing photoinhibition are frequently employed in genetic modification of microalgae to increase biofuel production (Tandon and Jin, 2017; Abdullah et al., 2019).

Genetically modified organisms make use of water, CO_2, and solar energy to produce organic compounds, which are converted into biofuels. Production of fourth-generation biofuels is still primarily at the research level. However, biofuels produced by bioengineering methods exhibit higher photon-to-fuel conversion efficiency compared to other generations of biofuels.

Fifth-Generation Biofuels

Fifth generation biofuels are the latest development in the field of biofuels; characterized by new technology and sustainable practices that ensure maximum efficiency and positive environmental impact. Unlike preceding generations, fifth generation biofuels employ synthetic biology and nanotechnology for better ways of converting biomass to fuel. This is aimed at attaining carbon neutrality as well as minimizing environmental effects and maximizing on resource use (Padder et al., 2024).

This makes such non-food biomass feedstocks as agricultural residues, waste biomass and even CO_2 from industrial processes not compete with food production thereby ensuring sustainability. Integration of synthetic biology, genetic engineering and nanotechnology throughout the process of producing biofuels involves improving feedstock traits, fermentation efficiencies and developing new catalysts for conversion. The process of cultivating, processing, distributing so as to minimize environmental footprint while decreasing greenhouse gas emissions via carbon neutral methods enhances environmental sustainability. Moreover, production of fifth-generation biofuels is intended to achieve higher energy yields per unit biomass input than its predecessors, thereby improving overall energy efficiency and cost-effectiveness. Beyond biofuels, fifth-generation technologies aim to produce valuable co-products such as bio-based chemicals, materials, and pharmaceuticals, enhancing the economic viability and sustainability of the entire bioeconomy. Fifth-generation biofuels have potential as a sustainable replacement for traditional fossil fuels, even if they are still in the research and development stage.

Pretreatment Techniques

Pretreatment techniques for biofuel production are essential processes that prepare biomass feedstocks for subsequent conversion into biofuels. These methods are crucial because they break down the polymeric complex structure of biomass materials into smaller molecules such as sugars, acids, oils, and amino acids making them more accessible to enzymatic or chemical processes that convert them into biofuels efficiently (Kumar and Sharma, 2017; Onumaegbu et al., 2018). Lignocellulosic feedstock is considered a promising source of biofuel due to its abundant availability globaly. Similarly, starch-containing feedstocks such as corn, cassava, barley, wheat, potatoes, sorghum, and rice are widely used for biofuel production. However, both types of feedstocks need pretreatment processes to break them down into basic sugars before they are metabolized and fermented into ethanol or other biofuels. The most commonly used pretreatment methods are discussed as follows:

Chemical Pretreatment

In chemical pretreatment methods, acidic and alkali substances, water or solvents are used to modify structure of biomass (Bay et al., 2020; Sołowski et al., 2020). Acid pretreatment use dilute or concentrated acids to break down hemicellulose and cellulose into fermentable sugars. Contrarily, alkaline pretreatment is employed to remove lignin and improves the digestibility of cellulose (Bay et al., 2020). On another hand, solvent based pretreatments dissolve lignin and allows the easy separation of cellulose and hemicelluloses from biomass (Chandel et al., 2024).

Biological Pretreatment

Unlike conventional physical or chemical techniques of pre-treating feedstocks, biological stripping is known for its efficiency, safety to the environment as well as minimal energy consumption. Biological means involve microbes or enzymes that break complex biomass substrates such as starches, lignins and celluloses into simpler forms – sugars. For example, bioethanol production involves fermentation of these sugars by using microorganisms plus enzymes. However, microorganisms are not capable of directly metabolizing either starch or cellulose. Initially however, polysaccharides present in biomass like starches or celluloses have to go through hydrolysis process so as to breakdown them into smaller sugars i.e.

glucose. This enzymatic process is crucial for making these sugars accessible for subsequent fermentation into bioethanol or other biofuels.

Microorganisms such as white-rot fungi, soft-rot fungi and brown-rot fungi together with enzymes like amylolytic and cellulolytic hydrolytic are the primary biological pretreatment agents.

During biofuel production microbial enzymes from fungal and bacterial strains have been found to be useful in biological pretreatment processes. Among other things, this is because of their high cellulase and hemi-cellulases synthesis efficiency which is important in the degradation of cellulose and hemicellulose major plant biomass constituents. Bacteria produce a wide range of enzymes, including proteases, lipases, amylases among others that are involved in decomposition of different kinds of organic matter. After enzymatic hydrolysis plant biomass is converted into fermentable sugars that can be fermented by yeast or bacteria to make ethanol. The microbial enzymes in biogas production have a vital role of hydrolyzing the organic waste material to simpler compounds. This enzymatic action intensifies anaerobic digestion process thereby increased CH_4 yield is realized. In biodiesel production lipases are employed in the transesterification of fats and oils, which is a critical step. Enzymes are also essential during pretreatment of algal biomass whereby cellulose, hemicelluloses, xylans, and other polysaccharides make up the algal biomass cell walls. These enzymes may help decompose the algal cell wall without harming interior cellular components leading to easy retrieval of valuable substances that can be used as biofuel (Mahapatra and Singh 2024; El-Sayed et al. 2024).

α-amylase enzymes catalyze organic matter rich with plenty of starch transforming branched polysaccharides with α-1,6-glucosidic linkages into linear oligosaccharide units through hydrolysis. Fermentable sugars like sucrose, glucose, maltose and fructose are produced by glucoamylase enzymes during saccharification. Biomass with high cellulose content undergoes hydrolysis with the involvement of three groups of cellulolytic enzymes: exoglucanase, which degrades and removes cellobiose residues from free chain-ends; β-glucosidase, hydrolyzing cellobiose into glucose; and endoglucanase, which acts on the low crystallinity regions of cellulose fibers, leading to the formation of free chain-ends.

Enzymatic hydrolysis offers several advantages over chemical or physical methods. Firstly, it yields a higher amount of sugars due to its

specificity in targeting substrates, requiring lower energy consumption and operating at moderate temperatures. This method is also generally more cost-effective. Enzymatic hydrolysis, compared to other methods, is more time-consuming method and needs to be performed in a more controlled manner. Furthermore, since enzymes can act on specific substrates, biomass composed of different materials may require additional pretreatment steps to expose the active sites for efficient enzymatic action (Champagne, 2007).

Thermochemical Pretreatment - Pyrolysis

Thermochemical treatments take place by heating biomass under controlled conditions of temperature, pressure, and residence time. This pretreatment includes steam explosion, microwave-assisted heating, and torrefaction (Camani et al., 2020; Nhuchhen et al., 2014). Biomass structure is distrupted, enzymatic accessibility is improved, and the release of fermentable sugars is enhanced by these methods.

Pyrolysis is one of the thermochemical treatments that increase the energy density of feedstock through deoxygenation by emitting water and volatile compounds to yield an oil for the production of fuels. The process of pyrolysis occurs at a high temperature of 400 to 600 °C and involves the application of catalysts sometimes (Hoang et al., 2021).

Hydrothermal Liquefaction

Hydrothermal pretreatment is based on the processing of biomass reacting in a water-containing medium to form either gases or liquids. The process occurs under high temperatures and high pressures, normally ranging between 200-600 °C and 5-40 MPa, thus keeping the water in a supercritical and liquid phase simultaneously. Reactions between temperatures of 200–350 °C are suitable for liquid products, while those above 400 °C for gaseous products. The result of this process is a mixture of biomass, bio oils, and many other compounds (Sivakumar et al., 2010).

Combined Pretreatment:

Most of the pretreatment strategies in efficient biomass conversion combine more than one method for maximum output. For instance, it has been proven that the combination of physical and chemical methods acts synergistically in improving the digestibility of biomass and reduces the

overall processing cost. This will, therefore, draw on the power of each of these techniques to achieve better results than using any single technique alone.

The single pretreatment operations can face several challenges, such as technological issues, environmental pollution, high energy consumption, long reaction times, and stringent requirements for reaction equipment corrosion resistance. At the same time, these methods may fail to meet the standards required for industrial production, ultimately falling short of the desired effect.

To solve the above-mentioned problems, many combined pretreatment methods have been developed (Shirkavand et al., 2016). For example, mechanical crushing can be followed by chemical or biological treatments. Electronic radiation and alkali treatment or microwave and chemical processing can be paired with mechanical breakdown. Each of these combinations is tailored to different lignocellulosic materials, greatly improving the efficacy of enzymatic hydrolysis (Aguilar-Reynosa et al., 2017; Areepak et al., 2022).

The combination of multiple pretreatment methods offers a number of advantages. Mechanical breakdown reduces particle size and increase the surface area for following treatments. Chemical treatments break down complex polymeric compounds into simpler molecules and make them more accessible for enzymatic reactions. Physical treatments, such as electronic radiation and microwave processing, can disrupt the structure of biomass and enhance the effect of chemical treatments. Biological treatments can further break down biomass and facilitate easier conversion to biofuels. Combining several of these methods may overcome the limitations of single pretreatment techniques. For example, mechanical crushing followed by chemical treatment will certainly enhance the accessibility of biomass and reduce the need for harsh chemical use and extreme conditions. Similarly, integrating microwave processing with chemical treatments will accelerate reactions and improve overall efficiency (Ma'mun et al., 2024).

Techniques for Transforming Feedstock into Biofuel

Pretreated feedstocks can be transformed into biofuel through several thermochemical and biochemical methods, such as pyrolysis, gasification,

hydrothermal liquefaction, alcoholic fermentation, anaerobic digestion, and transesterification.

One of the prevalently used transforming technique is pyrolysis. It which is performed at temperatures ranging from 400 to 600 °C under atmospheric pressure. In pyrolysis, organic materials break down into vapor, gaseous compounds, and a carbon-rich solid residue known as char. The vapor phase is subsequently condensed into a liquid product referred to as bio-oil (which are refined into biodiesel), syngas (a mixture of CO and H_2), and biochar (a carbon-rich solid) (Chen et al., 2015; Hoang et al., 2021).

Another transforming process is gasification. In gasification, the feedstock is partially oxidized at elevated temperatures and it is an essential method for the thermochemical transformation of biomass. Biomass is changed into a versatile gaseous mixture which is known as syngas or synthesis gas with the help of a gasifying agent. Syngas is composed of a mixture of primary components, including CO, H_2, CO_2, and CH_4, along with secondary ingredients such as H_2O, H_2S, NH_3, tar, and other trace elements. Its composition varies based on the type and characteristics of the feedstock, operating conditions (such as gasifying agent, gasifier temperature and pressure, and type of bed materials), and the gasification technology used. Biomass-derived syngas serves as a feedstock in various thermochemical processes to produce second-generation biofuels. These biofuels can be either liquid, such as ethanol, methanol, dimethyl ether, and Fischer-Tropsch diesel, or gaseous, like synthetic natural gas and H_2 (Molino et al., 2018).

In hydrothermal liquefaction, wet biomass is heated in water under high pressure (typical pressures ranging from 5 to 21 MPa) turning it into liquid bio-crude (Han et al., 2019). Wet biomass can be directly processed without drying and therefore, it offers an energy-efficient technique for certain feedstocks. The wet biomass feedstock is converted into four main products: biocrude oil, an aqueous phase, solid residue, and a gaseous phase. This process uses typically water as a solvent, under an inert atmosphere The yield and quality of biocrude oil, the primary product of hydrothermal liquefaction, are significantly affected by various factors like the biomass composition, method of feedstock preparation, type of solvent used, reaction temperature, particle size distribution, heating rate, residence time, operating pressure, and both the type and amount of catalyst used (Shahbeik et al., 2024).

Biochemical conversion methods involve fermentation, anaerobic digestion and transesterification methods. Fermentation utilize microorganisms to convert monomeric units of sugars in feedstock into ethanol, which can be blended with gasoline to produce bioethanol. Although yeasts, fungi, and bacteria can carry out fermentation, yeasts are preferred for fermentation due to their high alcohol tolerance and yield. *Saccharomyces* and *Zymomonas* species are the most frequently used yeasts for bioethanol production (Robak and Balcerek, 2018; Samantaray et al., 2024).

Anaerobic digestion is a widely used technology for the generation of biofuels, specifically biogas, from organic materials. Through the anaerobic digestion process, organic material is broken down by microorganims without the presence of oxygen. This process produces biogas, which is mostly made up of CO_2 and CH_4, along with other trace gases. It is also an effective waste management solution that produces renewable energy and valuable by-products like fertilizer. Organic wastes such as food and agricultural residues, sewage sludge, manure, and industrial organic residues or energy crops grown specifically for energy production, such as maize and grass silage can be used as feedstock. The anaerobic digestion process involves several stages: Hydrolysis, in which complicated organic materials like carbohydrates, fats, and proteins, are decomposed into basic small molecules like sugars, fatty acids, and amino acids; Acidogenesis, that converts these simpler molecules into volatile fatty acids, alcohols, H_2, and CO_2; Acetogenesis, where volatile fatty acids are further broken down into acetic acid, CO_2, and H_2; and Methanogenesis, where methanogenic archaea convert them (acetic acid, H_2, and CO_2) into CH_4 and water (Gong et al., 2021).

Transesterification is a chemical process widely used for the production of biodiesel. This process involves the transformation of triglycerides in fats and oils into biodiesel and glycerol through the reaction with an alcohol, typically methanol or ethanol, facilitated with a catalyst. Vegetable oils (soybean oil, rapeseed oil, palm oil, and sunflower oil), animal fats (lard, tallow, and other rendered fats), used cooking oils, algal oils are used as feedstocks. Frequently base catalysts like sodium hydroxide (NaOH) or potassium hydroxide (KOH) are utilized for their efficiency and low cost. As acid catalysts, sulfuric acid (H_2SO_4) is preferred for feedstocks with high free fatty acid content. Enzymatic catalysts like lipases, offering milder reaction conditions and easier product separation, can also be used (Kalita et al., 2022).

Transesterification includes several steps. In the first step, the oil or fat feedstock is mixed with the alcohol in the presence of the catalyst. The mixture is then heated and stirred to allow the transesterification reaction to occur. This reaction converts triglycerides into fatty acid methyl esters and glycerol. The resulting mixture is let to settle, and then biodiesel is separated from the glycerol byproduct during separation step. The biodiesel is ultimately cleaned by washing and purified to eliminate the remaining catalyst, alcohol, and other contaminants. The separated glycerol is also a valuable byproduct, and is purified and can be used in a number of industries such as pharmaceuticals and cosmetics.

Challenges in Biofuel Production

There are a number of issues that must be resolved to increase the sustainability, efficiency, and economic viability of biofuel production. The global scarcity of biomass and the difficulties in maintaining a consistent and reliable feedstock supply is one of the primary challenges. Biomass limitation is prevalent worldwide due to varying economic circumstances and hinders its utilization for biofuel production. For the production of biofuels, the use of food crops may put the food supply in jeopardy, raising ethical and economic concerns. In certain countries, biomass, such as wood and cattle dung, serves as the primary fuel source, while potential biofuel feed-stocks like certain grasses are utilized as animal feed instead. Furthermore, food waste, including bread, oil, and rice, remains insufficiently utilized in less developed countries, often serving as feed for domestic animals such as chickens and dogs.

Non-edible food crops are used in the production of second-generation biofuels. However, this poses another problem. Widespread cultivation of these crops for biofuel production can results in deforestation and also loss of biodiversity. In addition, plants cultivated for biofuels often require large amounts of water and fertilizer, which will have negative impacts on the environment.

Recent Advances and Emerging Technologies for Sustainable Biofuel Production

Emerging technologies will make a significant contribution to reducing the challenges of biofuel production and increasing efficiency, sustainability and financial feasibility (Gurkok, 2024).

Second-generation biofuels provide an encouraging substitute for first-generation biofuels. Conversion of agricultural residues, forestry waste, and dedicated energy crops into biofuels are promising approaches for effective feedstock utilization. Developed and developing pretreatment methods like steam explosion, acid hydrolysis, and enzymatic breakdown are improving the efficiency of converting cellulose and hemicellulose into fermentable sugars.

Because of their oil-rich content and fast growing nature, research on algae as a feedstock is developing and can be considered as an advanced feedstock utilization. Recent advances in the development of algal cultivation systems like photobioreactors and open pond systems (Peter et al., 2022; Razzak et al., 2023), as well as genetic engineering to increase lipid production, are making algal biofuels more viable (Tandon and Jin, 2017; Chaturvedi et al., 2020; Shokravi et al., 2022).

As previously mentioned, fourth-generation biofuels are derived from genetically modified algae biomass to enhance biofuel production and strategies such as improving photosynthetic efficiency, increasing light penetration, and reducing photoinhibition are commonly employed in the genetic modification of microalgae (Abdullah et al., 2019). To enhance light penetration in dense microalgae cultures, one approach involves truncating the chlorophyll antenna of chloroplasts (Buitrón et al., 2017). Strategies also include reducing the size of chlorophyll antennas (Mussgnug et al., 2007), minimizing light absorption, and manipulating pigments to enhance light penetration into microalgae cultures (Nakajima and Ueda, 1997). Another method is expanding the spectrum range absorbed during photosynthesis to improve the photosynthetic efficiency of genetically modified microalgae (Wolf et al., 2018).

Modification of the genes required for the biosynthesis of triacylglycerol and fatty acids by genetic engineering, is another key strategy to increase lipid content in microalgae (Sun et al., 2019). Fatty acid oxidation-related genes (acyl CoA oxidase, acyl CoA synthase, carnitineacyltransferase I, and fatty acyl CoA dehydrogenase) and acetyl-CoA carboxylase (ACCase), which is involved in fatty acid synthesis, have been the focus of research for years.

Applications of synthetic biology for re-engineering metabolic pathways or microorganisms is one of the recent advances for more efficient biofuel production. Synthetic biology enables the design and introduction of

custom metabolic pathways into microorganisms to produce biofuels. Synthetic biology can create new pathways that do not exist in nature, providing the production of novel biofuels or improving the efficiency of already existing ones. Construction of synthetic genetic circuits and regulatory networks, scientists now can properly control the genes expressions involved in biofuel production. Precise control mechanism can optimize the timing and level of enzyme production, leading to more efficient biofuel synthesis. Synthetic biology provides the development of optimized microbial host organisms specifically tailored for biofuel production. Tailored host organisms can be engineered to have enhanced tolerance to biofuel toxicity, improved substrate utilization, and increased production rates. Metabolic pathways can also be optimized to minimize by-products and maximize the yield of desired biofuels. This involves fine-tuning the activity of enzymes and regulatory elements within the pathway. Also, synthetic biology employs a modular approach, where biological parts (like promoters, ribosome binding sites, and enzymes) can be standardized and combined in various different ways to create new pathways or optimize already existing ones. This facilitates rapid prototyping and testing of biofuel production pathways.

Metabolic engineering can significantly increase lipid or carbohydrate content, which are crucial factors for improving biomass yield efficiency of microalgae (Hsieh and Wu, 2009) and other microorganisms (Liang and Jiang, 2013). Engineered yeast and bacteria can directly ferment sugars into advanced biofuels like butanol, isobutanol, and even jet fuel precursors.

The application of CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9) technology has enabled precise genetic modifications in plants and microorganisms used for biofuel production. In algal biotechnology, targeted genome editing with RNA-guided endonucleases is a new approach. This approach can lead to crops with higher yield, better resistance to pests and diseases, and enhanced tolerance to environmental stresses. CRISPR-Cas systems have been frequently employed recently to modify the genomes of several marine and freshwater microalgae, allowing simultaneous editing of multiple genes (Patel et al., 2019).

S. cerevisiae is a popular and commonly employed yeast species in the industrial production of bioethanol from sucrose or starch-based glucose. Although *S. cerevisiae* can ferment glucose and other hexose sugars such as galactose and mannose into ethanol, it is unable to utilize the primary five-

carbon sugars, D-xylose and L-arabinose. *S. cerevisiae* could be engineered to utilize pentose sugars from lignocellulosic biomass, which are not naturally fermentable by wild-type strains. This involves incorporation of functional pathways for xylose and arabinose utilization (Madhavan et al., 2012).

For the synthesis of butanol and isobutanol, metabolic pathways have been optimized in various microbial hosts. Certain *Clostridium* species have been engineered to increase the butanol production (Tian et al., 2019; Wen et al., 2019). n-Butanol production from *C. cellulovorans* was improved by integrated metabolic and evolutionary engineering. By introducing the adhE1 and ctfA-ctfB-adc genes from *C. acetobutylicum* ATCC 824, researchers constructed a coenzyme A (CoA)-dependent acetone-butanol-ethanol (ABE) pathway in *C. cellulovorans*. This modification allowed the organism to generate n-butanol utilizing the plentiful and inexpensive agricultural residue of alkali-extracted, deshelled corn cobs as its only carbon source. A new adaptive laboratory evolution method was also employed to enhance the n-butanol tolerance of *C. cellulovorans*, enabling it to fully realize its potential for n-butanol production. For additional boost in n-butanol production, researchers combined metabolic engineering with evolutionary engineering by employing the improved strain as the host for metabolic modifications (Wen et al., 2019).

Certain butanol production routes have been implemented in *Escherichia coli*, an even more practical host compared to *Clostridium* species, to enhance butanol yields and production yields. Metabolic engineering attempts in *E. coli* aimed to enhance butanol production by optimizing central carbon metabolism, removing competitive pathways, balancing cofactors, creating novel pathways, utilizing homologous enzymes, exploring diverse substrates, and employing advanced molecular biology techniques (Ferreira et al., 2020). *E. coli* is also an important microorganism for fatty acid biosynthesis and has been engineered to enhance the production of free fatty acids by expressing thioesterases and optimizing the fatty acid biosynthesis pathway (Handke et al., 2011).

Optimizing various production conditions are very crucial for the efficiency and economic viability of biofuel production. Key factors affecting the biofuel production efficiency, including feedstock selection, microbial strain improvement, process parameters, and technological innovations, are analyzed firstly by aiming to determine strategies for optimizing biofuel production.

Optimization of process parameters such as nutrient availability, temperature, pH, and reactor design is crucial for maximizing biofuel yields. For instance, in anaerobic digestion, maintaining optimal temperatures (mesophilic or thermophilic conditions) can enhance microbial activity and CH_4 production. Similarly, controlling the pH during fermentation processes can prevent microbial inhibition and improve biofuel yields. When designing photobioreactors for microalgae cultivation, it is essential to consider multiple key factors such as the ratio of surface to volume, temperature, the composition and mixing of the culture media, light exposure, levels of dissolved oxygen, CO_2 and air supply (Razzak et al., 2023). Increasing lipid production in algal can also be achieved by the adjustment of cultivation conditions (Lv et al., 2010).

Biosurfactants are surface active molecules produced primarily by microorganisms. They contain hydrophilic and hydrophobic groups partitioning at physical interfaces and decrease the surface and interfacial tension between different phases. Therefore, they are used in various fields of application (Gurkok, 2022; Gurkok and Ozdal, 2023), including the biofuel industry (Oyetunji et al., 2023).

In biofuel production, biosurfactants can be applied in different ways such as promoting microbial growth and activity, improving fermentation, and facilitating biomass pretreatment. Some inhibitor molecules such as phenols, furans, and organic acids are produced during the hydrolysis of lignocellulosic biomass. Biosurfactants can be used to reduce the toxicity of these compounds and enhance the microbial growth and activity, and thus improve the efficiency of biofuel production processes (Zhou et al., 2011; Bolan et al., 2023). By improving the mass transfer of substrates and oxygen in the fermentation medium through the use of biosurfactants, fermentation processes can be improved and thus fermentation efficiency and ethanol yields can be increased. Biosurfactants reduce the surface tension between oil and water and can be applied in the recovery of residual oils from biomass feedstocks. With enhanced oil recovery through biosurfactants, more oil can be extracted from feedstocks such as algae, waste oils and other oil-rich biomass. These oils can then be converted into biofuels. Especially in biodiesel production, fats and oils in biomass feedstocks can be emulsified by biosurfactants and become more accessible for microbial degradation, where they are converted into fatty acid methyl esters (Oyetunji et al., 2023). Biosurfactants can offer several advantages for improving the efficiency of

biofuel production. However, further research is still needed to fully realize their potential in biofuel production.

Conclusion

Biofuel production offers numerous benefits, one being the minimal production of greenhouse gases since biofuels are derived from pure carbon, essentially biomass, an organic compound. When biofuel is burned, it releases CO_2, which is absorbed again by plants, algae, and photosynthetic bacteria, completing the carbon cycle and minimizing greenhouse gas emissions and pollution, thereby preserving the environment. Additionally, biofuel production presents an alternative to crude oil that could be utilized in the future.

However, there are a number of obstacles associated with biofuel production, including a shortage of skilled professionals, insufficient tools and infrastructure, lack of public awareness, inadequate biomass supply, productivity, scalability, cost-effectiveness, quality of biofuels, poor communication between biofuel producers and consumers, and a shortage of vehicles equipped with biofuel engines.

To address these challenges, it is essential to prepare biomass and raw materials for biofuel production and simultaneously provide training people for this purpose, thus increasing the number of technical experts. Furthermore, systems and tools need to be developed and implemented.

While showing promise, there are still hurdles to overcome in improving the productivity, scalability, cost-effectiveness, and quality of biofuels before they can become economically feasible substitutes for fossil fuels.

REFERENCES

Abdullah, B., Muhammad, S. A. F. A. S., Shokravi, Z., Ismail, S., Kassim, K. A., Mahmood, A. N., & Aziz, M. M. A. (2019). Fourth generation biofuel: A review on risks and mitigation strategies. *Renewable and Sustainable Energy Reviews*, *107*, 37-50.

Aguilar-Reynosa, A., Romaní, A., Rodriguez-Jasso, R. M., Aguilar, C. N., Garrote, G., & Ruiz, H. A. (2017). Microwave heating processing as alternative of pretreatment in second-generation biorefinery: An overview. *Energy Conversion and Management*, *136*, 50-65.

Areepak, C., Jiradechakorn, T., Chuetor, S., Phalakornkule, C., Sriariyanun, M., Raita, M., ... & Laosiripojana, N. (2022). Improvement of lignocellulosic pretreatment efficiency by combined chemo-Mechanical pretreatment for energy consumption reduction and biofuel production. *Renewable Energy*, *182*, 1094-1102.

Bay, M. S., Karimi, K., Esfahany, M. N., & Kumar, R. (2020). Structural modification of pine and poplar wood by alkali pretreatment to improve ethanol production. *Industrial Crops and Products*, *152*, 112506.

Bolan, S., Padhye, L. P., Mulligan, C. N., Alonso, E. R., Saint-Fort, R., Jasemizad, T., ... & Bolan, N. (2023). Surfactant-enhanced mobilization of persistent organic pollutants: potential for soil and sediment remediation and unintended consequences. *Journal of hazardous materials*, *443*, 130189.

Buitrón, G., Carrillo-Reyes, J., Morales, M., Faraloni, C., & Torzillo, G. (2017). Biohydrogen production from microalgae. In Microalgae-based Biofuels and Bioproducts (pp. 209-234). Woodhead Publishing.

Camani, P. H., Anholon, B. F., Toder, R. R., & Rosa, D. S. (2020). Microwave-assisted pretreatment of eucalyptus waste to obtain cellulose fibers. *Cellulose*, *27*, 3591-3609.

Champagne, P. (2007). Feasibility of producing bio-ethanol from waste residues: a Canadian perspective: feasibility of producing bio-ethanol from waste residues in Canada. *Resources, Conservation and Recycling*, *50*(3), 211-230.

Chandel, H., Kumar, P., Chandel, A. K., & Verma, M. L. (2024). Biotechnological advances in biomass pretreatment for bio-renewable production through nanotechnological intervention. *Biomass Conversion and Biorefinery*, *14*(3), 2959-2981.

Chaturvedi, V., Goswami, R. K., & Verma, P. (2020). Genetic engineering for enhancement of biofuel production in microalgae. Biorefineries: A Step Towards Renewable and Clean Energy, 539-559.

Chen, H., Zhou, D., Luo, G., Zhang, S., & Chen, J. (2015). Macroalgae for biofuels production: Progress and perspectives. *Renewable and Sustainable Energy Reviews*, *47*, 427-437.

dos Santos, R. G., & Alencar, A. C. (2020). Biomass-derived syngas production via gasification process and its catalytic conversion into fuels by Fischer Tropsch synthesis: A review. *International Journal of Hydrogen Energy*, *45*(36), 18114-18132.

Dunkle, M. N., & Winniford, W. L. (2020). Environmental Impact of Emissions Originating from the Petroleum Industry. Analytical Techniques in the Oil and Gas Industry for Environmental Monitoring, 347-378.

El-Sayed, M. H., Gomaa, A. E. R. F., Atta, O. M., & Hassane, A. M. (2024). Characteristics and kinetics of thermophilic actinomycetes' amylase production on agro-wastes and its application for ethanol fermentation. *World Journal of Microbiology and Biotechnology*, *40*(8), 255.

Ferreira, S., Pereira, R., Wahl, S. A., & Rocha, I. (2020). Metabolic engineering strategies for butanol production in *Escherichia coli*. *Biotechnology and Bioengineering*, *117*(8), 2571-2587.

Golomb, D. (2020). Fossil Fuel Combustion: Air pollution and global warming. In *Managing Air Quality and Energy Systems* (pp. 177-190). CRC Press.

Gong, C., Singh, A., Singh, P., & Singh, A. (2021). Anaerobic digestion of agri-food wastes for generating biofuels. *Indian Journal of Microbiology*, *61*(4), 427-440.

Gurkok, S. (2022). Screening of high yield biosurfactant producing strains of agribiotechnological importance. In *Applications of Biosurfactant in Agriculture* (pp. 163-180). Academic Press.

Gurkok, S., & Ozdal, M. (2023). Screening Methods for Biosurfactant-Producing Microorganisms. In *Multifunctional Microbial Biosurfactants* (pp. 1-24). Cham: Springer Nature Switzerland.

Gurkok, S. (2024). Exploring Cost-Effective Pathways for Future Biofuel Production. In *Solid-Gaseous Biofuels Production* (pp. 119-147). Scrivener Publishing LLC.

Han, W., Liu, Y., Xu, X., Huang, J., He, H., Chen, L., ... & Hou, P. (2020). Bioethanol production from waste hamburger by enzymatic hydrolysis and fermentation. *Journal of Cleaner Production*, *264*, 121658.

Han, Y., Hoekman, S. K., Cui, Z., Jena, U., & Das, P. (2019). Hydrothermal liquefaction of marine microalgae biomass using co-solvents. *Algal Research*, *38*, 101421.

Handke, P., Lynch, S. A., & Gill, R. T. (2011). Application and engineering of fatty acid biosynthesis in *Escherichia coli* for advanced fuels and chemicals. *Metabolic Engineering*, *13*(1), 28-37.

Hoang, A. T., Ong, H. C., Fattah, I. R., Chong, C. T., Cheng, C. K., Sakthivel, R., & Ok, Y. S. (2021). Progress on the lignocellulosic biomass pyrolysis for biofuel production toward environmental sustainability. Fuel Processing Technology, *223*, 106997.

Hsieh, C. H., & Wu, W. T. (2009). Cultivation of microalgae for oil production with a cultivation strategy of urea limitation. *Bioresource Technology*, *100*(17), 3921-3926.

Ingale, S., Joshi, S. J., & Gupte, A. (2014). Production of bioethanol using agricultural waste: banana pseudo stem. *Brazilian Journal of Microbiology*, *45*, 885-892.

Ismail, M. M., Ismail, G. A., & El-Sheekh, M. M. (2024). Potential assessment of some micro-and macroalgal species for bioethanol and biodiesel production. *Energy Sources, Part A: Recovery, Utilization, and Environmental Effects*, *46*(1), 7683-7699.

Kalita, P., Basumatary, B., Saikia, P., Das, B., & Basumatary, S. (2022). Biodiesel as renewable biofuel produced via enzyme-based catalyzed transesterification. *Energy Nexus*, *6*, 100087.

Kougias, P. G., & Angelidaki, I. (2018). Biogas and its opportunities—A review. *Frontiers of Environmental Science & Engineering*, *12*, 1-12.

Kulikova, Y., Ilinykh, G., Sliusar, N., Babich, O., & Bassyouni, M. (2024). Life cycle assessments of biofuel production from beach-cast seaweed by pyrolysis and hydrothermal liquefaction. *Energy Conversion and Management: X*, 100647.

Kumar, A. K., & Sharma, S. (2017). Recent updates on different methods of pretreatment of lignocellulosic feedstocks: A review. *Bioresources and Bioprocessing*, *4*, 7.

Liang, M. H., & Jiang, J. G. (2013). Advancing oleaginous microorganisms to produce lipid via metabolic engineering technology. *Progress in Lipid Research*, *52*(4), 395-408.

Lv, J. M., Cheng, L. H., Xu, X. H., Zhang, L., & Chen, H. L. (2010). Enhanced lipid production of *Chlorella vulgaris* by adjustment of cultivation conditions. *Bioresource Technology*, *101*(17), 6797-6804.

Madhavan, A., Srivastava, A., Kondo, A., & Bisaria, V. S. (2012). Bioconversion of lignocellulose-derived sugars to ethanol by engineered *Saccharomyces cerevisiae*. *Critical Reviews in Biotechnology*, *32*(1), 22-48.

Mahapatra, S., & Singh, L. K. (2024). crude microbial enzymes (fungal enzymes) from agro-wastes as a potent agent for biological pretreatment of algal biomass processing to biofuel production. In Biofuels (pp. 335-349). CRC Press.

Ma'mun, S., Prasetio, M. W., Anugrah, A. R., Ruliandi, A. P., & Pramuwardani, D. (2024). Bioethanol from *Arthrospira platensis* biomass using a combined pretreatment. *Chemical Engineering Journal Advances*, *19*, 100616.

Molino, A., Larocca, V., Chianese, S., & Musmarra, D. (2018). Biofuels production by biomass gasification: A review. *Energies*, *11*(4), 811.

Mussgnug, J. H., Thomas-Hall, S., Rupprecht, J., Foo, A., Klassen, V., McDowall, A., ... & Hankamer, B. (2007). Engineering photosynthetic light capture: impacts on improved solar energy to biomass conversion. *Plant Biotechnology Journal*, *5*(6), 802-814.

Najafi, G., Ghobadian, B., Tavakoli, T., & Yusaf, T. (2009). Potential of bioethanol production from agricultural wastes in Iran. *Renewable and Sustainable Energy Reviews*, *13*(6-7), 1418-1427.

Nakajima, Y. & Ueda, R. (1997). Improvement of photosynthesis in dense microalgal suspension by reduction of light harvesting pigments. *Journal of Applied Phycology*, *9*, 503-510.

Nhuchhen, D. R., Basu, P., & Acharya, B. (2014). A comprehensive review on biomass torrefaction. *International Journal of Renewable Energy & Biofuels*, *2014*, 1-56.

Onumaegbu, C., Mooney, J., Alaswad, A., & Olabi, A. G. (2018). Pre-treatment methods for production of biofuel from microalgae biomass. *Renewable and Sustainable Energy Reviews*, *93*, 16-26.

Oyetunji, O. E., Kotun, B. C., Thonda, O. A., & Ademola, E. A. (2023). The Role of Biosurfactants in Biofuel Production. In *Advancements in Biosurfactants Research* (pp. 371-395). Cham: Springer International Publishing.

Padder, S. A., Khan, R., & Rather, R. A. (2024). Biofuel generations: New insights into challenges and opportunities in their microbe-derived industrial production. *Biomass and Bioenergy*, *185*, 107220.

Patel, V. K., Soni, N., Prasad, V., Sapre, A., Dasgupta, S., & Bhadra, B. (2019). CRISPR–Cas9 system for genome engineering of photosynthetic microalgae. *Molecular biotechnology*, *61*, 541-561.

Peng, L., Fu, D., Chu, H., Wang, Z., & Qi, H. (2020). Biofuel production from microalgae: a review. *Environmental Chemistry Letters*, *18*, 285-297.

Peter, A. P., Koyande, A. K., Chew, K. W., Ho, S. H., Chen, W. H., Chang, J. S., ... & Show, P. L. (2022). Continuous cultivation of microalgae in photobioreactors as a source of renewable energy: Current status and future challenges. *Renewable and Sustainable Energy Reviews*, *154*, 111852.

Rafa, N., Ahmed, S. F., Badruddin, I. A., Mofijur, R. M., & Kamangar, S. (2021). Strategies to produce cost-effective third-generation biofuel from microalgae. *Frontiers in Energy Research*, *9*, 749968.

Rasapoor, M., Young, B., Brar, R., Sarmah, A., Zhuang, W. Q., & Baroutian, S. (2020). Recognizing the challenges of anaerobic digestion: Critical steps toward improving biogas generation. *Fuel*, *261*, 116497.

Razzak, S. A., Bahar, K., Islam, K. O., Haniffa, A. K., Faruque, M. O., Hossain, S. Z., & Hossain, M. M. (2023). Microalgae cultivation in photobioreactors: sustainable solutions for a greener future. *Green Chemical Engineering* (pp, 418-439).

Robak, K., & Balcerek, M. (2018). Review of second generation bioethanol production from residual biomass. *Food Technology and Biotechnology*, *56*(2), 174.

Samantaray, B., Mohapatra, S., Mishra, R. R., Behera, B. C., & Thatoi, H. (2024). Bioethanol production from agro-wastes: a comprehensive review with a focus on pretreatment, enzymatic hydrolysis, and fermentation. *International Journal of Green Energy*, *21*(6), 1398-1424.

Shahbeik, H., Panahi, H. K. S., Dehhaghi, M., Guillemin, G. J., Fallahi, A., Hosseinzadeh-Bandbafha, H., ... & Aghbashlo, M. (2024). Biomass to biofuels using hydrothermal liquefaction: A comprehensive review. *Renewable and Sustainable Energy Reviews*, *189*, 113976.

Shirkavand, E., Baroutian, S., Gapes, D. J., & Young, B. R. (2016). Combination of fungal and physicochemical processes for lignocellulosic biomass pretreatment–a review. *Renewable and Sustainable Energy Reviews*, *54*, 217-234.

Shokravi, H., Heidarrezaei, M., Shokravi, Z., Ong, H. C., Lau, W. J., Din, M. F. M., & Ismail, A. F. (2022). Fourth generation biofuel from genetically modified algal biomass for bioeconomic development. *Journal of Biotechnology*, 360, 23-36.

Singh, A., Prajapati, P., Vyas, S., Gaur, V. K., Sindhu, R., Binod, P., ... & Varjani, S. (2023). A comprehensive review of feedstocks as sustainable substrates for next-generation biofuels. *BioEnergy Research*, *16*(1), 105-122.

Sivakumar, G., Vail, D. R., Xu, J., Burner, D. M., Lay, J. O., Ge, X., & Weathers, P. J. (2010). Bioethanol and biodiesel: Alternative liquid fuels for future generations. *Engineering in Life Sciences*, *10*(1), 8-18.

Sołowski, G., Konkol, I., & Cenian, A. (2020). Production of hydrogen and methane from lignocellulose waste by fermentation. A review of chemical pretreatment for enhancing the efficiency of the digestion process. *Journal of Cleaner Production*, *267*, 121721.

Suhartini, S., Pangestuti, M. B., Elviliana, Rohma, N. A., Junaidi, M. A., Paul, R., ... & Melville, L. (2024). Valorisation of macroalgae for biofuels in Indonesia: an integrated biorefinery approach. *Environmental Technology Reviews*, *13*(1), 269-304.

Sun, D., Chen, J., Wang, Y., Li, M., Rao, D., Guo, Y., ... & Ma, Y. (2019). Metabolic engineering of Corynebacterium glutamicum by synthetic small regulatory RNAs. *Journal of Industrial Microbiology and Biotechnology*, *46*(2), 203-208.

Tandon, P., & Jin, Q. (2017). Microalgae culture enhancement through key microbial approaches. *Renewable and Sustainable Energy Reviews*, *80*, 1089-1099.

Tian, L., Conway, P. M., Cervenka, N. D., Cui, J., Maloney, M., Olson, D. G., & Lynd, L. R. (2019). Metabolic engineering of *Clostridium thermocellum* for n-butanol production from cellulose. *Biotechnology for Biofuels*, *12*, 1-13.

Twigg, G., Fedenko, J., Hurst, G., Stanley, M. S., & Hughes, A. D. (2024). A review of the current potential of European brown seaweed for the production of biofuels. *Energy, Sustainability and Society*, *14*(1), 21.

Wang, M., Ye, X., Bi, H., & Shen, Z. (2024). Microalgae biofuels: illuminating the path to a sustainable future amidst challenges and opportunities. *Biotechnology for Biofuels and Bioproducts*, *17*(1), 10.

Wen, Z., Ledesma-Amaro, R., Lin, J., Jiang, Y., & Yang, S. (2019). Improved n-butanol production from *Clostridium cellulovorans* by integrated metabolic and evolutionary engineering. *Applied and environmental microbiology*, *85*(7), e02560-18.

Wolf, B. M., Niedzwiedzki, D. M., Magdaong, N. C. M., Roth, R., Goodenough, U., & Blankenship, R. E. (2018). Characterization of a newly isolated freshwater Eustigmatophyte alga capable of utilizing far-red light as its sole light source. *Photosynthesis research*, *135*, 177-189.

Zhou, M. F., Yuan, X. Z., Zhong, H., Liu, Z. F., Li, H., Jiang, L. L., & Zeng, G. M. (2011). Effect of biosurfactants on laccase production and phenol biodegradation in solid-state fermentation. *Applied biochemistry and biotechnology*, *164*, 103-114.

BACTERIAL DEGRADATION OF PLASTICS

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Introduction

The use of plastic is a growing problem around the world. Plastic pollution goes hand in hand with the increasing use of plastic. Many alternative methods have been tried to reduce the ever-increasing plastic pollution. One of these alternative methods is the microbial degradation of plastics. With the development of biotechnology, the microbial degradation of plastics was first reported in 1968 when it was discovered that fungi could degrade polyurethane (PUR) (Darby and Kaplan, 1968). In the 1970s, studies continued with the investigation of the microbial degradation of polyethylene (PE) and polystyrene (PS); however, the biodegradation efficiency was much lower than expected (Albertsson, 1978). In 1993, it was reported that polypropylene (PP) can be microbially degraded (Cacciari et al., 1993). With the increasing research on microbial degradation of plastics, bacteria with higher efficiency in degrading plastics were reported. Hadar and Sivan (2004) reported that the isolated *Rhodococcus ruber* strain (*R. ruber* C208) was able to degrade PE by 8% in a 30-day incubation, and that the addition of mineral oil to the synthetic medium resulted in a 50% increase in dry weight loss of PE compared to the medium without mineral oil. In the following years, Yoshida et al. (2016) isolated *Ideonella sakaiensis* 201-F6, which possesses the enzymes monohydroxyethyl terephthalate hydrolase (MHETase) and PET hydrolase (PETase) for the degradation of polyethylene terephthalate (PET).

It is now known that many microorganisms and enzymes can degrade plastics. However, little information is available on the degradation mechanisms and degradation efficiency of these bacteria and enzymes. In particular, recent studies have mainly focused on increasing the ability of these enzymes to degrade plastics (the degradation efficiency) (Rübsam et al., 2018; Apitius et al., 2019). This chapter discusses bacteria that can degrade plastics and the microbial degradation mechanisms of plastics.

The Types of Plastics

Plastics are a common material found in food packaging, decorative materials, furniture and laboratory equipment that are commonly used in everyday life (Rosato, 2011). For this reason, the production of synthetic plastics is a rapidly growing area of the industry. Although plastic products have been used in everyday life for more than 100 years, large-scale production of plastic products began in the 1950s and increased 20-fold after

1964 (Geyer et al., 2017). Global plastics production rose from 370.5 million tonnes (Mt) in 2018 to 400.3 Mt in 2022, while European plastic production fell from 62.3Mt in 2018 to 58.7Mt in 2022 (Europe Plastics, 2023). Particularly with the Covid 19 pandemic that we have experienced in recent years, the use of plastics has increased significantly due to the increased use of disinfectant bottles, face masks and gloves (de Sousa 2021). Petrochemical plastics such as PE, polyvinyl chloride (PVC), polypropylene (PP), PET, and PS account for around 80% of global plastic consumption (Figure 1). In contrast to these types of plastics, plastics that remain unchanged during product storage and meet the requirements of use, biodegrade after use, and disappear from the natural environment are called biodegradable plastics (Picó and Barceló, 2019). Biodegradable plastics, which have a similar structure but more functional groups that can be hydrolysed, are less popular than non-biodegradable plastics. Although some biodegradable plastics have shown good mechanical, degradable and physicochemical properties in many industrial applications, it is known that biodegradable plastics do not currently replace conventional (non-biodegradable) plastics (Rujnić-Sokele and Pilipović, 2017). For this reason, researchers have focused on discovering microorganisms and enzymes that degrade non-biodegradable plastics that are more difficult to biodegrade than biodegradable plastics, increasing the efficiency of plastic degradation efficiency of existing microorganisms and enzymes, and making non-biodegradable plastics more susceptible to microbial degradation.

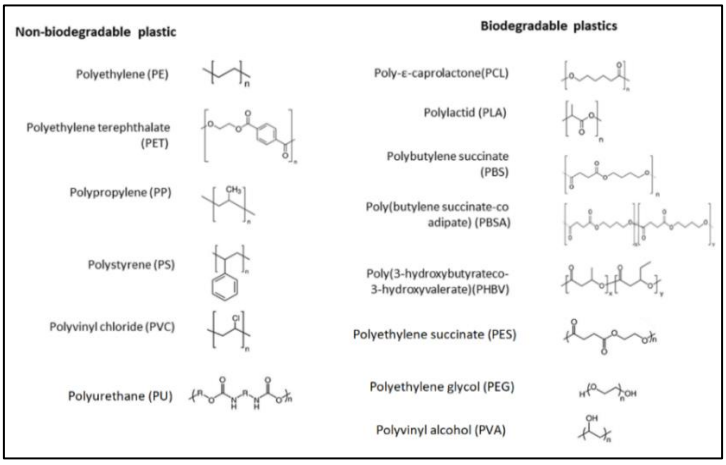

Figure 1. Structures of non-biodegradable and biodegradable plastics (Wilkes and Aristilde, 2017; Urbanek et al., 2018)

The widespread use of plastic around the world has led to pollution. The fact that it takes years to break down plastic waste in the environment has become a threat to many living beings, especially humans. Plastic pollution and waste management are just two of the many environmental problems resulting from decades of uncontrolled plastic use (Moshood et al., 2022). Almost all plastics enter wastewater treatment plants from primary and secondary sources (Mrowiec, 2017). The most common primary sources are microbeads and fragments of plastics found in industry, toothpaste, household cleaners and personal care products such as cosmetics, while the most common secondary sources are microplastics from the degradation of waste such as synthetic fabrics, household items and fibre production waste (Çevik et al., 2022). Wastewater treatment plants are not sufficient to eliminate micro- and nanoplastic pollution, and plastics enter the aquatic environment such as oceans and rivers with wastewater (Altuğ and Erdoğan, 2022). Plastic waste entering the aquatic environment causes pollution by accumulating in rivers and oceans, leading to the formation of plastic islands such as the Great Pacific Garbage Patch (Lebreton et al., 2018). In one of the worst-case scenarios, it is estimated that for the future of microplastics, the amount of plastic in the aquatic environment will exceed the amount of fish by 2050 (MacArthur, 2017). The fact that plastic pollution is so widespread has physical and chemical effects on organisms in the ecosystem (Markic et al., 2020). Recent studies have shown that microplastics are found in many seafood, especially edible algae, table salt and shellfish (Gündoğdu, 2018; Gündoğdu et al., 2020).

Plastic Degrading Bacteria and Their Mechanisms of Action

In the natural environment, biodegradable plastics break down very slowly. This slow degradation of biodegradable plastics can be accelerated by environmental factors. Events such as mechanical abrasion, sunlight, waves, wind stress and thermal oxidation trigger polymer chain reactions and chain breaks in plastics, which can transform plastics into microplastics (Zhao et al. 2022). The microplastics formed have the potential to be removed from the environment through microbial degradation and metabolism. The anaerobic or aerobic microbial metabolism of plastics produces CH_4, CO_2, and H_2O (Chidambarampadmavathy et al., 2017). Microbial hydrolases, depolymerases, and peroxidases are the key enzymes of microbial degradation (Amobonye et al., 2021).

Biofilm formation is one of the main mechanisms for the degradation of plastics by microorganisms. Plastic waste, particularly in the aquatic ecosystem, favours a biofilm on the surface due to the microbiome in the environment (Wright et al., 2020). This microbial community, which can form a biofilm on the surface of plastic waste, is influenced by environmental conditions (Marsay et al., 2023). Chitinophagaceae, Burkholderiaceae, Hyphomicrobiaceae, Pseudoalteromonadaceae, Opitutaceae and Xanthobacteraceae are the most common families found on PU and PS surfaces in the Northeast Atlantic (Kelly et al., 2022). The Rhodobacteraceae and Flavobacteriaceae families are commonly found in PE, PP, PVC and PET in the Fal Estuary (Lear et al., 2022). Dudek et al. (2020) showed that the Flavobacteriaceae, Pirellulaceae, Saprospiraceae and Rhodobacteraceae families were commonly found in biofilms on PE, PS, PP, PVC and PET incubated for 6 weeks at a depth of 1m in the Caribbean Sea. Pollet et al. (2018) reported that the Colwelliaceae, Flavobacteriaceae and Planctomucetaceae families were common in biofilms on the surface of PVC found in the Mediterranean. It was also reported that bacterial diversity in biofilms on plastic surfaces can vary indifferent points in the same region (Jiang et al., 2018).

Today, there are many strains of bacteria that are known to be able to break down microplastics. Recent studies have shown that many species of bacteria belonging to the Actinobacteria, Firmicutes and Proteobacteria have the ability to degrade plastics. It has also been reported that many bacteria that can degrade plastics have been isolated from contaminated areas such as wastewater and landfills (Ali et al., 2021). *Bacillus megaterium*, *Pseudomonas aeruginosa* and *R. ruber*, as well as bacterial species belonging to the genera *Pseudomonas*, *Bacillus*, and *Escherichia*, have been shown to give good results in the degradation of recalcitrant plastics such as PE, PET and PS (Amobonye et al., 2021).

PET is the most widely produced plastic in the world and is widely used in the packaging and textile industries (Maity et al., 2021). It is now known that there are only a few phyla of bacteria that can degrade PET and that most of the bacterial isolates that can degrade PET belong to *Actinobacteria*, including species belonging to the genera *Thermomonospora* and *Thermobifida* (Danso et al., 2018). PET can be directly degraded to ethylene glycol (EG) and terephthalic acid (TPA) by enzymatic hydrolysis. Intermediates from the partial hydrolysis of PET contain bis-(2-hydroxyethyl)

terephthalate (BHET) and MHET, which can be further hydrolysed to EG and TPA (Khairul Anuar et al., 2022). TPA, which enters the cell via a transporter, is converted into protocatechuic acid (PCA), which is then taken up into bacterial metabolism via the tricarboxylic acid (TCA) cycle. Similarly, EG can be incorporated into the TCA cycle via the glyoxylate shunt (Carr et al., 2020). The discovery of *I. sakaiensis* 201-F6, which uses plastics as its sole food source, can induce morphological changes on PET and has 2 different enzymes that degrade PET, is very important for the microbial degradation of PET (Yoshida et al., 2016). However, a recent study reported that the PETase from the photosynthetic microalgae *Phaeodactylum tricornutum* was more successful at degrading PET than that from *I. sakaiensis* (Moog et al., 2019). In their study, Yan et al. (2023) reported that *P. monteilii* S17 and *P. nitroreducens* S8 colonised the PET surface by forming a biofilm layer with a constant population density, which allows them to utilise PET as a carbon source. They also showed that the combination of these two strains had a synergistic effect and increased PET degradation.

PUR consists of polyols and di- or polyisocyanate, which are linked by urethane bonds (Seymour and Kauffman, 1992). Depending on the chemical composition of the polyols used, PUR is referred to as polyester-PUR, which is made from polyester-polyol, and polyether-PUR which is made from polyether polyol. The first study on the microbial degradation of PUR was carried out by Darby and Kaplan in 1968 when they showed that 7 different fungi could grow on solid polyester PUR. They also stated that the sensitivity of polyether PUR to fungal colonisation is determined by the number of contiguous methylene groups (Darby and Kaplan, 1968). Along with this, many studies have shown that PUR can also be degraded by bacteria (Howard et al., 1999; Vega et al., 1999; Rowe and Howard, 2002). Microbial proteases, ureases, and esterases can hydrolyse the ester and urethane bonds of PUR. The ester and urethane bonds of PUR can be hydrolysed by microbial proteases, ureases and esterases (Cregut et al., 2013). Table 1 shows some of the enzymes responsible for the microbial degradation of PUR. It is assumed that proteases target urethane and amide bonds, ureases target urea bonds and esterases target ester bonds (Wei and Zimmermann, 2017). Membrane-bound enzymes adhere directly to the surface of PUR, hydrolyse the urethane bonds and enable the formation of PUR monomers (Cregut et al., 2013). Moreover, insoluble PUR is degraded by the release of secretases (Wei and Zimmermann, 2017).

Among the synthetic plastics PP, PE, PVC, and PS are more resistant to biodegradation than PET and PUR, which have a hydrolysable backbone due to the C-C backbone (Chen et al., 2020). PS is a high-molecular-weight synthetic hydrophobic polymer that is not easily broken down by microorganisms (Kaplan et al., 1979). Similarly, Krueger et al. (2015) stated that the molecular weight and the thickness ratio of the plastic are the decisive factors for the microbial degradation of PS. Tischler et al. (2009) showed that *R. opacus* 1CP, an actinobacterium species, can mineralise styrene and styrene oxide, which are released during the pyrolysis of PS, via aerobic phenylacetic acid (PAA). This pathway involves the conversion of styrene oxide to phenylacetaldehyde by styrene oxide isomerase (SOI), phenylacetaldehyde to phenylacetate by phenylacetaldehyde dehydrogenase (PAD), and phenylacetate to acetyl CoA by β-oxidation with the enzymes of phenylacetate (PAA#). The resulting acetyl-CoA is involved in the TCA cycle (Tischler et al., 2009). Another proposed pathway for the microbial degradation of PS involves the conversion of styrene to pyruvate and pyruvate to acetyl-CoA as a result of a series of different enzymatic reactions and its involvement in the TCA cycle (Yang et al., 2023). O'Leary et al. (2002) reported that oxygenation of the sidechain is a fairly common pathway for the aerobic degradation of styrene, as in the genera *Xanthobacter* and *Pseudomonas*. Brandon et al. (2018) found in their study that there are bacterial species (*Kosakonia* sp. and *Citrobacter* sp.) that can degrade both PS and PE in mealworms (*Tenebrio molitor*) and can be engineered to degrade chemically distinct plastics.

PE is a plastic that is highly resistant to degradation due to its covalent bonds, which cannot be hydrolysed, and its C-C backbone. Studies shown that the microbial degradation of PE involves the enzymatic degradation of PE and the bio-assimilation of the lytic fragments by microorganisms and that the use of a microbial consortium may be more effective in the degradation of PE (Montazer et al., 2019; Gao and Sun, 2021). Although PP, PE, and PVC, which have similar C-C backbone chains, are degraded by many microorganisms, the main depolymerases responsible for the breakdown process and the final products are not fully known (Ru et al., 2020). The microbial degradation mechanism of PE and PP is similar. In both types of plastics, the -C-C- groups in the C-C backbone are oxidised to carbonyl groups (-C=O-) to form aliphatic hydrocarbons that bacteria can take up directly into the cell (Ru et al., 2020; Yang et al., 2023). Bacterial species such as *Bacillus* sp., *Pseudomonas* sp., *Nocardia* sp. and *Mycobacterium* sp. are known to degrade PE (Khandare and Chaudhary, 2021). Lee et al. (1991) found that

Streptomyces badius 252, *S. viridosporus* T7A and *S. setonii* 75Vi2, which have the ability to degrade lignocellulose, decreased the molecular weight and elongation of PE. Shahnawaz et al. (2016) investigated the effect of *Lysinibacillus fusiformis* and *B. cereus* on the microbial degradation of PE and showed that *L. fusiformis* can reduce the weight of PE by up to 21.9% at pH 3.5 and 25 °C. Studies have also shown that some strains of bacteria isolated from seawater, landfills, sewage sludge, and petroleum-contaminated soil can degrade untreated PE. Azeko et al. (2015) reported that a strain of *Serratia marcescens* isolated from soil reduced the weight of untreated PE by 36% within 70 days. The researchers also reported that both bacterial cells and the cell-free supernatant of *S. marcescens* were able to degrade PE, and even the cell-free supernatant degraded PE faster than bacterial cells. In their study, Sarmah and Rout (2018) reported that the cyanobacteria *Oscillatoria subbrevis* and *Phormidium lucidum* degraded 30% of PE in 42 days of incubation.

There are many studies on bacteria that degrade PE. However, the number of enzymes responsible for the biodegradation of PE is quite small. Santo et al. (2013) reported that extracellular laccase, which can reduce the molecular weight of PE and form carbonyl groups, isolated from *R. ruber* C208, which is able to degrade PE, can oxidise PE films. Researchers also shown that recombinant *E. coli* cells cloned *alkB, alkB1*, and *alkB2* genes, which synthesise alkane hydroxylase, can degrade low molecular weight PE (Yoon et al., 2012). In addition, a study based on quantum mechanical calculations claims that enzymatic cleavage of the C-C bonds of PS and PE by oxygenases or oxidases may be possible (Xu et al., 2019).

PVC is a synthetic plastic that is resistant to both chemical and hydrophobic abrasion and is much more difficult to degrade than PE and PP due to the chlorinated hydrocarbons it contains (Shah et al., 2008; Yang et al., 2023). Although the microbial degradation of PVC is difficult, bacterial strains that can degrade PVC have been reported. Sah et al. (2011) showed that *Bacterium* Te68R, *Microbacterium* sp., *B. aerius, B. cereus, P. aeruginosa, P. otitidis, P. putida* and *Acanthopleurobacter pedis* isolated from soil can cause morphological damage to the PVC surface after 70 days of incubation. In their study, Anwar et al. (2013) reported that a bacterial consortium consisting of *A. pedis, B. aerius, B. cereus*, and *P. otitidis* degrade PVC better than LDPE after 70 days of incubation. Currently, the only enzyme known to be capable of degrading PVC is laccase, which can break the double

bonds of PVC and form new C=O bonds (Sumathi et al., 2016). It is speculated that the short chain formed as a result of the reaction triggered by laccase is taken up by microorganisms and utilised in the TCA cycle (Yang et al., 2023). For these reasons, studies are needed to discover other microbial enzymes that degrade PVC and to understand their mechanisms of action.

Table 1. Plastics, degrading bacteria and related enzymes

Non-Biodegradable Plastic Degrading Microbial Enzymes			
Plastic	**Bacteria**	**Enzyme/s**	**Reference**
PS	*Azotobacter beijerinckii* HM121	Hydroquinone peroxidase	Nakamiya et al., 1997
PUR	*Comamonas acidovorans*	Esterase	Allen et al., 1999
PUR	*P. chlororaphis*	Esterase	Howard et al., 1999
PUR	*P. fluorescens*	PU esterase	Vega et al., 1999
PUR	*B. subtilis*	Lipase	Rowe and Howard, 2002
PS	*P. putida* AJ	Alkene monooxygenase	Danko et al., 2004
PET	*T. fusca*	TfH	Müller et al., 2005
PET	*P. mendocina*	PmC	Ronkvist et al., 2009
PE	*Pseudomonas* sp. AKS2	Hydrolase	Tribedi and Sil, 2013
PET	*T. fusca* KW3	TfCut2	Barth et al., 2016
PS	*Pseudomonas* sp.	Esterase	Mohan et al., 2016
PUR	Metagenomic library from plant compost, *T. fusca* KW3 and *T. curvata* DSM43183	LC cutinase, Tcur0390, TfCut2 and Tcur1278	Schmidt et al., 2017
Several types of plastic	Halophilic, alkaliphilic, thermophilic, and psychrophilic bacteria	Bacteriophilic enzyme	Atanasova et al., 2021
PE, PS, PP, PVC	*Arthrobacter*, *Pseudomonas*	PME hydrolases	Carmen, 2021
PC (BPA)	*Bacillus* sp. (GZB)	A spore laccase	Das et al., 2019
PUR	*E. coli*	Esterase (E3576)	Magnin et al., 2019
PET	*P. aestusnigri*	Polyester hydrolase	Bollinger et al., 2020
PET	*S. scabies*	Cutinase	Jabloune et al., 2020

PET	*Streptomyces* sp. SM14	Hydrolase	Carr et al., 2023
PET	*Stenotrophomonas maltophilia* PRS8	Cutinase like enzyme	Din et al., 2023
Biodegradable Plastic Degrading Microbial Enzymes			
Polyethylene glycol (PEG)	*P. stutzeri*	PEG dehydrogenase	Obradors and Aguilar, 1991
Polyvinyl alcohol (PVA)	*P. vesicularis* PD	Esterase	Kawai and Hu, 2009
Polyethylene succinate (PES)	*Pseudomonas* sp. AKS2	Esterase	Tribedi et al., 2012
poly(3-hydroxybutyrate) (PHB)	*Agrobacterium* sp. DSGZ	Depolymerase	Guo et al., 2016
poly(butylene adipate-*co*-terephthalate) (PBAT)	*B. pumilus*	Hydrolase	Muroi et al., 2017
PHB	*Pseudomonas* sp. DSDY0501	Depolymerase	Di et al., 2019

Conclusion

Plastics have a wide range of uses in our daily lives. Plastics are used in many different areas such as packaging, textiles and cosmetics. The overuse of plastics in such a wide range has led to plastic pollution. The increase in plastic pollution has prompted researchers to look for other ways to combat it. The production of biodegradable plastics is one of such study. However, biodegradable plastics are not favoured over to synthetic plastics. For this reason, studies have been carried out to eliminate the pollution caused by synthetic plastics. Today, many bacteria and enzymes have been discovered that can degrade synthetic plastics. However, many of them are not used commercially because the microbial degradation capacity is not sufficient. Therefore, it is necessary to make the commercially widely used synthetic plastics suitable for microbial degradation, to discover new microorganisms that can degrade plastics, to increase the microbial degradation efficiency of existing bacteria by biotechnological methods or by forming microbial consortia, and to determine the mechanisms of action of bacteria or enzymes.

REFERENCES

Albertsson, A. C. (1978). Biodegradation of synthetic polymers. II. A limited microbial conversion of 14C in polyethylene to 14CO2 by some soil fungi. *Journal of Applied Polymer Science*, *22*(12), 3419-3433.

Ali, S. S., Elsamahy, T., Al-Tohamy, R., Zhu, D., Mahmoud, Y. A. G., Koutra, E., ... & Sun, J. (2021). Plastic wastes biodegradation: Mechanisms, challenges and future prospects. *Science of The Total Environment*, *780*, 146590.

Allen, A. B., Hilliard, N. P., & Howard, G. T. (1999). Purification and characterization of a solublepolyurethane degrading enzyme from *Comamonasacidovorans*. *International biodeterioration & biodegradation*, *43*(1-2), 37-41.

Altuğ, H., & Erdoğan, Ş. (2022). Wastewater treatment plants as a point source of plastic pollution. *Water, Air, & Soil Pollution*, *233*(12), 488.

Amobonye, A., Bhagwat, P., Singh, S., & Pillai, S. (2021). Plastic biodegradation: Frontline microbes and their enzymes. *Science of the Total Environment*, *759*, 143536.

Anwar, M., Negi, H., Zaidi, M., Haider, G., Gupta, S., & Goel, R. (2013). Biodeterioration studies of thermoplastics in nature using indigenous bacterial consortium. *Brazilian Archives of Biology and Technology*, *56*, 475-484.

Apitius, L., Rübsam, K., Jakesch, C., Jakob, F., & Schwaneberg, U. (2019). Ultrahigh-throughput screening system for directed polymer binding peptide evolution. *Biotechnology and Bioengineering*, *116*(8), 1856-1867.

Atanasova, N., Stoitsova, S., Paunova-Krasteva, T., & Kambourova, M. (2021). Plastic degradation by extremophilic bacteria. *International Journal of Molecular Sciences*, *22*(11), 5610.

Azeko, S. T., Etuk-Udo, G. A., Odusanya, O. S., Malatesta, K., Anuku, N., & Soboyejo, W. O. (2015). Biodegradation of linear low density polyethylene by *Serratia marcescens* subsp. *marcescens* and its cell free extracts. *Waste and Biomass Valorization*, *6*, 1047-1057.

Barth, M., Honak, A., Oeser, T., Wei, R., Belisário-Ferrari, M. R., Then, J., ... & Zimmermann, W. (2016). A dual enzyme system composed of a polyester hydrolase and a carboxylesterase enhances the biocatalytic degradation of polyethylene terephthalate films. *Biotechnology Journal*, *11*(8), 1082-1087.

Bollinger, A., Thies, S., Knieps-Grünhagen, E., Gertzen, C., Kobus, S., Höppner, A., ... & Jaeger, K. E. (2020). A novel polyester hydrolase from the marine bacterium *Pseudomonas aestusnigri*–structural and functional insights. *Frontiers in Microbiology*, *11*, 114.

Brandon, A. M., Gao, S. H., Tian, R., Ning, D., Yang, S. S., Zhou, J., ... & Criddle, C. S. (2018). Biodegradation of polyethylene and plastic mixtures in mealworms (larvae of *Tenebrio molitor*) and effects on the gut microbiome. *Environmental Science & Technology*, *52*(11), 6526-6533.

Cacciari, I., Quatrini, P., Zirletta, G., Mincione, E., Vinciguerra, V., Lupattelli, P., & Giovannozzi Sermanni, G. (1993). Isotactic polypropylene biodegradation by a microbial community: physicochemical characterization of metabolites produced. *Applied and Environmental Microbiology*, *59*(11), 3695-3700.

Carmen, S. (2021). Microbial capability for the degradation of chemical additives present in petroleum-based plastic products: A review on current status and perspectives. *Journal of Hazardous Materials*, *402*, 123534.

Carr, C. M., Clarke, D. J., & Dobson, A. D. (2020). Microbial polyethylene terephthalate hydrolases: current and future perspectives. *Frontiers in Microbiology*, *11*, 571265.

Carr, C. M., Keller, M. B., Paul, B., Schubert, S. W., Clausen, K. S., Jensen, K., ... & Dobson, A. D. (2023). Purification and biochemical characterization of SM14est, a PET-hydrolyzing enzyme from the marine sponge-derived *Streptomyces* sp. SM14. *Frontiers in Microbiology*, *14*, 1170880.

Chen, C. C., Dai, L., Ma, L., & Guo, R. T. (2020). Enzymatic degradation of plant biomass and synthetic polymers. *Nature Reviews Chemistry*, *4*(3), 114-126.

Chidambarampadmavathy, K., Karthikeyan, O. P., & Heimann, K. (2017). Sustainable bio-plastic production through landfill methane recycling. *Renewable and Sustainable Energy Reviews*, *71*, 555-562.

Cregut, M., Bedas, M., Durand, M. J., & Thouand, G. (2013). New insights into polyurethane biodegradation and realistic prospects for the development of a sustainable waste recycling process. *Biotechnology Advances*, *31*(8), 1634-1647.

Çevik, C., Kıdeyş, A. E., Tavşanoğlu, Ü. N., Kankılıç, G. B., & Gündoğdu, S. (2022). A review of plastic pollution in aquatic ecosystems of Turkey. *Environmental Science and Pollution Research*, 1-20.

Danko, A. S., Luo, M., Bagwell, C. E., Brigmon, R. L., & Freedman, D. L. (2004). Involvement of linear plasmids in aerobic biodegradation of vinyl chloride. *Applied and Environmental Microbiology*, *70*(10), 6092-6097.

Danso, D., Schmeisser, C., Chow, J., Zimmermann, W., Wei, R., Leggewie, C., ... & Streit, W. R. (2018). New insights into the function and global distribution of polyethylene terephthalate (PET)-degrading bacteria and enzymes in marine and terrestrial metagenomes. *Applied and Environmental Microbiology*, *84(8)*, e02773-17.

Darby, R. T., & Kaplan, A. M. (1968). Fungal susceptibility of polyurethanes. *Applied Microbiology*, *16*(6), 900-905.

Das, R., Liang, Z., Li, G., Mai, B., & An, T. (2019). Genome sequence of a spore-laccase forming, BPA-degrading *Bacillus* sp. GZB isolated from an electronic-waste recycling site reveals insights into BPA degradation pathways. *Archives of Microbiology*, *201*, 623-638.

de Sousa, F. D. B. (2021). Plastic and its consequences during the COVID-19 pandemic. *Environmental Science and Pollution Research*, *28*(33), 46067-46078.

Di, Y., Xia, H., Jiao, Y., Zhang, X., Fang, Q., Li, F., & Chen, S. (2019). Biodegradation of polyhydroxybutyrate by *Pseudomonas* sp. DSDY0501 and purification and characterization of polyhydroxybutyrate depolymerase. *3 Biotech*, *9*, 1-9.

Din, S. U., Kalsoom, Satti, S. M., Uddin, S., Mankar, S. V., Ceylan, E., ... & Shah, A. A. (2023). The purification and characterization of a cutinase-like enzyme with activity on polyethylene terephthalate (PET) from a newly isolated bacterium *Stenotrophomonas maltophilia* PRS8 at a mesophilic temperature. *Applied Sciences*, *13*(6), 3686.

Dudek, K. L., Cruz, B. N., Polidoro, B., & Neuer, S. (2020). Microbial colonization of microplastics in the Caribbean Sea. *Limnology and Oceanography Letters*, *5*(1), 5-17.

Europe Plastics (2023). Plastics – the fast Facts 2023. Available at: https://plasticseurope.org/knowledge-hub/plastics-the-fast-facts-2023/ (Accessed July 05, 2024).

Gao R., & Sun C. A. (2021). marine bacterial community capable of degrading poly(ethylene terephthalate) and polyethylene. *Journal of Hazardous Materials*, *416*, 125928.

Geyer, R., Jambeck, J. R., & Law, K. L. (2017). Production, use, and fate of all plastics ever made. *Science Advances*, *3*(7), e1700782.

Guo, Z., Li, F., Liu, D., Xia, H., Yang, C., Chen, S., & Yang, Y. (2016). Biodegradation of poly (3-hydroxybutyrate-co-4-hydroxybutyrate) by a novel P3/4HB depolymerase purified from *Agrobacterium* sp. DSGZ. *Journal of Applied Polymer Science*, *133*(1).

Gündoğdu, S. (2018). Contamination of table salts from Turkey with microplastics. *Food Additives & Contaminants: Part A*, *35*(5), 1006-1014.

Gündoğdu, S., Çevik, C., & Ataş, N. T. (2020). Stuffed with microplastics: Microplastic occurrence in traditional stuffed mussels sold in the Turkish market. *Food Bioscience*, *37*, 100715.

Hadar, Y., & Sivan, A. (2004). Colonization, biofilm formation and biodegradation of polyethylene by a strain of *Rhodococcus ruber*. *Applied microbiology and biotechnology*, *65*, 97-104.

Howard, G. T., Ruiz, C., & Hilliard, N. P. (1999). Growth of *Pseudomonas chlororaphis* on apolyester–polyurethane and the purification andcharacterization of a polyurethanase–esterase enzyme. *International Biodeterioration & Biodegradation*, *43*(1-2), 7-12.

Jabloune, R., Khalil, M., Moussa, I. E. B., Simao-Beaunoir, A. M., Lerat, S., Brzezinski, R., & Beaulieu, C. (2020). Enzymatic degradation of p-nitrophenyl esters, polyethylene terephthalate, cutin, and suberin by Sub1, a suberinase encoded by the plant pathogen *Streptomyces scabies*. *Microbes and Environments*, *35*(1), ME19086.

Jiang, P., Zhao, S., Zhu, L., & Li, D. (2018). Microplastic-associated bacterial assemblages in the intertidal zone of the Yangtze Estuary. *Science of the Total Environment*, *624*, 48-54.

Kaplan, D. L., Hartenstein, R. O. Y., & Sutter, J. (1979). Biodegradation of polystyrene, poly (metnyl methacrylate), and phenol formaldehyde. *Applied and Environmental Microbiology*, *38*(3), 551-553.

Kawai, F., & Hu, X. (2009). Biochemistry of microbial polyvinyl alcohol degradation. *Applied Microbiology and Biotechnology*, *84*(2), 227-237.

Kelly, M. R., Whitworth, P., Jamieson, A., & Burgess, J. G. (2022). Bacterial colonisation of plastic in the Rockall Trough, North-East Atlantic: An improved understanding of the deep-sea plastisphere. *Environmental Pollution*, *305*, 119314.

Khairul Anuar, N. F. S., Huyop, F., Ur-Rehman, G., Abdullah, F., Normi, Y. M., Sabullah, M. K., & Abdul Wahab, R. (2022). An overview into polyethylene terephthalate (PET) hydrolases and efforts in tailoring enzymes for improved plastic degradation. *International Journal of Molecular Sciences*, *23*(20), 12644.

Khandare S. D., Chaudhary D. R., Jha B. (2021). Marine bacterial biodegradation of low-density polyethylene (LDPE) plastic. *Biodegradation*, *32*, 127–143.

Krueger, M. C., Hofmann, U., Moeder, M., & Schlosser, D. (2015). Potential of wood-rotting fungi to attack polystyrene sulfonate and its depolymerisation by *Gloeophyllum trabeum* via hydroquinone-driven Fenton chemistry. *PloS One*, *10*(7), e0131773.

Lear, L., Padfield, D., Dowsett, T., Jones, M., Kay, S., Hayward, A., & Vos, M. (2022). Bacterial colonisation dynamics of household plastics in a coastal environment. *Science of The Total Environment*, *838*, 156199.

Lebreton, L., Slat, B., Ferrari, F., Sainte-Rose, B., Aitken, J., Marthouse, R., ... & Reisser, J. (2018). Evidence that the Great Pacific Garbage Patch is rapidly accumulating plastic. *Scientific Reports*, *8*(1), 1-15.

Lee, B., Pometto III, A. L., Fratzke, A., & Bailey Jr, T. B. (1991). Biodegradation of degradable plastic polyethylene by *Phanerochaete* and *Streptomyces* species. *Applied and Environmental Microbiology*, *57*(3), 678-685.

MacArthur, E. (2017). The new plastics economy: Rethinking the future of plastics & catalysing action. *Ellen MacArthur Foundation: Cowes, UK*, *68*.

Magnin, A., Pollet, E., Perrin, R., Ullmann, C., Persillon, C., Phalip, V., & Avérous, L. (2019). Enzymatic recycling of thermoplastic polyurethanes: Synergistic effect of an esterase and an amidase and recovery of building blocks. *Waste Management*, *85*, 141-150.

Maity, W., Maity, S., Bera, S., & Roy, A. (2021). Emerging roles of PETase and MHETase in the biodegradation of plastic wastes. *Applied Biochemistry and Biotechnology*, *193*, 2699-2716.

Markic, A., Gaertner, J. C., Gaertner-Mazouni, N., & Koelmans, A. A. (2020). Plastic ingestion by marine fish in the wild. *Critical Reviews in Environmental Science and Technology*, *50*(7), 657-697.

Marsay, K. S., Ambrosino, A. C., Koucherov, Y., Davidov, K., Figueiredo, N., Yakovenko, I., ... & Oren, M. (2023). The geographical and seasonal effects on the composition of marine microplastic and its microbial communities: The case study of Israel and Portugal. *Frontiers in Microbiology*, *14*, 1089926.

Mohan, A. J., Sekhar, V. C., Bhaskar, T., & Nampoothiri, K. M. (2016). Microbial assisted high impact polystyrene (HIPS) degradation. *Bioresource Technology*, *213*, 204-207.

Montazer Z., Habibi Najafi M. B., Levin D. B. (2019). Microbial degradation of low-density polyethylene and synthesis of polyhydroxyalkanoate polymers. *Canadian Journal of Microbiology*, 65, 224–234.

Moog, D., Schmitt, J., Senger, J., Zarzycki, J., Rexer, K. H., Linne, U., ... & Maier, U. G. (2019). Using a marine microalga as a chassis for polyethylene terephthalate (PET) degradation. *Microbial Cell Factories*, *18*, 1-15.

Moshood, T. D., Nawanir, G., Mahmud, F., Mohamad, F., Ahmad, M. H., & AbdulGhani, A. (2022). Sustainability of biodegradable plastics: New problem or solution to solve the global plastic pollution?. *Current Research in Green and Sustainable Chemistry*, *5*, 100273.

Mrowiec, B. (2017). Plastic pollutants in water environment. *Environmental Protection and Natural Resources*, *28*(4), 51-55.

Muroi, F., Tachibana, Y., Soulenthone, P., Yamamoto, K., Mizuno, T., Sakurai, T., ... & Kasuya, K. I. (2017). Characterization of a poly (butylene adipate-co-

terephthalate) hydrolase from the aerobic mesophilic bacterium *Bacillus pumilus*. *Polymer Degradation and Stability*, *137*, 11-22.

Müller, R. J., Schrader, H., Profe, J., Dresler, K., & Deckwer, W. D. (2005). Enzymatic degradation of poly (ethylene terephthalate): rapid hydrolyse using a hydrolase from *T. fusca*. *Macromolecular Rapid Communications*, *26*(17), 1400-1405.

Nakamiya, K., Ooi, T., & Kinoshita, S. (1997). Non-heme hydroquinone peroxidase from *Azotobacter beijerinckii* HM121. *Journal of Fermentation and Bioengineering*, *84*(1), 14-21.

Obradors, N., & Aguilar, J. (1991). Efficient biodegradation of high-molecular-weight polyethylene glycols by pure cultures of *Pseudomonas stutzeri*. *Applied and Environmental Microbiology*, *57*(8), 2383-2388.

O'Leary, N. D., O'Connor, K. E., & Dobson, A. D. (2002). Biochemistry, genetics and physiology of microbial styrene degradation. *FEMS Microbiology Reviews*, *26*(4), 403-417.

Picó, Y., & Barceló, D. (2019). Analysis and prevention of microplastics pollution in water: current perspectives and future directions. *ACS Omega*, *4*(4), 6709-6719.

Pollet, T., Berdjeb, L., Garnier, C., Durrieu, G., Le Poupon, C., Misson, B., & Briand, J. F. (2018). Prokaryotic community successions and interactions in marine biofilms: the key role of *Flavobacteriia*. *FEMS Microbiology Ecology*, *94*(6), fiy083.

Ronkvist, Å. M., Xie, W., Lu, W., & Gross, R. A. (2009). Cutinase-catalyzed hydrolysis of poly (ethylene terephthalate). *Macromolecules*, *42*(14), 5128-5138.

Rosato, D. V. (2011). Plastics end use applications. Springer Science & Business Media.

Rowe, L., & Howard, G. T. (2002). Growth of *Bacillus subtilis* on polyurethane and the purification and characterization of a polyurethanase-lipase enzyme. *International Biodeterioration & Biodegradation*, *50*(1), 33-40.

Ru, J., Huo, Y., & Yang, Y. (2020). Microbial degradation and valorization of plastic wastes. *Frontiers in Microbiology*, *11*, 442.

Rujnić-Sokele, M., & Pilipović, A. (2017). Challenges and opportunities of biodegradable plastics: A mini review. *Waste Management & Research*, *35*(2), 132-140.

Rübsam, K., Weber, L., Jakob, F., & Schwaneberg, U. (2018). Directed evolution of polypropylene and polystyrene binding peptides. *Biotechnology and Bioengineering*, *115*(2), 321-330.

Sah, A., Negi, H., Kapri, A., Anwar, S., & Goel, R. (2011). Comparative shelf life and efficacy of LDPE and PVC degrading bacterial consortia under bioformulation. *Ekologija*, *57*(2), 55-61.

Santo, M., Weitsman, R., & Sivan, A. (2013). The role of the copper-binding enzyme–laccase–in the biodegradation of polyethylene by the actinomycete *Rhodococcus ruber*. *International Biodeterioration & Biodegradation*, *84*, 204-210.

Sarmah, P., & Rout, J. (2018). Efficient biodegradation of low-density polyethylene by cyanobacteria isolated from submerged polyethylene surface in domestic sewage water. *Environmental Science and Pollution Research*, *25*, 33508-33520.

Schmidt, J., Wei, R., Oeser, T., Dedavid e Silva, L. A., Breite, D., Schulze, A., & Zimmermann, W. (2017). Degradation of polyester polyurethane by bacterial polyester hydrolases. *Polymers*, *9*(2), 65.

Seymour R. B., Kauffman G. B. (1992). Polyurethanes: A class of modern versatile materials. *Journal of Chemical Education*, *69*, 909.

Shah, A. A., Hasan, F., Hameed, A., & Ahmed, S. (2008). Biological degradation of plastics: a comprehensive review. *Biotechnology Advances*, *26*(3), 246-265.

Shahnawaz, M., Sangale, M. K., & Ade, A. B. (2016). Rhizosphere of *Avicennia marina* (Forsk.) Vierh. as a landmark for polythene degrading bacteria. *Environmental Science and Pollution Research*, *23*, 14621-14635.

Sumathi, T., Viswanath, B., Sri Lakshmi, A., & SaiGopal, D. V. R. (2016). Production of laccase by *Cochliobolus* sp. isolated from plastic dumped soils and their ability to degrade low molecular weight PVC. *Biochemistry Research International*, *2016*(1), 9519527.

Tischler, D., Eulberg, D., Lakner, S., Kaschabek, S. R., van Berkel, W. J., & Schlömann, M. (2009). Identification of a novel self-sufficient styrene monooxygenase from *Rhodococcus opacus* 1CP. *Journal of Bacteriology*, *191*(15), 4996-5009.

Tribedi, P., Sarkar, S., Mukherjee, K., & Sil, A. K. (2012). Isolation of a novel *Pseudomonas* sp. from soil that can efficiently degrade polyethylene succinate. *Environmental Science and Pollution Research*, *19*, 2115-2124.

Tribedi, P., & Sil, A. K. (2013). Low-density polyethylene degradation by *Pseudomonas* sp. AKS2 biofilm. *Environmental Science and Pollution Research*, *20*, 4146-4153.

Urbanek, A. K., Rymowicz, W., & Mirończuk, A. M. (2018). Degradation of plastics and plastic-degrading bacteria in cold marine habitats. *Applied microbiology and biotechnology*, *102*, 7669-7678.

Vega, R. E., Main, T., & Howard, G. T. (1999). Cloning and expression in *Escherichia coli* of apolyurethane-degrading enzyme from *Pseudomonas fluorescens*. *International Biodeterioration & Biodegradation*, *43*(1-2), 49-55.

Wei, R., & Zimmermann, W. (2017). Microbial enzymes for the recycling of recalcitrant petroleum-based plastics: how far are we?. *Microbial Biotechnology*, *10*(6), 1308-1322.

Wilkes, R. A., & Aristilde, L. (2017). Degradation and metabolism of synthetic plastics and associated products by *Pseudomonas* sp.: capabilities and challenges. *Journal of Applied Microbiology*, *123*(3), 582-593.

Wright, R. J., Erni-Cassola, G., Zadjelovic, V., Latva, M., & Christie-Oleza, J. A. (2020). Marine plastic debris: a new surface for microbial colonization. *Environmental Science & Technology*, *54*(19), 11657-11672.

Xu, J., Cui, Z., Nie, K., Cao, H., Jiang, M., Xu, H., ... & Liu, L. (2019). A quantum mechanism study of the CC bond cleavage to predict the bio-catalytic polyethylene degradation. *Frontiers in Microbiology*, *10*, 489.

Yan, Z. F., Xu, K. W., & Wu, J. (2023). Synergism between multi-*Pseudomonas* and cutinase for biodegradation of crude oil-based derivatives. *Current Microbiology*, *80*(1), 30.

Yang, X. G., Wen, P. P., Yang, Y. F., Jia, P. P., Li, W. G., & Pei, D. S. (2023). Plastic biodegradation by in vitro environmental microorganisms and in vivo gut microorganisms of insects. *Frontiers in Microbiology*, *13*, 1001750.

Yoon, M. G., Jeon, H. J., & Kim, M. N. (2012). Biodegradation of polyethylene by a soil bacterium and AlkB cloned recombinant cell. *Journal of Bioremediation and Biodegradation*, *3*(4), 1-8.

Yoshida, S., Hiraga, K., Takehana, T., Taniguchi, I., Yamaji, H., Maeda, Y., ... & Oda, K. (2016). A bacterium that degrades and assimilates poly (ethylene terephthalate). *Science*, *351*(6278), 1196-1199.

Zhao, X., Wang, J., Yee Leung, K. M., & Wu, F. (2022). Color: an important but overlooked factor for plastic photoaging and microplastic formation. *Environmental Science & Technology*, *56*(13), 9161-9163.

HYPERACCUMULATOR PLANTS AND THEIR USE IN PHYTOREMEDIATION

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Introduction

A significant issue of the twenty-first century is environmental pollution, which is caused by a variety of factors including urbanisation and industrialisation, which emit different types of pollutants into the atmosphere. Some inorganic pollutants such as heavy metals like cadmium (Cd), copper (Cu), lead (Pb), manganese (Mn), mercury (Hg), nickel (Ni) and zinc (Zn), are extremely harmful to the environment due to their toxicity, persistence, and ability to bioaccumulate in ecosystems (Rascio and Navari-Izzo, 2011). Unlike organic pollutants, heavy metals are not degradable, which makes their removal from contaminated soils and water bodies particularly difficult (Aziz et al., 2023; Kumar, 2024).

Conventional heavy metal removal techniques, like chemical extraction and physical removal, can be costly, harmful to the environment, and ineffectual for large-scale or low-concentration pollution. For these reasons, interest in ecological and sustainable alternative clean-up routes has increased, which has stimulated the development of phytoremediation, a biological process that utilises the capacity of plants to absorb, trap and detoxify environmental pollutants (Liu et al., 2023).

Using plants and related microbial communities to lessen pollution levels and their harmful effects on the environment is known as phytoremediation. This method utilises the natural and various processes by which organic and inorganic pollutants are broken down and sequestered by plants and the microorganisms in their rhizosphere. Phytoremediation is a remediation technique that works well for a variety of environmental pollutants (Pilon-Smits, 2005). It takes advantage of plants' natural ability to absorb, break down, and neutralise a range of environmental contaminants, such as heavy metals, organic pollutants, and radioactive materials. Hyperaccumulator species are distinguished from other plant species employed in phytoremediation by their capacity to grow in contaminated settings and to collect very high quantities of metals in their tissues (Ali et al., 2013; Zhakypbek et al., 2024).

The term ‘hyperaccumulator’ was first coined in a 1976 study to denote *Pycnandra acuminata*, which exhibited nickel (Ni) concentrations significantly higher (often hundreds or thousands of times) than those typically found in plants growing in most common soils (Jaffré et al., 1976; Reeves et al., 2021; Reeves, 2024). Plants known as hyperaccumulators are

able to withstand and accumulate heavy metals at far higher concentrations in their aboveground tissues than in the soil or other non-accumulating plants (McGrath et al., 2001; Memon et al., 2001; Memon and Schröder, 2009). In their shoots and leaves, they can concentrate metals like zinc (Zn), nickel (Ni), cadmium (Cd), and arsenic (As), frequently at concentrations that would be lethal to the majority of other plant species (Rascio and Navari-Izzo, 2011). Because of this feature, they are especially useful for cleaning up polluted sites, where traditional techniques might be too costly or impractical (Reeves et al., 2018). An increasingly popular method for cleaning up groundwater and restoring contaminated soils is the use of hyperaccumulators in phytoremediation, which is gaining attention in environmental research (Vangronsveld et al., 2009; Yan et al., 2020).

Mechanisms of Hyperaccumulation

Hyperaccumulation is a complex process involving various physiological, biochemical, and molecular mechanisms (Clemens, 2006; Verbruggen et al., 2009; Krämer, 2010; Maestri et al., 2010). Various physiological, biochemical, and molecular processes are involved in the intricate process of hyperaccumulation. It is essential to comprehend these mechanisms to maximise the application of hyperaccumulator plants in phytoremediation (Peer et al., 2006; Pollard et al., 2014).

The main processes involved in the hyperaccumulation of organic or inorganic pollutants by plants from soil or water to shoots are the following:

- activation of metals in the rhizosphere through interactions between roots and microbes,
- facilitated and increased uptake due to metal carriers present in the plasma membrane,
- detoxification of metals by binding to cell walls and diffusing into apoplasts using various ligands such as phytochelatins, metallothionins and metal-binding proteins, including chelation within the cytoplasm,
- sequestration of metals in vacuoles via transporters located in the tonoplast (Yang et al., 2005; Zhakypbek et al., 2024).

Metal Uptake and Transport

Hyperaccumulators are able to selectively absorb metals from the soil thanks to the development of specialised root systems (Dakora and Phillips, 2002; Kumar, 2024). The process begins with the release of root exudates,

which are organic acids like citrate and malate that increase the bioavailability of metals for absorption and improve metal solubility in the rhizosphere (Osmolovskaya et al., 2018). Metals are absorbed by particular transport proteins, found in the membranes of the root cells, such as members of the ZIP (Zinc-Iron Permease) family, once they have become soluble, and they are actively transported into the root cells by these proteins through the plasma membrane (Grotz and Guerinot, 2006; Milner and Kochian, 2008). Metals enter the root cells and are either stored there or move through the xylem to the shoots (Ueno et al., 2008). Most heavy metals absorbed by non-hyperaccumulator plants remain in the root cells, where they are either chelated in the cytoplasm to remove them or stored in vacuoles (Ansari et al., 2016). On the other hand, these metals are swiftly and effectively transferred to the shoots through the xylem by hyperaccumulators (Dar et al., 2015). Hyperaccumulators differ from non-hyperaccumulators in that they efficiently translocate metals to above-ground tissues, whereas non-hyperaccumulators usually restrict metal movement to prevent toxicity. Metal-binding ligands, such as organic acids and metallothionein, which chelate the metals and shield the plant from their harmful effects, enable this translocation in hyperaccumulators (Callahan et al., 2006; White and Broadley, 2009; Claus et al., 2013).

In hyperaccumulators, metals are detoxified and sequestered by a variety of metabolic processes (Hall, 2002; Yang et al., 2005; Verbruggen et al., 2013; Sharma et al., 2021). After metals are transferred to the shoots, they are frequently chelated by peptides like phytochelatins or organic acids and kept in membrane-bound vacuoles within plant cells (Figure 1). This compartmentalization is necessary to keep metals from interfering with essential biological functions (Küpper et al., 2000; Sarret et al., 2002; Sharma et al., 2016; Sharma et al., 2022).

For instance, in *Noccaea caerulescens*, a well-known hyperaccumulator of cadmium (Cd) and zinc (Zn), high concentrations of citrate in the xylem sap facilitate the translocation of zinc from roots to shoots. Once in the shoots, zinc (Zn) is sequestered in vacuoles, where it is bound to malate or other organic acids, rendering it non-toxic to the plant (Gupta et al., 2016). Similarly, in *Pteris vittata*, a hyperaccumulator of arsenic (As), the reduction of arsenate to arsenite and its subsequent complexation with thiol-rich peptides like glutathione are critical steps in arsenic detoxification (Abbas et al., 2018)

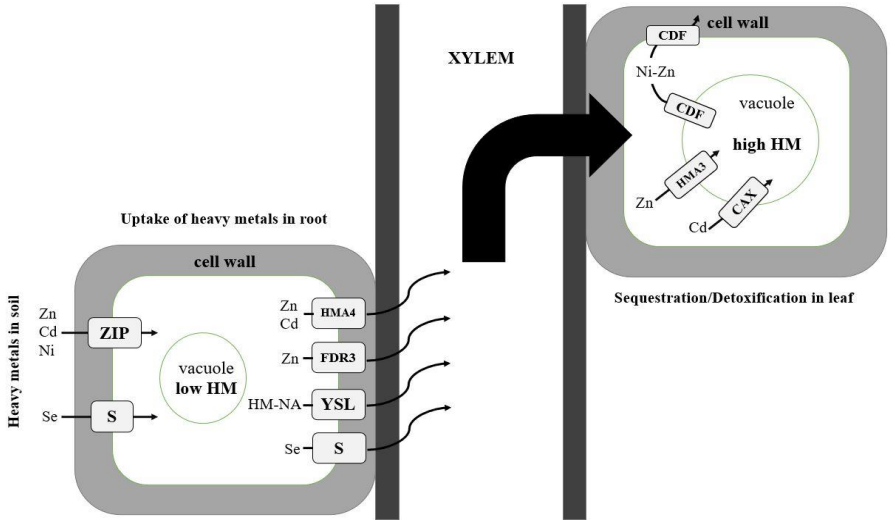

Figure 1. Schematic representation of uptake, transport, and sequestration/detoxification of heavy metals in plants (adapted from Rascio and Navari-Izzo, 2011 and Dar et al., 2015). HM: heavy metal, ZIP: zinc-regulated and iron-regulated transporter proteins, S: sulphate transporters, HMA: heavy metal transporting ATPases, FDR3: a member of the multidrug and toxin efflux family, NA: nicotianamine, YSL: yellow strip 1-like proteins, CAX: cation exchangers, CDF: cation diffusion facilitators.

Common Hyperaccumulator Plants

Hyperaccumulator plants are distributed across various plant families and ecological regions. The diversity of these plants reflects their adaptation to different environmental conditions and metal types. Currently, over 450 species of hyperaccumulator plants in 45 families exist that satisfy the requirements for hyperaccumulation. These plants are mostly hyperaccumulators (%75) of nickel (Ni). Moreover, a single metal can be overaccumulated and overtolerant in the majority of hyperaccumulator plant species. However, some plant species, including cadmium (Cd), copper (Cu) and zinc (Zn), can accumulate and tolerate several metals in large quantities (Güneş and Bozkurt, 2021; Kocaman, 2022).

Plants showing hyperaccumulator properties mostly belong to families such as “Brassicaceae, Asteraceae, Cyperaceae, Caryophyllaceae, Lamiaceae, Fabaceae, Violaceae, Poaceae and Euphorbiaceae”. Brassicaceae is recognised as the largest family of hyperaccumulator plants with 87 hyperaccumulator species and 11 genera. In Brassicaceae, 7 genera and 72

species can accumulate nickel (Ni) (Güneş and Bozkurt, 2021). Table 1 lists some hyperaccumulator plants and their properties.

Table 1. List of some hyperaccumulator plants

Family	**Species**	**Metals**	**References**
Amaranthaceae	*Amaranthus dubius*	Zn, Cu, Mn	Subramanian et al., 2012
	Beta vulgaris	Cd, Ni	Chen et al. 2013; Papazoglou and Fernando, 2017
Amaryllidaceae	*Allium roseum*	Pb, Zn,Cd	Chaabani et al., 2017
Araliaceae	*Hedera helix*	As, Hg, Cd, Zn	ur Rehman et al., 2019
Asteraceae	*Achillea millefolium*	Cd, Zn	Antoniadis et al., 2021
	Artemisia vulgaris		
	Helianthus annuus	Cu, Mn	Tokalıoğlu, 2012
Betulaceae	*Alnus nepalensis*	Zn, Pb and Cd	Jing et al., 2014
Brassicaceae	*Brassica napus*	Cu, Mn	Özcan, 2006
	Noccaea praecox	Zn, Cd	Nečemer et al., 2008
	Odontarrhena chalcidica	Ni	Salihaj et al., 2016
Caryophyllaceae	*Dianthus deltoides*	Cd, Pb	Qiao et al., 2023
	Rabelera holostea	Cd, Zn	Antoniadis et al., 2021
	Sabulina verna	Cu, Ni, Pb, Zn	Ernst, 2005
	Silene vulgaris		
	Spergularia rubra	Cd, Zn	Medina-Díaz et al., 2024
Convolvulaceae	*Calystegia hederacea*	Pb, Zn, As, Cd	Sun et al., 2016
Crassulaceae	*Crassula helmsii*	Cu	Kupper et al., 2009
	Sedum alfredi	Cd, Zn, Pb	Deng et al., 2008
Ericaceae	*Erica lusitanica*	Mn	de la Fuente et al., 2010
Euphorbiaceae	*Euphorbia amygdaloides*	Zn, Mn, Ni, Cu, Pb, Mo, Co	Salihaj et al., 2016
Fabaceae	*Acacia dealbata*	Mn, Zn	De La Calle et al., 2013
	Robinia pseudoacacia	Zn, Cu, Cd, Pb	Lovynska et al., 2023
	Trifolium pratense	Cs	Wu et al., 2009

Hypericaceae	*Hypericum perforatum*	Mn, Zn	Tokalıoğlu, 2012
Iridaceae	*Iris versicolor*	Cd	Mohsin et al., 2023
Lamiaceae	*Stachys sylvatica*	As	Antosiewicz et al., 2008
	Thymus praecox	Cd, Hg, Fe	Rajsz et al., 2021
Plantaginaceae	*Callitriche platycarpa*	Cr	Augustynowicz et al., 2010
	Linaria dalmatica	Se	Quinn et al., 2008
	Plantago lanceolata	Ni, Cd, Cu	Pietrelli et al., 2022
Poaceae	*Agrostis capillaris*	Cd, Cu, Mn, Ni, Pb, Zn	Ernst, 2005
	Agrostis stolonifera	As, Cd, Cu, Pb, Zn	Pérez-de-Mora et al., 2006
	Agrostis tenuis	Pb	Anguilano et al., 2022
	Alopecurus pratensis	Cd, Zn	Antoniadis et al., 2021
	Arrhenatherum elatius		
	Bromus inermis		
Polygonaceae	*Rumex acetosa*	Zn	Barrutia et al., 2009
Potamogetonaceae	*Potamogeton crispus*	Cd	Sivaci et al., 2008
Ranunculaceae	*Ranunculus penicillatus*	U	Cordeiro et al., 2016
Rubiaceae	*Galium verum*	Zn, Ni, Cu	Salihaj et al., 2018
	Galium mollugo	Cd, Zn	Antoniadis et al., 2021
Salicaceae	*Populus alba*	Cd, Cr, Cu, Ni, Zn	Guerra et al., 2011; Rafati et al., 2011
Solanaceae	*Lycium barbarum*	Ni, Cu, Cd, Cr, Co	Lu et al., 2017

Criteria for Classifying Hyperaccumulators

To classify a plant as a hyperaccumulator, it must demonstrate the ability to concentrate metals in its tissues at significantly higher levels compared to non-hyperaccumulating species. For different heavy metals, the exact threshold values typically used to detect hyperaccumulators are as follows:

Table 2. Threshold level for definition of hyperaccumulator plants (Lepp, 1981; Markert, 1996; McIntyre, 2003; Debnath et al., 2015)

Element	Average range in soils (µg g^{-1} dry weight)	Average range in plant tissues (µg g^{-1} dry weight)	Threshold for hyperaccumulators (µg g^{-1} dry weight)
Mercury (Hg)	<0.1	0.005-0.2	1000
Selenium (Se)	1-2	0.01-0.2	1000
Cadmium (Cd)	1-2	0.03-0.5	100
Copper (Cu)	2-60	2-20	1000
Nickel (Ni)	2-200	0.4-4	1000
Chromium (Cr)	5-1000	0.2-1	1000
Lead (Pb)	10-150	0.1-5	1000
Zinc (Zn)	25-200	15-150	10000
Manganese (Mn)	100-4000	1-7000	10000

These thresholds, however, are not absolute and may change based on the species and the surrounding circumstances. The ability of plants to tolerate and accumulate metals to these levels reflects their potential use in phytoremediation and bioremediation efforts.

Phytoremediation Techniques Involving Hyperaccumulators and Applications

Using the inherent qualities of plants, phytoremediation is a sustainable and economically feasible method of cleaning contaminated soils, sediments, and water bodies. This method involves the uptake, translocation, stabilization, or degradation of a wide array of environmental pollutants. The process is categorised into various techniques, each meticulously designed to address specific contaminants and adapt to distinct ecological contexts. Hyperaccumulator plants play an important role in multiple phytoremediation techniques due to their extraordinary ability to absorb, concentrate and trap high levels of pollutants from the environment.

Phytoremediation utilises various mechanisms, such as degradation (including rhizo-degradation and phytodegradation), accumulation (phytoextraction and rhizofiltration), dissipation (phytovolatilization), and immobilization (hydraulic control and phytostabilization) to degrade, extract, or immobilise contaminants (Figure 2) (Kafle et al., 2022).

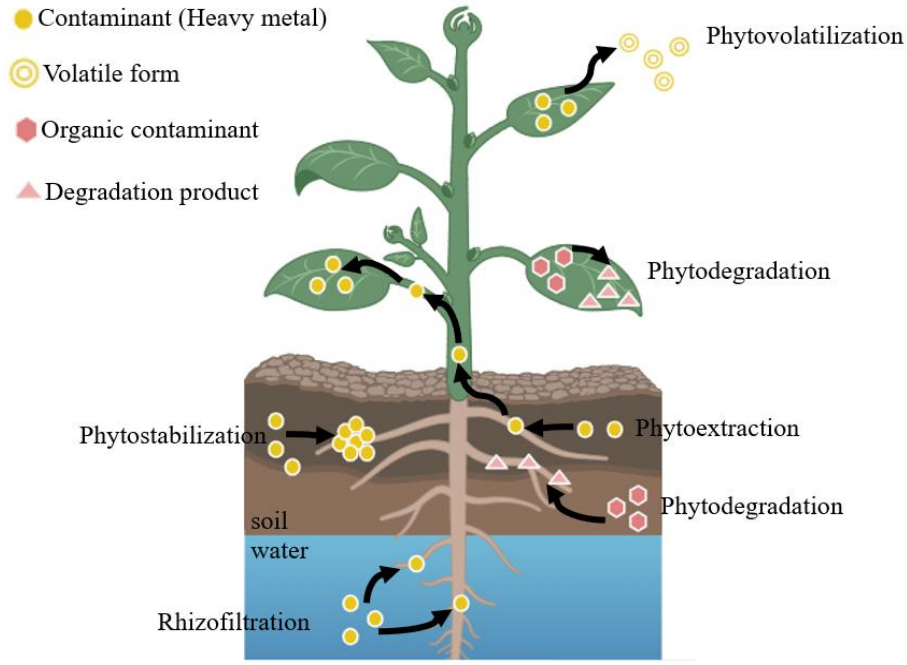

Figure 2. Schematic representation of phytoremediation techniques (created in Biorender) (adapted from Rigoletto et al., 2020). "Phytoextraction: use of plants to clean up pollutants via accumulation in harvestable tissues; Rhizofiltration: use of plants in hydroponic setup for filtering polluted water; Phytodegradation: breakdown of pollutants by plant enzymes, usually inside tissues; Phytovolatilization: release of pollutants by plants in volatile form; Phytostabilization: denotes the use of plants to stabilise pollutants in soil" (Pilon-Smits, 2005).

Phytoextraction

Phytoextraction, which is sometimes referred to as phytoaccumulation, phytoabsorption, or phytosequestration, is the most common phytoremediation technique involving hyperaccumulators (Chandra et al., 2017). It is the process through which contaminants are absorbed from the soil by plant roots and accumulate in the biomass above ground. Eventually, concentrated metals detected in harvested biomass can be removed and disposed of or processed (a procedure called phytomining) to recover precious metals (Kumar, 2024). Because hyperaccumulators easily absorb heavy metals like cadmium (Cd), nickel (Ni), and zinc (Zn), phytoextraction is especially useful for these elements (Mc Grath and Zhao, 2003; Ali et al., 2013, Sinha et al., 2021).

Studies show that numerous plant species can be employed in phytoextraction. *Helianthus annuus* and *Brassica juncea* are popular species for phytoextraction because of their high biomass, quick growth, and high tolerance to metals and other inorganic substances (Pilon-Smits, 2005). In a phytoextraction experiment, nickel (Ni) was extracted from serpentine soils using *Odontarrhena muralis* (Kanso et al., 2018). This technique can be economically applied to contaminated sites by recovering the metal through the harvesting and processing of the biomass that is rich in nickel (Ni). While *Noccaea caerulescens* (formerly *Thlaspi caerulescens*) is well-known for its capacity to hyperaccumulate nickel (Ni) and zinc (Zn) (Seregin et al., 2014), *Brassica juncea* is frequently used for the extraction of cadmium (Cd), lead (Pb) and zinc (Zn) (Anamika et al., 2009). *Pteris vittata* works well for removing arsenic (As) (Yang et al., 2022). In addition, woody plant species like *Populus* and *Salix* are also utilised in phytoextraction because of their high biomass in comparison to shrubs and herbs, as well as their capacity to absorb large amounts of metals from their aboveground shoots (Khan et al., 2023).

Phytostabilization

Phytostabilization is a phytoremediation method that uses plants to stabilise pollutants in the soil, preventing their spread through water, wind, or biological uptake (Kumar, 2024). In this technique, plant roots immobilise pollutants by binding them to the soil and changing their chemical form, reducing their mobility and bioavailability. Therefore, it is particularly useful to prevent the spread of pollutants through erosion or leaching into groundwater (Bolan et al., 2011; Ansari et al., 2016; Shackira and Puthur, 2019).

This approach is often used in combination with soil amendments, such as lime or organic matter, to enhance metal immobilization and reduce phytotoxicity. Phytostabilization is commonly applied in sites where contamination is widespread, and complete removal of contaminants is not feasible (Solomou et al., 2022).

Some of the contaminants commonly targeted by phytostabilization include arsenic (As), cadmium (Cd), lead (Pb) and zinc (Zn). For instance, *Chrysopogon zizanioides* (formerly *Vetiveria zizanioides*) has a deep root system that stabilises soil and immobilises lead by binding it in the root zone (Abaga et al., 2021). *Deschampsia caespitosa* and *Lolium perenne* can

stabilise zinc (Zn) in contaminated soils by accumulating it in root tissues, limiting its movement (Korzeniowska and Stanislawska-Glubiak, 2023). *Salix* and *Populus* can stabilise heavy metals in contaminated soils by reducing its mobility through root exudates (Hrkić Ilić et al 2020).

Rhizofiltration

Rhizofiltration or phytofiltration is a phytoremediation technique that involves the use of plant roots to remove contaminants, particularly heavy metals, from aqueous environments such as groundwater, surface water, or wastewater. Unlike other phytoremediation methods that focus on soil, hemofiltration specifically targets water-based contaminants (Dushenkov et al., 1995).

In rhizofiltration, both terrestrial and aquatic plants are used to absorb, concentrate, and precipitate pollutants from polluted water (Yadav et al., 2011). Hyperaccumulators with high root biomass and fast growth rates, such as *Helianthus annuus* and *Brassica juncea*, are often used in this technique. Rhizofiltration is particularly effective for removing heavy metals such as cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), nickel (Ni) and zinc (Zn), from wastewater and contaminated groundwater (Dushenkov et al., 1995; Ansari et al., 2016).

This technique is utilised across diverse contexts, including the treatment of industrial effluents to address wastewater contaminated with heavy metals, the remediation of groundwater to lower heavy metal concentrations, and the treatment of radioactive wastewater to eliminate radioactive elements such as uranium (Lee and Yang, 2010; Kumar, 2024).

In practical applications, plants are grown in hydroponic systems or directly in contaminated water bodies. The metal-laden roots are then harvested and treated, effectively removing the contaminants from the aquatic environment (Woraharn et al., 2021, Banerjee and Roychoudhury, 2022).

Rhizofiltration involves aeration and is therefore not only limited to aquatic species, but also benefits terrestrial species with large roots and the capacity to accumulate inorganic substances (Pilon-Smits, 2005). For instance, *Pontederia crassipes* (formerly *Eichhornia crassipes*) is effective in absorbing cadmium (Cd), lead (Pb), uranium (U) and zinc (Zn) from water and storing it in its root system (Bhainsa, 2001; Yadav et al., 2015). *Azolla*

species can absorb and concentrate lead (Pb) and nickel (Ni) from contaminated water (Thayaparan et al., 2013; Valderrama et al., 2016). *Phragmites australis* can remove arsenic (As) from water by accumulating it in its root system and shoots (Álvarez-Robles et al., 2022). *Ceratophyllum demersum* a floating aquatic plant, can accumulate heavy metals such as cadmium (Cd), iron (Fe) and nickel (Ni) in its biomass and roots (Parnian et al., 2022; Nasution, 2024).

Phytodegradation

Phytodegradation, or phytotransformation (Kumar, 2024), is a phytoremediation technique that uses plants to break down organic pollutants in the environment, mostly by biochemical processes (Pilon-Smits, 2005). Unlike other phytoremediation techniques that concentrate on either gathering or stabilizing contaminants, in phytodegradation, plant enzymes breaking down organic pollutants play in role. These enzymes convert the contaminants into stable intermediates that the plant stores, or they partially break them down into inorganic molecules. This process depends on the plant's ability to metabolise contaminants into less harmful substances, involving several key mechanisms. Enzymes that plants can secrete into the soil or water help break down organic contaminants. Furthermore, plants' root systems have the ability to stimulate rhizosphere microbial populations, which improves microbial activity and the breakdown of pollutants. Some plants also possess intrinsic metabolic pathways that enable them to transform pollutants internally, thereby reducing their toxicity (Pilon-Smits, 2005; Abhilash et al., 2009; Kumar, 2024).

For the treatment of organic pollutants including pesticide residue, petroleum hydrocarbons, and medication leftovers, phytodegradation is particularly effective. Because of their intricate chemical structures and enduring presence in the environment, these pollutants are often challenging to eliminate through traditional means. It is possible to use plants that can break down these compounds in a variety of environments, such as wastewater treatment systems, agricultural areas, and oil spill sites (Al-Baldawi et al., 2015; Kaur et al., 2021; Banerjee et al., 2022; Hussein et al., 2022).

For example, the aquatic *Elodea canadensis* and the terrestrial *Pueraria montana var. lobata* (formerly *Pueraria thunbergiana*) enabled reductive dehalogenation of DTT and its degradation catalyzed by biomolecules or cofactors of enzymes (Garrison et al., 2000). *Phragmites australis* degrades

ibuprofen in water and is therefore a suitable species for application in constructed wetlands to treat wastewater effluents containing ibuprofen and possibly other micropollutants (He et al., 2017). *Spirodela polyrhiza* may be an effective biotool for removing environmentally important concentrations of antibiotics from wastewater (Singh et al., 2019).

Despite its advantages in terms of cost-effectiveness and environmental benefits, phytodegradation has disadvantages. It is primarily suited for organic contaminants (Asante-Badu et al., 2020). The process can be relatively slow compared to chemical treatments (Al-Baldawi et al., 2015), and the efficacy depends on selecting appropriate plant species that can metabolise specific pollutants (Kafle et al., 2022). Therefore, for implementation to be successful, thorough consideration of plant species and environmental conditions is required.

Phytovolatilization

Phytovolatilization is a type of phytoremediation technique in which plants absorb organic pollutants, especially volatile organic compounds (VOCs) and metals, such as arsenic (As), mercury (Hg) and selenium (Se), from soil or water and the common groundwater contaminants, trichloroethylene (TCE) and tetrachloroethylene (PCE), through their roots and subsequently release them into the atmosphere through their leaves in a volatile form. This process works especially well for some pollutants that can change into less harmful gaseous compounds and then be released from the plant (Pilon-Smits, 2005; Pilon-Smits and Freeman, 2006; Limmer and Burken, 2016; Kumar, 2024).

Populus and *Salix* species are the most commonly utilised for phytovolatilization of volatile organic compounds because of their high transpiration rate, which facilitates the chemicals' movement through the plant and into the atmosphere (Pilon-Smits, 2005; Limmer and Burken, 2016). The best-known species for selenium (Se) phytovolatilization is *Brassica juncea*. More than 20 species, including *Myriophyllum aquaticum* (formerly *Myriophyllum brasiliense*), *Juncus xiphioides*, *Typha latifolia*, *Typha angustifolia*, and *Bolboschoenus robustus* (formerly *Scirpus robustus*) are also plants with great potential for selenium (Se) phytoremediation in wetlands (Pilon-Smits et al., 1999; Schiavon and Pilon-Smits, 2017). Additionally, *Astragalus racemosus* can convert selenium (Se) to dimethyl diselenide via

phytovolatylation, while *Arabidopsis thaliana* can also convert Hg^{2+} to Hg^{0} and thus increase mercury (Hg) volatilization (Awa and Hadibarata, 2020).

Despite the beneficial effects of phytovolatilization, a recent study has emphasised the need for risk assessment in urban areas due to poor air quality, given the high dilution and photochemical degradation of pollutants in large volumes of air (Ionata et al., 2024).

Future Prospects

Hyperaccumulators in phytoremediation have a bright future because there is continuous research and technological advancements aimed at resolving present issues and broadening the scope of this environmentally friendly technology.

Genetic Engineering and Plant Biotechnology

The genetic engineering of hyperaccumulator plants to enhance metal uptake, transport, and tolerance capabilities is one of the most fascinating research topics. The goal of these investigations is to continue to develop 'super hyperaccumulators' or organisms that can survive in a greater variety of environments and accumulate higher concentrations of metals, by introducing or altering particular genes involved in metal transport, chelation, and sequestration (Halder and Anirban, 2021).

Studies have provided evidence that several of these properties have been successfully modified. For instance, to increase the iron (Fe) content of rice (*Oryza sativa*), the soya bean ferritin gene was transferred into the plant. By utilising the rice seed storage protein glutelin promoter (GluB-1), ferritin expression in developing seeds could be targeted. Thus, it was found that the iron (Fe) content in transformed seeds was 3 times higher than normal (Goto et al., 1999). In another study, *Nicotiana tabacum* and *Arabidopsis thaliana* were transformed with barley (*Hordeum vulgare*) NAS1 gene encoding nicotinamine (NA) synthase enzyme. *Arabidopsis thaliana* had increased tolerance to cadmium (Cd), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni) and zinc (Zn), while *Nicotiana tabacum* had increased tolerance to nickel (Ni) and increased manganese (Mn) accumulation (Kim et al., 2005; Fasani et al., 2018).

Additionally, a different study indicated that it is now feasible to identify and modify gene function to improve phytoremediation capacity due to the development of new technologies like in situ editing and CRISPR/Cas9 gene silencing. This study also noted that long non-coding RNAs and micro-RNAs that control plant stress will be the focus of additional research in the future. In addition to genetic engineering techniques like enhancing the absorption of pollutants by an enhanced root system, promoting synergies between transgenic plants and gene-modified microbes, and discovering new genes to improve xenobiotic chelating ability, it has also been reported to hold good promise for developing plants that are more adept at handling heavy metal stress (Kumar et al., 2023).

Conclusion

As global concerns about environmental pollution and sustainability increase, interest and orientation towards green technologies such as phytoremediation is likely to grow. Phytoremediation, a good biological approach to environmental pollution removal, can be applied to both organic (e.g., herbicides, pesticides, and polyaromatic hydrocarbons) and inorganic pollutants (e.g., metals such as cadmium (Cd), chromium (Cr), lead (Pb), and uranium (U)) through various techniques. Hyperaccumulator plants are very powerful and promising tools for phytoremediation of contaminated environments. Their ability to uptake, accumulate and tolerate high concentrations of toxic pollutants in their tissues makes them uniquely suited for environmentally friendly and sustainable clean-up of contaminated sites. Although limitations such as slow growth rates, limited biomass production and the need for meticulous disposal of contaminated biomass hinder their widespread use to a certain extent, these obstacles can be overcome in time with increasing studies and technological advances.

Studies in genetic engineering and plant biotechnology offer promising avenues for enhancing plant growth, metal accumulation and tolerance, and for developing more efficient biomass disposal methods. Moreover, the integration of hyperaccumulator plants into more comprehensive environmental management strategies, the application of phytomining to recover precious metals from contaminated soils, and the application of phytoremediation in combination with other breeding and remediation methods will contribute to more effective and economical utilisation of phytoremediation.

As industrialisation and environmental pollution continue to increase globally, hyperaccumulator plants will play a crucial role in the search for sustainable and long-term solutions to environmental pollution.

REFERENCES

Abaga, N. O. Z., Dousset, S., & Munier-Lamy, C. (2021). Phytoremediation potential of vetiver grass (*Vetiveria zizanioides*) in two mixed heavy metal contaminated soils from the Zounrascidweogo and Boulkiemde regions of Burkina Faso (West Africa). *Journal of Geoscience and Environment Protection*, 9(11), 73-88.

Abbas, G., Murtaza, B., Bibi, I., Shahid, M., Niazi, N. K., Khan, M. I., Amjad, M., Hussain, M., & Natasha. (2018). Arsenic uptake, toxicity, detoxification, and speciation in plants: physiological, biochemical, and molecular aspects. *International Journal of Environmental Research and Public Health*, 15(1), 59.

Abhilash, P. C., Jamil, S., & Singh, N. (2009). Transgenic plants for enhanced biodegradation and phytoremediation of organic xenobiotics. *Biotechnology Advances*, 27(4), 474-488.

Al-Baldawi, I. A., Abdullah, S. R. S., Anuar, N., Suja, F., & Mushrifah, I. (2015). Phytodegradation of total petroleum hydrocarbon (TPH) in diesel-contaminated water using *Scirpus grossus*. *Ecological Engineering*, 74, 463-473.

Ali, H., Khan, E., & Sajad, M. A. (2013). Phytoremediation of heavy metals—concepts and applications. *Chemosphere*, 91(7), 869-881.

Álvarez-Robles, M. J., Bernal, M. P., De Brasi-Velasco, S., Sevilla, F., & Clemente, R. (2022). Response of *Phragmites australis* to increasing As (V) concentrations: Accumulation and speciation of As, and plant oxidative stress. *Chemosphere*, 302, 134937.

Anamika, S., Eapen, S., & Fulekar, M. H. (2009). Phytoremediation of cadmium, lead and zinc by *Brassica juncea* L. Czern and Coss. *Journal of Applied Biosciences*, 13, 726-736.

Anguilano, L., Onwukwe, U., Dekhli, A., Venditti, S., Aryani, D., & Reynolds, A. (2022). Hyperaccumulation of lead using *Agrostis tenuis*. *Environmental Systems Research*, 11(1), 30.

Ansari, A. A., S. S. Gill, R. Gill, G. Lanza, & L. Newman (Eds.). (2016). *Phytoremediation* (pp. 12-18). Berlin, Germany. Springer International Publishing.

Antoniadis, V., Shaheen, S. M., Stärk, H. J., Wennrich, R., Levizou, E., Merbach, I., & Rinklebe, J. (2021). Phytoremediation potential of twelve wild plant species for toxic elements in a contaminated soil. *Environment International*, 146, 106233.

Antosiewicz, D. M., Escudě-Duran, C., Wierzbowska, E., & Skłodowska, A. (2008). Indigenous plant species with the potential for the phytoremediation of arsenic and metals contaminated soil. *Water, Air, and Soil Pollution*, 193, 197-210.

Asante-Badu, B., Kgorutla, L. E., Li, S. S., Danso, P. O., Xue, Z., & Qiang, G. (2020). Phytoremediation of organic and inorganic compounds in a natural and an agricultural environment: a review. *Applied Ecology & Environmental Research*, 18(5).

Augustynowicz, J., Grosicki, M., Hanus-Fajerska, E., Lekka, M., Waloszek, A., & Kołoczek, H. (2010). Chromium (VI) bioremediation by aquatic macrophyte *Callitriche cophocarpa* Sendtn. *Chemosphere*, 79(11), 1077-1083.

Awa, S. H., & Hadibarata, T. (2020). Removal of heavy metals in contaminated soil by phytoremediation mechanism: a review. *Water, Air, and Soil Pollution*, 231(2), 47.

Aziz, K. H. H., Mustafa, F. S., Omer, K. M., Hama, S., Hamarawf, R. F., & Rahman, K. O. (2023). Heavy metal pollution in the aquatic environment: efficient and low-cost removal approaches to eliminate their toxicity: a review. *RSC Advances*, 13(26), 17595-17610.

Banerjee, A., & Roychoudhury, A. (2022). Assessing the rhizofiltration potential of three aquatic plants exposed to fluoride and multiple heavy metal polluted water. *Vegetos*, 35(4), 1158-1164.

Banerjee, A., Cardozo, S., Khuntia, S. P., & Upadhyay, A. K. (2022). Phytoremediation of pharmaceutical wastes. *Phytoremediation* (pp. 387-414). Academic Press.

Barrutia, O., Epelde, L., García-Plazaola, J. I., Garbisu, C., & Becerril, J. M. (2009). Phytoextraction potential of two *Rumex acetosa* L. accessions collected from metalliferous and non-metalliferous sites: effect of fertilization. *Chemosphere*, 74(2), 259-264.

Bhainsa, K. C., & D'Souza, S. F. (2001). Uranium (VI) biosorption by dried roots of *Eichhornia crassipes* (water hyacinth). *Journal of Environmental Science and Health*, Part A, 36(9), 1621-1631.

Bolan, N. S., Park, J. H., Robinson, B., Naidu, R., & Huh, K. Y. (2011). Phytostabilization: a green approach to contaminant containment. *Advances in Agronomy*, 112, 145-204.

Callahan, D. L., Baker, A. J., Kolev, S. D., & Wedd, A. G. (2006). Metal ion ligands in hyperaccumulating plants. *Journal of Biological Inorganic Chemistry*, 11, 2-12.

Chaabani, S., Abdelmalek-Babbou, C., Ben Ahmed, H., Chaabani, A., & Sebei, A. (2017). Phytoremediation assessment of native plants growing on Pb–Zn mine site in Northern Tunisia. *Environmental Earth Sciences*, 76, 1-15.

Chandra, R., Dubey, N. K., & Kumar, V. (2017). *Phytoremediation of environmental pollutants*. CRC Press.

Chen, S., Chao, L., Sun, L., & Sun, T. (2013). Effects of bacteria on cadmium bioaccumulation in the cadmium hyperaccumulator plant *Beta vulgaris* var. *cicla* L. *International Journal of Phytoremediation*, 15(5), 477-487.

Claus, J., Bohmann, A., & Chavarría-Krauser, A. (2013). Zinc uptake and radial transport in roots of *Arabidopsis thaliana*: a modelling approach to understand accumulation. *Annals of Botany*, 112(2), 369-380.

Clemens, S. (2006). Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. *Biochimie*, 88(11), 1707-1719.

Cordeiro, C., Favas, P. J., Pratas, J., Sarkar, S. K., & Venkatachalam, P. (2016). Uranium accumulation in aquatic macrophytes in an uraniferous region: relevance to natural attenuation. *Chemosphere*, 156, 76-87.

Dakora, F. D., & Phillips, D. A. (2002). Root exudates as mediators of mineral acquisition in low-nutrient environments. *Food Security in Nutrient-Stressed Environments: Exploiting Plants' Genetic Capabilities*, 201-213.

Dar, M. I., Khan, F. A., Rehman, F., Masoodi, A., Ansari, A. A., Varshney, D., Naushin, F., & Naikoo, M. I. (2015). Roles of Brassicaceae in phytoremediation of metals and metalloids. *Phytoremediation: Management of Environmental Contaminants*, 1, 201-215.

De La Calle, I., Costas, M., Cabaleiro, N., Lavilla, I., & Bendicho, C. (2013). Fast method for multielemental analysis of plants and discrimination according to the anatomical part by total reflection X-ray fluorescence spectrometry. *Food Chemistry*, 138(1), 234-241.

de la Fuente, V., Rufo, L., Rodríguez, N., Amils, R., & Zuluaga, J. (2010). Metal accumulation screening of the Río Tinto flora (Huelva, Spain). *Biological Trace Element Research*, 134, 318-341.

Debnath, S., Ray, P., & Rakshit, R. (2015). Remediation of heavy metal polluted soil: phytoremediation vis-à-vis chemical immobilization. *Pop Kheti*, 3, 262-267.

Deng, D. M., Deng, J. C., Li, J. T., Zhang, J., Hu, M., Lin, Z., & Liao, B. (2008). Accumulation of zinc, cadmium, and lead in four populations of *Sedum alfredii* growing on lead/zinc mine spoils. *Journal of Integrative Plant Biology*, 50(6), 691-698.

Dushenkov, V., Kumar, P. N., Motto, H., & Raskin, I. (1995). Rhizofiltration: the use of plants to remove heavy metals from aqueous streams. *Environmental Science & Technology*, 29(5), 1239-1245.

Ernst, W. H. (2005). Phytoextraction of mine wastes–options and impossibilities. *Geochemistry*, 65, 29-42.

Fasani, E., Manara, A., Martini, F., Furini, A., & DalCorso, G. (2018). The potential of genetic engineering of plants for the remediation of soils contaminated with heavy metals. *Plant, Cell & Environment*, 41(5), 1201-1232.

Garrison, A. W., Nzengung, V. A., Avants, J. K., Ellington, J. J., Jones, W. J., Rennels, D., & Wolfe, N. L. (2000). Phytodegradation of p, p ‘-DDT and the enantiomers of o, p ‘-DDT. *Environmental Science & Technology*, 34(9), 1663-1670.

Goto, F., Yoshihara, T., Shigemoto, N., Toki, S., & Takaiwa, F. (1999). Iron fortification of rice seed by the soybean ferritin gene. *Nature Biotechnology*, 17(3), 282-286.

Guerra, F., Gainza, F., Pérez, R., & Zamudio, F. (2011). Phytoremediation of heavy metals using poplars (*Populus* spp.): a glimpse of the plant responses to copper, cadmium and zinc stress. *Handbook of Phytoremediation*, 387-413.

Güneş, F., & Bozkurt, S. (2021). Bazi hiperakümülatör bitkiler ve özellikleri. *Türk Bilimsel Derlemeler Dergisi*, 14(1), 67-90.

Gupta, N., Ram, H., & Kumar, B. (2016). Mechanism of zinc absorption in plants: uptake, transport, translocation and accumulation. *Reviews in Environmental Science and Bio/Technology*, 15, 89-109.

Halder, S., & Anirban, A. (2021). Phytoremediation of EMS induced *Brassica juncea* heavy metal hyperaccumulator genotypes. B*ioRxiv*, 2021-12.

Hall, J. Á. (2002). Cellular mechanisms for heavy metal detoxification and tolerance. *Journal of Experimental Botany*, 53(366), 1-11.

He, Y., Langenhoff, A. A., Sutton, N. B., Rijnaarts, H. H., Blokland, M. H., Chen, F., Huber, C., & Schröder, P. (2017). Metabolism of ibuprofen by *Phragmites australis*: uptake and phytodegradation. *Environmental Science & Technology*, 51(8), 4576-4584.

Hrkić Ilić, Z., Pajević, S., Borišev, M., & Luković, J. (2020). Assessment of the phytostabilization potential of two *Salix* L. clones based on the effects of heavy metals on the root anatomical traits. *Environmental Science and Pollution Research*, 27, 29361-29383.

Hussein, Z. S., Hamido, N., Hegazy, A. K., El-Dessouky, M. A., Mohamed, N. H., & Safwat, G. (2022). Phytoremediation of crude petroleum oil pollution: a review. *Egyptian Journal of Botany*, 62(3), 611-640.

Ionata, E., Caputo, E., Mandrich, L., & Marcolongo, L. (2024). Moving towards biofuels and high-value products through phytoremediation and biocatalytic processes. *Catalysts*, 14(2), 118.

Jaffré, T., Brooks, R. R., Lee, J., & Reeves, R. (1976). *Sebertia acuminata*: a hyperaccumulator of nickel from New Caledonia. *Science*, 193(4253), 579-580.

Jing, Y., Cui, H., Li, T., & Zhao, Z. (2014). Heavy metal accumulation characteristics of *Nepalese alder* (*Alnus nepalensis*) growing in a lead-zinc spoil heap, Yunnan, south-western China. *iForest-Biogeosciences and Forestry*, 7(4), 204.

Kafle, A., Timilsina, A., Gautam, A., Adhikari, K., Bhattarai, A., & Aryal, N. (2022). Phytoremediation: Mechanisms, plant selection and enhancement by natural and synthetic agents. *Environmental Advances*, 8, 100203.

Kanso, A., Azoury, S., Benizri, E., Kobaissi, A., Echevarria, G., & Sirguey, C. (2018). Improvement of Ni phytoextraction by *Alyssum murale* and its rhizosphere microbial activities by applying nitrogen fertilizer. *Ecological Research*, 33, 811-821.

Kaur, R., Singh, D., Kumari, A., Sharma, G., Rajput, S., Arora, S., & Kaur, R. (2021). Pesticide residues degradation strategies in soil and water: a review. *International Journal of Environmental Science and Technology*, 1-24.

Khan, S., Masoodi, T. H., Pala, N. A., Murtaza, S., Mugloo, J. A., Sofi, P. A., Zaman, M. U., Kumar, R., & Kumar, A. (2023). Phytoremediation prospects for restoration of contamination in the natural ecosystems. *Water*, 15(8), 1498.

Kim, S., Takahashi, M., Higuchi, K., Tsunoda, K., Nakanishi, H., Yoshimura, E., Mori, S., & Nishizawa, N. K. (2005). Increased nicotianamine biosynthesis confers enhanced tolerance of high levels of metals, in particular nickel, to plants. *Plant and Cell Physiology*, 46(11), 1809-1818.

Kocaman, A. (2022). Assessment of The use of *Artemisia dracunculus* L. and *Erigeron canadensis* in the remediation of heavy metal contaminated soils and their ability to phytoextraction and biomass yield. *Türk Doğa ve Fen Dergisi*, 11(4), 1-10.

Korzeniowska, J., & Stanislawska-Glubiak, E. (2023). The phytoremediation potential of local wild grass versus cultivated grass species for zinc-contaminated soil. *Agronomy*, 13(1), 160.

Krämer, U. (2010). Metal hyperaccumulation in plants. *Annual Review of Plant Biology*, 61(1), 517-534.

Kumar, K., Shinde, A., Aeron, V., Verma, A., & Arif, N. S. (2023). Genetic engineering of plants for phytoremediation: advances and challenges. *Journal of Plant Biochemistry and Biotechnology*, 32(1), 12-30.

Kumar, N. (Ed.). (2024). *Hydroponics and Environmental Bioremediation: Wastewater Treatment*. Springer Nature.

Kupper, H., Götz, B., Mijovilovich, A., Kupper, F. C., & Meyer-Klaucke, W. (2009). Complexation and toxicity of copper in higher plants. I. Characterization of copper accumulation, speciation, and toxicity in *Crassula helmsii* as a new copper accumulator. *Plant Physiology*, 151(2), 702-714.

Küpper, H., Lombi, E., Zhao, F. J., & McGrath, S. P. (2000). Cellular compartmentation of cadmium and zinc in relation to other elements in the hyperaccumulator *Arabidopsis halleri*. *Planta*, 212(1), 75-84.

Lee, M., & Yang, M. (2010). Rhizofiltration using sunflower (*Helianthus annuus* L.) and bean (*Phaseolus vulgaris* L. var. *vulgaris*) to remediate uranium contaminated groundwater. *Journal of Hazardous Materials*, 173(1-3), 589-596.

Lepp, N. W. (Ed.). (2012). *Effect of Heavy Metal Pollution on Plants: Metals in The Environment* (Vol. 2). Springer Science & Business Media.

Limmer, M., & Burken, J. (2016). Phytovolatilization of organic contaminants. *Environmental Science & Technology*, 50(13), 6632-6643.

Liu, C. J., Deng, S. G., Hu, C. Y., Gao, P., Khan, E., Yu, C. P., & Ma, L. Q. (2023). Applications of bioremediation and phytoremediation in contaminated soils and waters: CREST publications during 2018–2022. *Critical Reviews in Environmental Science and Technology*, 53(6), 723-732.

Lovynska, V., Holoborodko, K., Ivanko, I., Sytnyk, S., Zhukov, O., Loza, I., ... & Heilmeier, H. (2023). Heavy metal accumulation by *Acer platanoides* and *Robinia pseudoacacia* in an industrial city (Northern Steppe of Ukraine). *Biosystems Diversity*, 31(2), 246-253.

Lu, Y., Li, X., He, M., Zeng, F., & Li, X. (2017). Accumulation of heavy metals in native plants growing on mining-influenced sites in Jinchang: a typical industrial city (China). *Environmental Earth Sciences*, 76, 1-15.

Maestri, E., Marmiroli, M., Visioli, G., & Marmiroli, N. (2010). Metal tolerance and hyperaccumulation: costs and trade-offs between traits and environment. *Environmental and Experimental Botany*, 68(1), 1-13.

Markert, B. (1996). *Instrumental Element and Multi-element Analysis of Plant Samples: Methods and Applications*. John Wiley & Sons, Chichester.

McGrath, S. P., & Zhao, F. J. (2003). Phytoextraction of metals and metalloids from contaminated soils. *Current Opinion in Plant Biology*, 6(3), 439-446.

McGrath, S. P., Zhao, F. J., & Lombi, E. (2001). Plant and rhizosphere processes involved in phytoremediation of metal-contaminated soils. *Plant and Soil*, 232, 207-214.

McIntyre, T. C. (2003). Databases and protocol for plant and microorganism selection: hydrocarbons and metals. *Phytoremediation: Transformation and Control of Contaminants*, 887-904.

Medina-Díaz, H. L., López-Bellido, F. J., Alonso-Azcárate, J., Fernández-Morales, F. J., & Rodríguez, L. (2024). A new hyperaccumulator plant (*Spergularia rubra*) for the decontamination of mine tailings through electrokinetic-assisted phytoextraction. *Science of the Total Environment*, 912, 169543.

Memon, A. R., & Schröder, P. (2009). Implications of metal accumulation mechanisms to phytoremediation. *Environmental Science and Pollution Research*, 16, 162-175.

Memon, A. R., Aktoprakligil, D., Özdemir, A., & Vertii, A. (2001). Heavy metal accumulation and detoxification mechanisms in plants. *Turkish Journal of Botany*, 25(3), 111-121.

Milner, M. J., & Kochian, L. V. (2008). Investigating heavy-metal hyperaccumulation using *Thlaspi caerulescens* as a model system. *Annals of Botany*, 102(1), 3-13.

Minguzzi, C. (1948). Il contenuto di nichel nelle ceneri di *Alyssum bertolonii* Desv. *Atti della Società Toscana di Scienze Naturali Serie A*, 55, 49-74.

Mohsin, M., Nawrot, N., Wojciechowska, E., Kuittinen, S., Szczepańska, K., Dembska, G., & Pappinen, A. (2023). Cadmium accumulation by *Phragmites australis* and *Iris pseudacorus* from stormwater in floating treatment wetlands microcosms: Insights into plant tolerance and utility for phytoremediation. *Journal of Environmental Management*, 331, 117339.

Nasution, D. A. (2024). The effectiveness of Coontail Plant (*Ceratophyllum demersum* L.) for removal of iron (Fe) artificial waste. *Serambi Journal of Agricultural Technology*, 6(1).

Nečemer, M., Kump, P., Ščančar, J., Jaćimović, R., Simčič, J., Pelicon, P., Budnar, M., Jeran, Z., Pongrac, P., Regvar, M., & Vogel-Mikuš, K. (2008). Application of X-ray fluorescence analytical techniques in phytoremediation and plant biology studies. *Spectrochimica Acta Part B: Atomic Spectroscopy*, 63(11), 1240-1247.

Osmolovskaya, N., Dung, V. V., & Kuchaeva, L. (2018). The role of organic acids in heavy metal tolerance in plants. *Biological Communications*, 63(1), 9-16.

Özcan, M. M., (2006). Determination of the mineral compositions of some selected oil-bearing seeds and kernels using inductively coupled plasma atomic emissions spectrometry (ICP-AES). *Grasas Y Aceites*, 57(2), 211-218.

Papazoglou, E. G., & Fernando, A. L. (2017). Preliminary studies on the growth, tolerance and phytoremediation ability of sugarbeet (*Beta vulgaris* L.) grown on heavy metal contaminated soil. *Industrial Crops and Products*, 107, 463-471.

Parnian, A., Furze, J. N., Chorom, M., & Jaafarzadeh, N. (2022). Competitive bioaccumulation by *Ceratophyllum demersum* L. *Earth Systems Protection and Sustainability: Volume 2* (pp. 15-30). Cham: Springer International Publishing.

Peer, W. A., Baxter, I. R., Richards, E. L., Freeman, J. L., & Murphy, A. S. (2006). Phytoremediation and hyperaccumulator plants. *Molecular Biology of Metal Homeostasis and Detoxification: From Microbes to Man*, 299-340.

Pérez-de-Mora, A., Burgos, P., Madejón, E., Cabrera, F., Jaeckel, P., & Schloter, M. (2006). Microbial community structure and function in a soil contaminated by heavy metals: effects of plant growth and different amendments. *Soil Biology and Biochemistry*, 38(2), 327-341.

Pietrelli, L., Menegoni, P., & Papetti, P. (2022). Bioaccumulation of heavy metals by herbaceous species grown in urban and rural sites. *Water, Air, & Soil Pollution*, 233(4), 141.

Pilon-Smits, E. (2005). Phytoremediation. *Annual Review of Plant Biology*, 56, 15-39.

Pilon-Smits, E. A. H., De Souza, M. P., Hong, G., Amini, A., Bravo, R. C., Payabyab, S. T., & Terry, N. (1999). *Selenium Volatilization and Accumulation by Twenty Aquatic Plant Species* (Vol. 28, No. 3, pp. 1011-1018). American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America.

Pilon-Smits, E. A., & Freeman, J. L. (2006). Environmental cleanup using plants: biotechnological advances and ecological considerations. *Frontiers in Ecology and the Environment*, 4(4), 203-210.

Pollard, A. J., Reeves, R. D., & Baker, A. J. (2014). Facultative hyperaccumulation of heavy metals and metalloids. *Plant Science*, 217, 8-17.

Qiao, K., Wang, Q., Liu, X., Gong, S., & Wang, J. (2023). Cadmium/lead tolerance of six *Dianthus* species and detoxification mechanism in *Dianthus spiculifolius*. *Chemosphere*, 312, 137258.

Quinn, C. F., Freeman, J. L., Galeas, M. L., Klamper, E. M., & Pilon-Smits, E. A. (2008). The role of selenium in protecting plants against prairie dog herbivory: implications for the evolution of selenium hyperaccumulation. *Oecologia*, 155, 267-275.

Rafati, M., Khorasani, N., Moattar, F., Shirvany, A., Moraghebi, F., & Hosseinzadeh, S. (2011). Phytoremediation potential of *Populus alba* and *Morus alba* for cadmium, chromuim and nickel absorption from polluted soil. *International Journal of Environmental Research*, 5(4), 961-970.

Rajsz, A., Wojtuń, B., Samecka-Cymerman, A., Wąsowicz, P., Mróz, L., Rudecki, A., & Kempers, A. J. (2021). Metals in *Calluna vulgaris*, *Empetrum nigrum*, *Festuca vivipara* and *Thymus praecox* ssp. arcticus in the geothermal areas of Iceland. *Environmental Science and Pollution Research*, 28, 67224-67233.

Rascio, N., & Navari-Izzo, F. (2011). Heavy metal hyperaccumulating plants: How and why do they do it? And what makes them so interesting? *Plant Science*, 180(2), 169-181.

Reeves, R. D. (2024). The discovery and global distribution of hyperaccumulator plants: A personal account. *Ecological Research*, 39(4), 416-436.

Reeves, R. D., Baker, A. J. M., Jaffré, T., Erskine, P. D., Echevarria, G., & van der Ent, A. (2018). A global database for plants that hyperaccumulate metal and metalloid trace elements. *New Phytologist*, 218(2), 407-411.

Reeves, R. D., van Der Ent, A., Echevarria, G., Isnard, S., & Baker, A. J. (2021). Global distribution and ecology of hyperaccumulator plants. *Agromining: Farming for Metals: Extracting Unconventional Resources Using Plants*, 133-154.

Rigoletto, M., Calza, P., Gaggero, E., Malandrino, M., & Fabbri, D. (2020). Bioremediation methods for the recovery of lead-contaminated soils: a review. *Applied Sciences*, 10(10), 3528.

Salihaj, M., Bani, A., & Echevarria, G. (2016). Heavy metals uptake by hyperaccumulating flora in some serpentine soils of Kosovo. *Global Nest Journal*, 18(1), 214-222.

Salihaj, M., Bani, A., Shahu, E., Benizri, E., & Echevarria, G. (2018). Metal accumulation by the ultramafic flora of Kosovo. *Ecological Research*, 33(4), 687-703.

Sarret, G., Saumitou-Laprade, P., Bert, V., Proux, O., Hazemann, J. L., Traverse, A., Marcus, M. A., & Manceau, A. (2002). Forms of zinc accumulated in the hyperaccumulator *Arabidopsis halleri*. *Plant Physiology*, 130(4), 1815-1826. doi:10.1104/pp.011965.

Schiavon, M., & Pilon-Smits, E. A. (2017). Selenium biofortification and phytoremediation phytotechnologies: a review. *Journal of Environmental Quality*, 46(1), 10-19.

Seregin, I. V., Erlikh, N. T., & Kozhevnikova, A. D. (2014). Nickel and zinc accumulation capacities and tolerance to these metals in the excluder *Thlaspi arvense* and the hyperaccumulator *Noccaea caerulescens*. *Russian Journal of Plant Physiology*, 61, 204-214.

Shackira, A. M., & Puthur, J. T. (2019). Phytostabilization of heavy metals: understanding of principles and practices. *Plant-Metal Interactions*, 263-282.

Sharma, A., Kapoor, D., Gautam, S., Landi, M., Kandhol, N., Araniti, F., Ramakrishnan, M., Satish, L., Singh, V. P., Sharma, P., Bhardwaj, R., Tripathi, D. K., & Zheng, B. (2022). Heavy metal induced regulation of plant biology: Recent insights. *Physiologia Plantarum*, 174(3), e13688.

Sharma, P., Ngo, H. H., Khanal, S., Larroche, C., Kim, S. H., & Pandey, A. (2021). Efficiency of transporter genes and proteins in hyperaccumulator plants for metals tolerance in wastewater treatment: Sustainable technique for metal detoxification. *Environmental Technology & Innovation*, 23, 101725.

Sharma, S. S., Dietz, K. J., & Mimura, T. (2016). Vacuolar compartmentalization as indispensable component of heavy metal detoxification in plants. *Plant, Cell & Environment*, 39(5), 1112-1126.

Singh, V., Pandey, B., & Suthar, S. (2019). Phytotoxicity and degradation of antibiotic ofloxacin in duckweed (*Spirodela polyrhiza*) system. *Ecotoxicology and Environmental Safety*, 179, 88-95.

Sinha, R., Singh, A. K., Bauddh, K., Sharma, T. R., & Sharma, P. (2021). Phytomining: a sustainable approach for recovery and extraction of valuable metals. *Phytorestoration of Abandoned Mining and Oil Drilling Sites* (pp. 487-506). Elsevier.

Sivaci, A., Elmas, E., Gümüş, F., & Sivaci, E. R. (2008). Removal of cadmium by *Myriophyllum heterophyllum* Michx. and *Potamogeton crispus* L. and its effect on pigments and total phenolic compounds. *Archives of Environmental Contamination and Toxicology*, 54, 612-618.

Solomou, A. D., Germani, R., Proutsos, N., Petropoulou, M., Koutroumpilas, P., Galanis, C., Maroulis, G., & Kolimenakis, A. (2022). Utilizing mediterranean plants to remove contaminants from the soil environment: A short review. *Agriculture*, 12(2), 238.

Subramanian, R., Gayathri, S., Rathnavel, C., & Raj, V. (2012). Analysis of mineral and heavy metals in some medicinal plants collected from local market. *Asian Pacific Journal of Tropical Biomedicine*, 2(1), S74-S78.

Sun, Z., Chen, J., Wang, X., & Lv, C. (2016). Heavy metal accumulation in native plants at a metallurgy waste site in rural areas of Northern China. *Ecological Engineering*, 86, 60-68.

Thayaparan, M., Iqbal, S. S., Chathuranga, P. K. D., & Iqbal, M. C. M. (2013). Rhizofiltration of Pb by *Azolla pinnata*. *International Journal of Environmental Sciences*, 3(6), 1811-1821.

Tokalıoğlu, Ş. (2012). Determination of trace elements in commonly consumed medicinal herbs by ICP-MS and multivariate analysis. *Food Chemistry*, 134(4), 2504-2508.

Ueno, D., Iwashita, T., Zhao, F. J., & Ma, J. F. (2008). Characterization of Cd translocation and identification of the Cd form in xylem sap of the Cd-hyperaccumulator *Arabidopsis halleri*. *Plant and Cell Physiology*, 49(4), 540-548.

ur Rehman, K., Hamayun, M., Khan, S. A., Iqbal, A., & Hussain, A. (2019). Heavy metal analysis of locally available anticancer medicinal plants. *Biosciences Biotechnology Research Asia*, 16(1), 105.

Valderrama, A., Carvajal, D. E., Peñailillo, P., & Tapia, J. (2016). Accumulation capacity of cadmium and copper and their effects on photosynthetic performance in *Azolla filiculoides* Lam. under induced rhizofiltration. *Gayana Botánica*, 73(2), 283-291.

Vangronsveld, J., Herzig, R., Weyens, N., Boulet, J., Adriaensen, K., Ruttens, A., Thewys, T., Vassilev, A., Meers, E., Nehnevajova, E., Leile, D., & Mench, M. (2009). Phytoremediation of contaminated soils and groundwater: lessons from the field. *Environmental Science and Pollution Research*, 16(7), 765-794.

Verbruggen, N., Hanikenne, M., & Clemens, S. (2013). A more complete picture of metal hyperaccumulation through next-generation sequencing technologies. *Frontiers in Plant Science*, 4, 388.

Verbruggen, N., Hermans, C., & Schat, H. (2009). Molecular mechanisms of metal hyperaccumulation in plants. *New Phytologist*, 181(4), 759-776.

White, P. J., & Broadley, M. R. (2009). Biofortification of crops with seven mineral elements often lacking in human diets—iron, zinc, copper, calcium, magnesium, selenium, and iodine. *New Phytologist*, 182(1), 49-84.

Woraharn, S., Meeinkuirt, W., Phusantisampan, T., & Chayapan, P. (2021). Rhizofiltration of cadmium and zinc in hydroponic systems. *Water, Air, & Soil Pollution*, 232(5), 204.

Wu, H., Tang, S., Zhang, X., Guo, J., Song, Z., Tian, S., & Smith, D. L. (2009). Using elevated CO2 to increase the biomass of a *Sorghum vulgare×Sorghum vulgare* var. *sudanense* hybrid and *Trifolium pratense* L. and to trigger hyperaccumulation of cesium. *Journal of Hazardous Materials*, 170(2-3), 861-870.

Yadav, A. K., Pathak, B., & Fulekar, M. H. (2015). Rhizofiltration of heavy metals (cadmium, lead and zinc) from fly ash leachates using water hyacinth (*Eichhornia crassipes*). *International Journal of Environment*, 4(1), 179-196.

Yan, A., Wang, Y., Tan, S. N., Mohd Yusof, M. L., Ghosh, S., & Chen, Z. (2020). Phytoremediation: a promising approach for revegetation of heavy metal-polluted land. *Frontiers in Plant Science*, 11, 359.

Yang, C., Han, N., Inoue, C., Yang, Y. L., Nojiri, H., Ho, Y. N., & Chien, M. F. (2022). Rhizospheric plant-microbe synergistic interactions achieve efficient arsenic phytoextraction by *Pteris vittata*. *Journal of Hazardous Materials*, 434, 128870.

Yang, X., Feng, Y., He, Z., & Stoffella, P. J. (2005). Molecular mechanisms of heavy metal hyperaccumulation and phytoremediation. *Journal of Trace Elements in Medicine and Biology*, 18(4), 339-353.

Zhakypbek, Y., Kossalbayev, B. D., Belkozhayev, A. M., Murat, T., Tursbekov, S., Abdalimov, E., Pashkovskiy, P., Kreslavski, V., Kuznetsov, V., & Allakhverdiev, S. I. (2024). Reducing heavy metal contamination in soil and water using phytoremediation. *Plants*, 13(11), 1534.

UNVEILING NATURE'S POTENTIAL IN ENZYMES – INDUSTRIAL ENZYMES AND THEIR APPLICATION AREAS

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Abstract

Microbial enzymes play a critical role in the development of industrial bioprocesses. Their applications span a wide range of industries including pulp and paper, leather, detergents, textiles, pharmaceuticals, chemicals, food and beverages, biofuels, animal feed, and cosmetics. By leveraging microbial diversity and modern molecular techniques such as metagenomics and genomics, new enzymes with improved catalytic properties are being discovered through rational, semi-rational, and random directed evolution strategies. Most industrial enzymes are found in recombinant forms produced by bacteria and fungi. These advancements hold significant potential for enhancing the efficiency and sustainability of industrial bioprocesses. Progress in this field not only promotes environmental sustainability but also contributes to the development of economically competitive production processes. This chapter provides a theoretical guide for researchers and industry professionals on the discovery and application of new biocatalysts.

Introduction

Enzymes are ideal metabolic catalysts that facilitate various endogenous biochemical reactions through well-defined pathways. By lowering the activation energy without undergoing a permanent change, they accelerate a range of biological reactions that are vital for the survival of living organisms.

Classification of Enzymes

In the past, various systems were used to classify enzymes. Today, the classification system introduced by the International Union of Biochemistry and Molecular Biology Enzyme Commission (IUBMB) is used. This commission addresses the classification and nomenclature of enzymes and coenzymes, units of enzyme activity, standard assay methods, and symbols used in defining enzyme kinetics (Aszalos, 1978). Enzymes are previously classified into six categories based on the reactions they catalyze: oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases, and translocases, with the classification utilizing the Enzyme Commission (EC) number (de Souza Vanderberghe et al., 2020). Recently, another class of enzymes catalyzing the movement of ions or molecules across membranes or

their separation within membranes, has now been classified under a new EC class of translocases in 2018 (McDonald & Tipton, 2023).

These enzyme groups, along with the reactions they catalyze, are as follow.

EC1. Oxidoreductases: These enzymes facilitate electron transfer, catalyzing oxidation-reduction (redox) reactions. All enzymes that participate in redox reactions belong to this class. Oxidoreductases are involved in cellular respiration and photosynthesis. They include dehydrogenases, reductases, and oxidases. For instance, NADH dehydrogenase, involved in the electron transport chain, is a key oxidoreductase.

EC2. Transferases catalyze the transfer of functional groups between molecules. They enable the transfer of atoms or groups of atoms, excluding hydrogen, from one molecule to another. Kinases and aminotransferases are well known enzymes of this group. Kinases, such as hexokinase, catalyze phosphorylation reactions, they transfer phosphate groups from ATP to substrates, which is crucial in signal transduction and metabolism.

EC3. Hydrolases catalyze the hydrolysis of various chemical bonds, leading to the breakdown of complex molecules by the addition of water. They play roles in digestion and the breakdown of biomolecules including carbohydrates, proteins, lipids and nucleic acids.

EC4. Lyases catalyze the formation of double bonds through non-hydrolytic means. Enzymes in this group break chemical bonds such as C-O and C-N without the involvement of water. Decarboxylases and synthases belong to this group and are involved in processes such as the citric acid cycle and fatty acid synthesis.

EC5. Isomerases catalyze the transfer of groups within a molecule and make geometric or structural changes that result in isomerization. Isomerases, such as phosphoglucose isomerase, play roles in glycolysis and gluconeogenesis. They catalyze the rearrangement of molecules and facilitate the conversion of glucose-6-phosphate to fructose-6-phosphate.

EC6. Ligases catalyze dehydration reactions that occur in parallel with the breakdown of ATP. They play a role in joining two molecules together, usually with energy derived from the hydrolysis of ATP. DNA ligase is a well-known example and plays role in DNA replication and repair. Ligases often

participate in anabolic pathways and form new bonds using energy derived from ATP hydrolysis.

EC7. Translocases catalyze the movement of ions or molecules such as proteins or nucleic acids, across biological membranes or within the cell. They play key roles in processes like protein import into mitochondria, chloroplasts, and the endoplasmic reticulum. They are also important in the translocation of various ions and small molecules across cellular membranes.

Hydrolytic Enzymes

The hydrolases are worth mentioning in depth as they include many important enzymes which are crucial for various industrial areas. Among the hydrolases, the following enzyme subclasses are distinguished based on the specific bonds they act upon:

EC 3.1 (Hydrolysis of ester bonds): These enzymes, such as lipases and esterases, catalyze the hydrolysis of ester bonds in lipids and other ester-containing molecules. Lipases break down fats / triglycerides into fatty acids and glycerol.

EC 3.2 (Hydrolysis of glycosidic bonds): Glycosidases, including amylases and cellulases, fall into this category. They hydrolase complex carbohydrates like starch and cellulose into simpler sugars. Amylases hydrolyze starch into maltose and glucose, while cellulases degrade cellulose into glucose units.

EC 3.3 (Hydrolysis of ether bonds): Although they are less common compared to other hydrolases, these hydrolase enzymes act on ether bonds. Chorismatase, isochorismatase, and lysoplasmalogenase fall into this category.

EC 3.4 (Hydrolysis of peptide bonds): Proteases are a key group within hydrolases. They include enzymes like trypsin, chymotrypsin, and pepsin. They catalyze the hydrolysis of peptide bonds in proteins and facilitate protein digestion and processing.

EC 3.5 (Hydrolysis of carbon-nitrogen bonds, other than peptide bonds): This hydrolase class catalyzes the hydrolysis of amides (amidase enzyme) and nitriles (nitrilase enzyme). They are important in nitrogen metabolism and detoxification pathways.

EC 3.6 (Hydrolysis of acid anhydrides): Nucleotidases and phosphatases fall under this category. This group hydrolyzes acid anhydride bonds in molecules like ATP and nucleotides and plays crucial roles in energy metabolism and nucleotide turnover.

EC 3.7 (Hydrolysis of carbon-carbon bonds): This rare enzyme class hydrolyzes C-C linkages, which are often involved in specialized metabolic pathways.

EC 3.8 (Hydrolysis of halide bonds): These enzymes catalyze the cleavage of carbon-halogen linkages, such as in halogenated organic compounds. They are often important in bioremediation processes.

EC 3.9 (Hydrolysis of phosphorus-nitrogen bonds): Phosphoamidases, which hydrolyze phosphorus-nitrogen linkages, play roles in nucleotide metabolism and other biochemical pathways.

EC 3.10 (Hydrolysis of sulfur-nitrogen bonds): These enzymes act on S-N linkages, contributing to the metabolism of sulfur-containing compounds.

EC 3.11 (Hydrolysis of carbon-phosphorus bonds): Phosphonatases hydrolyze C-P linkages and play role in the degradation of phosphonates, which are a component of some pesticides and detergents.

EC 3.12 (Hydrolysis of sulfur-sulfur bonds): These enzymes target S-S linkages and play roles in the metabolism of disulfide-containing compounds.

EC 3.13 (Hydrolysis of carbon-sulfur bonds): Sulfatases and some other related enzymes hydrolyze C-S linkages and participate in the breakdown of sulfur-containing molecules.

Among the hydrolases, carbohydrases, proteases, and lipases are of significant industrial importance. Carbohydrases like amylases and cellulases are often used in the food and biofuel industries to hydrolyze starches and cellulose into fermentable sugars. Proteases are extensively used in the detergent, food, and pharmaceutical industries for protein degradation and modification. Lipases are crucial in the production of biodiesel and cheese, the detergent and the pharmaceutical industries. These kinds of hydrolases not only facilitate essential biochemical processes within the living organisms but also have broad applications in various biotechnological and industrial fields

due to their ability to catalyze the specific hydrolysis of diverse chemical bonds.

The Sources of Enzymes

Enzymes as biological catalysts are ubiquitous across all life forms, including bacteria, fungi, plants, and animals. Their roles in biochemical reactions are critical for numerous physiological processes. Despite the broad distribution of enzymes across these kingdoms, microorganisms have emerged as the primary source for industrial enzyme production. This preference is largely attributable to the ease with which microorganisms can be cultivated in a controlled environment, allowing for consistent and scalable enzyme production.

Microbial enzymes provide various advantages over their plant- and animal-derived counterparts. First of all, their high catalytic activities ensure efficient biochemical conversions, which is crucial for industrial applications where reaction efficiency directly impacts productivity and cost-effectiveness. Secondly, the production of microbial enzymes typically results in fewer undesirable by-products, thereby simplifying downstream processing and purification. Thirdly, enzymes from microorganisms exhibit high stability under various environmental conditions, including extremes temperature and pH values, which enhances their utility across diverse industrial processes.

The production of microbial enzymes is also cost-effective compared to others. Microorganisms, such as bacteria and fungi, can be cultivated on inexpensive substrates, often derived from agricultural and industrial wastes. This not only reduces production costs but also aligns with sustainable practices by utilizing waste products. Additionally, the rapid growth rates of microorganisms enable the production of large quantities of enzymes in a relatively short period, ensuring a steady supply to meet industrial demands.

The enhanced catalytic effectiveness, stability, and economic benefits of microbial enzymes underscore their preference and use in industrial applications. These attributes, coupled with the ability to produce them in controlled and sustainable manners, make microorganisms an indispensable resource for the biotechnology and enzyme production industries (Wiseman, 1987).

Industrial Enzyme Production and Fermentation Techniques

Industrial enzyme production process involves the large-scale cultivation of the source microorganisms, the extraction and purification of enzyme of interest, and their characterization for use in various applications. In the first stage of process, the selection and improvement (if necessary) of microorganism takes place. Microorganisms such as bacteria (e.g., *Bacillus* spp., *Pseudomonas* spp.), fungi (e.g., *Aspergillus* spp., *Penicillium* spp.), and yeast (e.g., *Saccharomyces cerevisiae*) are frequently preferred for their high yield and ease of cultivation. Recombinant DNA technology can be applied to further increase enzyme yield and overall productivity.

The next step of enzyme production is fermentation phase. Producing enzymes on an industrial scale requires meticulous attention to physical and chemical conditions to optimize the yield. Fermentation techniques are crucial in this process, and selecting the appropriate method is essential for achieving high efficiency and cost-effectiveness. The primary fermentation operating processes used for enzyme production include batch, fed-batch, and continuous fermentation types.

Batch fermentation is a closed system where all necessary components (substrate/nutrients and microorganisms) are introduced to the fermenter at the beginning of the process. The system is then sealed, and fermentation starts and proceeds without additional input until the target enzyme concentration is reached. Batch culture is simple and easy to control, suitable for small-scale production, bears lower risk of contamination due to the closed system, allows for the production of high enzyme concentrations. However, it is limited to the initial nutrient supply, which can limit microbial growth and enzyme production. It also requires downtime for cleaning, sterilization, and preparation between batches. Therefore, this system can be referred to be less efficient for large-scale production.

Feed batch fermentation is a semi-closed system in which substrates/nutrients are added to the fermenter at one time, several times or continuously over time. This fermentation technique facilitates better control over the growth environment and can lead to higher enzyme yields. It provides improved control over substrate/nutrient concentration, prevents substrate inhibition, prolongs the production phase (e.g. logarithmic phase) and leads to higher enzyme yields. It is proper to adjust nutrient feeding based on real-time

monitoring and efficient for medium to large-scale production. However, it requires more complex control systems and monitoring, bears potential risk of contamination due to frequent nutrient additions, and has higher operational complexity and cost.

Continuous fermentation is an open system. In this system, fresh substrate/nutrient is continuously added, and the culture broth containing the produced enzyme (in addition to microorganisms and wastes) is continuously removed. This ensures a steady state of microbial growth and enzyme production. Continuous fermentation provides consistent enzyme production over extended periods, higher productivity and efficiency, reduced downtime which are ideal for large-scale production. It also provides better utilization of bioreactor capacity. On the other hand, it carries a high risk of contamination as there will be continuous inflow and outflow, requires sophisticated control and monitoring systems, has potential difficulties in maintaining steady-state conditions, requires more complex process management and higher initial set-up costs.

Fermentation operating processes can be carried out through submerged fermentation (SmF) or solid-state fermentation (SSF) techniques. The choice between SmF and SSF techniques depends on factors such as the microorganism, operating process, desired end products, and available infrastructure.

SmF is a frequently used method for the industrial production of microbial enzymes, especially those derived from bacteria. This method involves cultivating microorganisms in a liquid nutrient medium within large bioreactors or fermenters. The medium is continuously mixed and aerated to ensure uniform distribution of nutrients and oxygen and promotes optimal microbial growth and enzyme production. The liquid medium is specifically formulated to enhance the production of target enzymes. SmF offers precise control over environmental parameters such as temperature, pH, and oxygen levels. These controlled conditions are vital for maximizing enzyme yield. However, SmF also presents challenges, including high operational costs, significant energy consumption, potential contamination risks, and complex waste management.

SSF, on the other hand, is often used when studying enzyme production with fungi as it better mimics their natural growth environment. Furthermore, SSF is particularly beneficial for fungi because it avoids the viscosity issues encountered in SmF, where fungal hyphae and mycelia can interfere with mixing and aeration. In SSF, microorganisms are cultivated on solid substrates, which can typically be agricultural residues or other natural materials. This method is advantageous due to lower operational costs, the use of inexpensive substrates, and reduced energy requirements. Nonetheless, SSF comes with its own set of challenges. Ensuring adequate aeration and mixing is more difficult in solid substrates and leads to potential localized variations in critical parameters like temperature and pH. Additionally, evenly distributing moisture and nutrients can be challenging and potentially impact the overall efficiency of enzyme production.

The choice between submerged and solid-state fermentation depends primarily on the microbial strain and the type of the target enzyme. Each method offers distinct advantages and poses unique challenges. SmF provides a controlled environment ideal for bacterial enzyme production but comes with higher costs and complexity. While SSF is more cost-effective and suitable for fungi, it requires careful management of aeration and nutrient distribution to ensure consistent enzyme yield and activity. Balancing these factors is key to optimizing industrial enzyme production (Wiseman, 1987).

Downstream processing is the third step of enzyme production. Firstly, microbial cells are separated from the fermentation broth using a method such as centrifugation or filtration. Depending on the location of the enzyme, different methods are employed to extract the enzyme. For intracellular enzymes, cells are disrupted by mechanical and/or chemical methods to release the enzymes. For extracellular enzymes, cells are removed, and the supernatant of the fermentation culture is used as crude enzyme.

The fourth stage of enzyme production is enzyme purification, which involves techniques such as precipitation, chromatography (ion exchange, affinity, gel filtration), and ultrafiltration. The required level of enzyme purity depends on its intended use. For industrial enzyme applications, partial purification may suffice, whereas high-purity enzymes are necessary for specialized uses.

The final step of enzyme production may involve characterization, formulation, stabilization, and quality control of the enzyme, depending on

the intended applications. Enzymes are formulated into stable forms, such as powders, liquids, or immobilized preparations and are tailored to specific industrial uses. Additives like polyols, sugars, and salts may be incorporated to enhance the stability of the enzyme during storage and application. Quality control is ensured through activity assays, purity tests, and stability assessments.

Industrial Enzymes and Specialty Enzymes

The fields of application of enzymes have significantly increased and diversified, leading to their categorization into industrial enzymes and specialty enzymes as shown in Figure 1. The primary differences between industrial and specialty enzymes can be clearly observed in their scale, cost, specificity, purity, customization, and regulatory requirements.

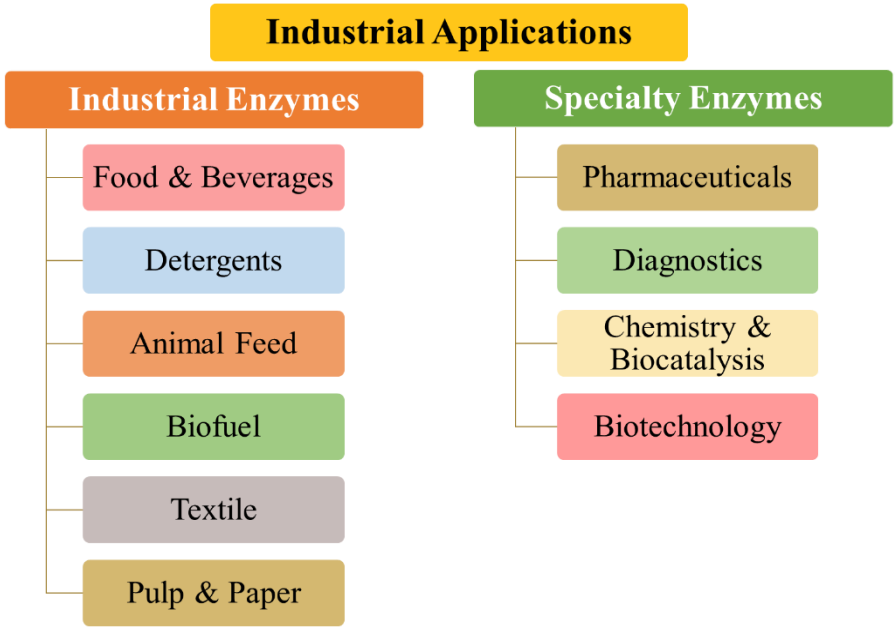

Figure 1. Application areas of industrial enzymes and specialty enzymes.

Industrial enzymes find extensive uses across various sectors. In the food and beverage industry, they enhance the brewing, baking, and dairy production processes. For instance, amylases break down starches into sugars and facilitate the fermentation process in brewing. In the detergent industry, proteases, lipases, and amylases are integral in breaking down proteins, fats,

and starches, respectively, and improve the cleaning efficiency of laundry and dishwashing detergents. The textile industry benefits from cellulases, which aid in fabric processing by removing excess fibers. Additionally, in the biofuel sector, cellulases, amylases, and other hydrolytic enzymes break down biomass into fermentable sugars and contribute to sustainable energy production. In the paper and pulp industry, enzymes such as xylanases are employed in pulp bleaching and reducing lignin content and enhance the quality of the final product.

Large-scale production and cost-effectiveness are characteristic features of industrial enzymes. Their robustness allows them to withstand harsh industrial operating conditions, including extreme pH levels and temperatures. They exhibit moderate specificity to adapt to a range of substrates, increasing efficiency and reducing production costs in industrial processes.

Specialty enzymes are essential in more precise, small-scale, and high-value applications. Examples of the use of specialty enzymes in the pharmaceutical industry include asparaginase, used as therapeutic agents in the treatment of leukemia, and lactase enzymes used in the production of lactose-free milk for lactose intolerant individuals. The biotechnology sector relies on enzymes like DNA polymerase for DNA replication processes, which is essential in research and diagnostics. In cosmetics, enzymes like hyaluronidase are utilized for their ability to break down hyaluronic acid, benefiting skin care products for exfoliation and anti-aging treatments.

Specialty enzymes are also known for their high specificity and purity. They are tailored to meet the precise needs of specific applications, especially in pharmaceuticals and diagnostics. The production of specialized enzymes often involves complex and tedious processes to ensure the highest levels of purity and activity. Consequently, they are typically more expensive compared to industrial enzymes due to the meticulous production and quality control measures. Specialty enzymes, especially those used in pharmaceuticals and diagnostics, are subject to stringent regulatory requirements to ensure their safety and efficacy. This contrasts with industrial enzymes, which have less stringent regulatory oversight due to their broader application scope.

Industrial Enzymes and Their Application Areas

It is clear that a wide variety of enzymes have diverse applications (Figure 2). This chapter will concentrate on key enzymes of industrial significance, specifically carbohydrases (amylases and cellulases), proteases, lipases, polymerases and nucleases as shown in Figure 3.

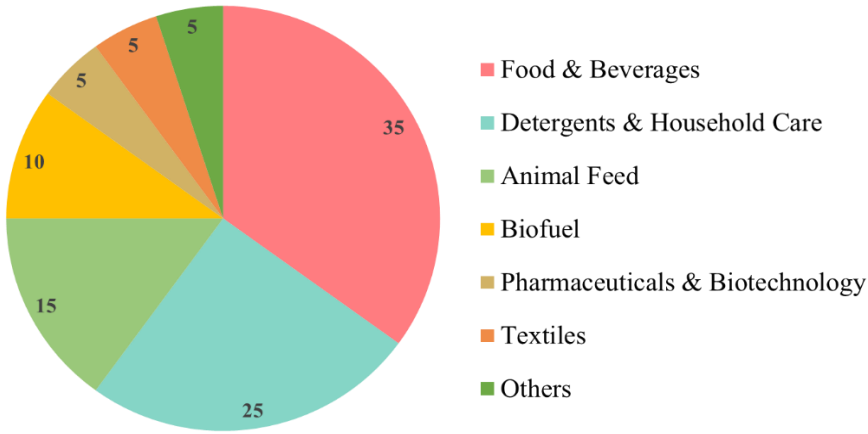

Figure 2. The pie chart illustrating the estimated market share of each sector in the enzyme industry.

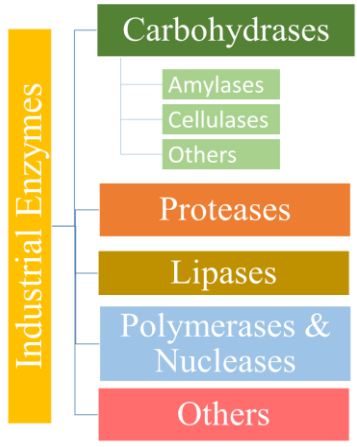

Figure 3. The most industrially significant enzyme types

Amylases

The industrial enzyme market is valued at $7 billion and is experiencing rapid growth with an annual increase of 4% (Zhu et al., 2023). Amylases represent a significant portion of the enzyme market due to their wide range of industrial applications.

In the food industry, they are commonly used for liquefying starch, producing high-fructose syrups and maltose, manufacturing oligosaccharide mixtures, and in baking and chocolate production (Wang et al., 2020; Farooq et al., 2021).

Amylases are frequently employed in the liquefaction of starch. These enzymes do not act directly on starch molecules but only affect gelatinized starch. Gelatinization, which is dependent on temperature, time, and molecular size, occurs when starch granules swell and burst upon heating, forming a starch gel (Brandam et al., 2003). The α-1,4 bonds in amylose and amylopectin are broken, rapidly converting gelatinized starch into glucose, maltose, and dextrins. After gelatinization, viscosity increases, and α-amylase enzymes are used to liquefy the starch and reduce viscosity (Saini et al., 2017).

In addition to being used directly as a food source, starch from plants is chemically or enzymatically converted into various products such as starch hydrolysates, glucose syrups, fructose, and maltose (De Baere, 1999). High fructose syrups are produced by converting glucose using glucose isomerase. This involves sequential steps of starch liquefaction, saccharification, and isomerization to produce glucose (Parker et al., 2010). Bacterial α-amylases are used for starch liquefaction, while fungal α-amylases are used in saccharification processes (Goyal et al., 2005). Maltose, resulting from starch hydrolysis, is an important disaccharide in syrups and is used as sweeteners due to its non-hygroscopic nature (Saini et al., 2017).

Amylases take part in the processing of starch and carbohydrate-containing products. In baking, they accelerate fermentation, thus providing better rise and texture to dough and preventing bread staleness (Tiwari et al., 2015). In sugar production, amylases convert starch into glucose and facilitate sugar production and improve the texture and consistency of confectionery and sweets (Gupta et al., 2003). They also help reduce cloudiness in fruit juice production (Li et al., 2005). In beer and alcoholic beverage production, amylases hydrolyze the starch in malt into glucose to be used for fermentation

(Chen et al., 2023). Amylases enhance the flavor of starch-based products and are effective in dairy product processing, including milk sweeteners and yogurt production. Additionally, amylases prevent ice crystal formation in ice cream and other frozen foods and extend their freshness. They are also used in processing starches and carbohydrates in canned and ready-to-eat foods (Demirkan et al., 2005; Hussain et al., 2013).

The detergent industry has long utilized various enzymes to improve laundry and dishwashing performance. Proteases, lipases, amylases, and cellulases are frequently used enzymes in this sector (Gurkok, 2019; Gurkok & Ozdal, 2021; Hemşinli, 2022; Yılmaz, 2023; Hemsinli & Gurkok, 2024; Akbaş, 2024). α-Amylases are crucial in the detergent industry, being the second most preferred enzymes after proteases. They are used to remove starch-based stains from rice, pasta, sauces, potatoes, corn, chocolate, and baby food (Gurkok, 2019; Sharma et al., 2022; Akbas, 2024). Due to their activity at low temperatures, amylases have been present in 90% of liquid detergents in the cleaning market since 1975. Typically, these enzymes are microbial in origin, mostly derived from *Bacillus* and *Aspergillus* species (Gupta et al., 2003; Vaidya et al., 2015).

In the bioenergy sector, amylase enzymes facilitate the fermentation of starch (after conversion to glucose) into ethanol and biofuel (Arya et al., 2022). Ethanol can be produced from both starchy and lignocellulosic substrates. Starchy feedstocks are commonly preferred as substrates for ethanol production due to their availability and low cost (Elyasi Far et al., 2020). Starchy materials such as grains, potatoes, and distiller's grains are treated with α-amylase to convert them into fermentable raw materials for ethanol production (Escaramboni et al., 2018). First, starch molecules are broken down into sugars by α-amylase enzyme or microorganisms. The yeast *S. cerevisiae* then ferments these sugars into ethanol (Mojsov, 2012).

In the textile industry, starch paste is used in the weaving process to enhance thread durability and prevent loss due to friction. To smooth the fabric, the starch coating is removed using α-amylase enzymes. α-Amylases are employed to remove starch before sizing and dyeing processes (Al-Amri et al., 2022).

In the paper industry, starch is used in coating processes to soften the paper surface, improve its durability and lead to better writing quality. Due to the high viscosity of starch during these processes, microbial amylases are

used. (Souza & Magalhães, 2010; Hussain et al., 2013). Papers coated with starch are hydrolyzed using α-amylases to remove the excess starch (Saini et al., 2017).

Food processing facilities generate wastewater with high levels of starch contamination. Microbial amylases are used for the biotechnological treatment of such wastewater (Aiyer, 2005).

In medical and pharmaceutical industry, α-amylases are utilized as targets in the treatments for various diseases, including diabetes mellitus, dental caries, and hyperlipidemia (da Silva et al., 2013). Additionally, measuring α-amylase activity in human serum and urine is a method used for diagnosing pancreatic disorders (Tran et al., 2014).

Cellulase

Cellulases constitute approximately 20% of the global enzyme market due to their use in various commercial sectors, including food, textiles, paper and pulp, laundry, beverages, agriculture, biofuel production, and detergent industries (Ejaz et al., 2021).

Cellulases are applied in the paper industry for recycling used paper and newspapers, refining and grinding woody raw materials, and improving the durability of paper through mechanical pulping processes. Refining with cellulases results in the formation of pulps with high amounts of fines, bulk, and stiffness. Biomechanical pulping with cellulases saves significant energy during refining (Bhat, 2000). Cellulases also remove wood components that reduce pulp quality (Zoppas et al., 2013). They are used for ink removal in paper, with acidic cellulases preferred for their ability to prevent yellowing, enhance brightness, and reduce environmental pollution (Ratnayake & Jayasekara, 2019). Additionally, cellulases improve the softness of paper towels (Sukumaran et al., 2005).

Cellulases are successfully used in textile wet processing, especially for finishing cellulose-based textiles. They are employed for bio-stoning of denim and bio-polishing of cotton and other cellulosic fabrics (Hebeish & Ibrahim, 2007). Using cellulases instead of pumice stones in denim processing prevents equipment damage and reduces labor (Schafer et al., 2007). In bio-polishing, cellulases enhance fabric smoothness, appearance, and color brightness without needing additional chemicals. They also improve wash

resistance, flexibility, and fabric softness (Bhat, 2000). The process increases the moisture content of treated products by about 6% (Doğan, 2015). For wool, cellulases clean vegetable residues in raw wool through a process called carbonization, reducing the need for sulfuric acid and offering an eco-friendlier method (Gouveia et al., 2008).

In fermented beverage production, such as beer, endoglucanases can be included to hydrolyze glucan, reducing wort viscosity and improving filterability. Cellulases are also used in red wine production, acting as maceration enzymes by affecting the polysaccharide fraction of grape skin cell walls, allowing better maceration (de Souza & Kawaguti, 2021).

Cellulases have broad applications in food processing. They are used with other enzymes, known collectively as maceration enzymes, to improve the extraction and clarification of fruit and vegetable juices. Maceration enzymes reduce viscosity and improve the stability and aromatic properties of juices during processing. Pre-treatment with enzymes reduces the risk of membrane fouling during juice filtration (Baker & Wicker, 1996).

In the animal feed industry, cellulases and hemicellulases are valued for their potential to enhance the nutritional value and performance of animal feed. Pre-treating agricultural silage and grain feed with cellulases or xylanases can improve nutritional value. Enzymes also remove anti-nutritional factors in feed grains and break down specific feed components to enhance nutritional value. Cellulases effectively hydrolyze cellulose, converting it into easily absorbable components, thus improving animal health and performance (Sharada et al., 2014). Cellulases produced from *Bacillus* species enhance digestion when added to high-cellulose feeds like barley and oats (Gado et al., 2007; Murad & Azzaz, 2010).

Protease

Proteases, which are a part of the industrial enzyme group, constitute 60% of the global market share (Rani et al., 2012). Their applications span various fields such as food processing, detergents, waste treatment, leather processing, heavy metal (silver) recovery, pharmaceuticals, and peptide synthesis (Silva, 2017).

In food industry, proteases are used in the hydrolysis of food proteins for improving nutritional quality, extending shelf life, modifying functional

properties, and enhancing flavor, aroma, and color (González et al., 2011). In the animal feed industry, proteases are added to plant-based feeds to improve digestibility and nutrient availability for monogastric animals (Çelik Algül, 2019). Chymosin, an enzyme used in cheese making, coagulates milk by targeting casein. In baking, proteases break down gluten to soften dough and facilitate kneading. They are also used to prevent excessive rising in some baked goods. Proteases improve bread quality by enhancing its color, softness, and texture through the hydrolysis of gluten (Kara et al., 2005; Buehler, 2006). Proteases, from both plant (papain, bromelain, actinidin, ficin) and microbial (*A. oryzae* and *B. subtilis*) sources, are used to tenderize meat. They also clarify alcoholic and non-alcoholic beverages by removing protein-induced haze. Trypsin and chymotrypsin hydrolyze β-lactoglobulin in cow and sheep milk to produce hypoallergenic foods (Kondo et al., 2007).

Proteases are the foremost detergent enzymes. The first enzyme used in detergents was derived from pancreatic extracts, later replaced by bacterial enzymes (Singh et al., 2016). Subtilisins from *Bacillus* species dominate 89% of enzymes used in detergents. They are stable at high temperatures. Proteases from *A. favus*, *A. niger*, *B. cereus*, *B. firmus* MTCC 7728, *Tritirachium album*, and *Penicillium chrysogenium* are common in laundry detergents (Nadeem et al., 2020). Proteases for detergents must remain active and stable at high pH and temperatures, tolerate other additives, and perform effectively on various stains (Gurkok, 2019). Therefore, the discovery of extremophilic protease-producing microbial sources such as the bacterium *Exiguobacterium alkaliphilum* has gained importance (Hemsinli and Gurkok, 2024). In dishwasher detergents, proteases combined with amylases and lipases effectively remove starch and protein-based stains, reduce phosphate usage, and save energy by working at lower temperatures (Contesini et al., 2018).

In the leather industry, different stages such as calcination, soaking, removal of hair and wool, tanning, strawing, degreasing and cleaning are used to obtain processed leather. These stages use highly toxic chemicals such as lime, salt, solvents and sodium sulfide, which cause environmental pollution (Sharma et al., 2019). Using proteases in leather processing reduces environmental pollution. Alkaline proteases hydrolyze non-collagenous components, enhancing leather softness and durability (Ellaiah et al., 2002). Enzymes from *A. tamarii*, *B. halodurans* JB 99, *Bacillus sp*. JB 99, *B. subtilis*, and *Streptomyces avermectinus* NRRL B-8165 improve leather quality and reduce environmental impact (Ahmed et al., 2008).

X-ray film waste containing metallic black silver is a very good source for silver recovery. Silver makes up about 1.5-2% of the gelatin layer of X-ray film. Silver recovered by incineration of X-ray films causes environmental pollution. Proteolytic enzymes can recover silver without pollution (Wahab & Ahmed, 2017). Proteases from *B. subtilis* ATCC 6633 and *Conidiobolus coronatus* help extract silver by degrading the gelatin layer on X-ray films (Nakiboglu et al., 2001; Shankar et al., 2010). Protease from *C. globosum* is also effective in this process (Nadeem et al., 2020).

Waste treatment industry, proteases break down protein-containing wastes, reducing the biological oxygen demand in water systems (Sharma et al., 2019). They convert fibrous proteins like horns, hair, nails, and feathers into useful biomass. Microbial proteases are crucial in hydrolyzing keratin waste from poultry and leather industries into environmentally friendly byproducts (Tapia & Simoes, 2008; Hemsinli, 2022). Enzymes from *A. oryzae* NRRL-447, *B. amyloliquefaciens*, *Bacillus* JB 99, *B. licheniformis* RG1, *B. licheniformis* ZJUEL31410, *B. pseudofirmus* FA30-01, *Bacillus* RV. B2.90, *Streptomyces*, and *Pseudomonas* sp., MS21 are used for feather hydrolysis (Kainoor & Naik, 2010).

In pharmaceutical industry, microbial proteases are utilized to treat various diseases, including skin wounds, cancer, cystic fibrosis, heart problems, digestive disorders, and infections (Chanalia et al., 2011). Subtilisin or collagenase combined with antibiotics treats burns and wounds. An enzyme from *A. oryzae* regulates digestion in patients with lytic enzyme deficiency syndrome. *E. coli*-derived asparaginase is used to treat lymphocytic leukemia by removing asparagine from the blood (Kudrya & Simonenko, 1994; Rao et al., 1998; Gupta et al., 2002).

In textile industry, proteases are used in the pre-treatment of textiles, processing wool and raw silk, preventing felting, improving dye uptake, and adding brightness and whiteness (Contesini et al., 2018). Protease-treated wool prevents shrinkage, offering an alternative to chlorinated resins. Proteases like papain, pronase, and pepsin enhance wool fiber flexibility and remove natural stains for a whiter appearance. Protease treatment of sericin in raw silk, a costly and time-consuming process, can be achieved quickly and cheaply with enzymes (Duman, 2008).

Proteases in the cosmetic industry catalyze the hydrolysis of peptide bonds in keratin, elastin, and collagen. They are used in toothpaste, hair

removal products, hair care products, and contact lens solutions. Hydrolysates of collagen and elastin, derived from bovine tendon hydrolysis, are used in skin and hair care products. Proteases like bromelain and papain are used for skin peeling, promoting cell renewal and removing dead cells from the epidermis (Silva, 2017). Keratinases from *B. licheniformis* ZJUEL31410 have potential applications in the cosmetic industry (Ni et al., 2011).

Lipases

Recent advancements in biotechnology have significantly expanded the application areas of the lipase enzyme in many aspects of daily life. These applications are especially prominent in sectors such as detergents, food, oil and oleochemicals, biodiesel, textiles, biosensors, paper, cosmetics, health, and pharmaceuticals (Sangeetha et al., 2011; Choudhury & Bhunia, 2015).

Lipases modify the nutritional value, aroma, and flavor of foods by altering the positions of fatty acids on the glycerol backbone, the length of fatty acid chains, the degree of unsaturated fatty acids, and the physical properties of triglycerides. Due to their roles in various fermentation processes, lipases are widely used to enhance the flavor of various foods, especially processed meat products (such as sausages) and cheese (Aravindan et al., 2007).

Lipases used as catalysts in organic chemistry work between hydrophilic and lipophilic surfaces and tolerate organic solvents in reaction mixtures. Lipases catalyze the hydrolysis of water-immiscible triglycerides at the water-oil interface. Under certain conditions, the amount of water in the reaction mixture determines the direction of the lipase-catalyzed reaction. In the absence or minimal presence of water, the reaction favors esterification or transesterification, while an excess of water favors hydrolysis. Additionally, lipases are used in biotransformations in both aqueous and organic environments, resolution of racemic acids and alcohols, regioselective acylations, ester synthesis, and especially in the synthesis of biopolymers due to their catalysis of esterification and transesterification reactions (Hasan et al., 2006).

Due to their catalysis of lipid hydrolysis and transesterification reactions, lipases have significant applications in the pharmaceutical and health sectors. The lipase enzyme from *Candida rugosa* is known to reduce

serum cholesterol levels, and the lipase from *Serratia marcescens* is widely used in the synthesis of lovastatin (Gurung, 2013).

Hydrophobic components like triglycerides and waxes in trees cause difficulties and reduce the quality of paper during production. Lipases are used in paper manufacturing to mitigate these negative effects. In Japan, Nippon Paper Industries has developed a method using *C. rugosa* lipase, which ensures 90% hydrolysis of triglycerides to address these problems (Demuner et al., 2011).

Immobilized lipases have applications as fast, efficient, and cost-effective biosensors for the quantitative determination of triacylglycerols in food, pharmaceuticals, and clinical settings (Ray, 2012).

In biodiesel production, lipases from species like *A. niger*, *C. antarctica*, *C. rugosa*, *C. viscosum*, *Mucor miehei*, *P. cepacia*, *P. fluorescens*, *P. lipolyticum*, *Rhizopus oryzae*, *Streptomyces sp.*, and *T. lanuginosus* are used as sources of lipase (Sharma et al., 2001).

Due to their ability to hydrolyze fats, lipases are key additives in detergents alongside proteases, amylases, and cellulases. Lipase enzymes are frequently used in laundry and dishwashing detergents, bleaching products, leather cleaning, and in clearing fat-clogged drains. Many microbial lipases are commercially produced for the detergent industry. Examples include Lumafast, formulated by Genencor International, America with *Pseudomonas mendocina* lipase enzyme; Lipomax, formulated by Genencor International, America, and Gist-Brocades, the Netherlands, with *P. alcaligenes* lipase enzyme; and Lipolase, formulated by Novo Nordisk with *Thermomyces lanuginosus* lipase enzyme (Jaeger et al., 1994; Jaeger & Reetz, 1998). The performance of lipase enzymes used in detergents is influenced by factors such as the composition of the detergent, pH value, ionic strength, hardness of the wash water, temperature, and procedure. Desired properties of lipases used in detergents include broad substrate specificity, stability in alkaline environments (pH 10-11), oxidative stability, and activity in the presence of surfactants and other enzymes (Gurkok, 2019; Gurkok & Ozdal, 2021).

Polymerases and Nucleases

Polymerases and nucleases are essential enzymes in molecular biology, and their industrial applications have grown substantially with the advancements in biotechnology. These enzymes take part in DNA replication, DNA repair, and recombination, which make them invaluable tools in various industries, including pharmaceuticals, chemistry, diagnosis, and biotechnology (Aschenbrenner & Marx 2017; Mota et al., 2020).

Polymerases are indispensable enzymes in numerous applications as they synthesize DNA and/or RNA molecules from nucleotides and are crucial for amplifying genetic material. Polymerase Chain Reaction (PCR) is a fundamental technique in molecular biology and is used to amplify DNA sequences *in vitro*. PCR has revolutionized genetic research, diagnostics, and forensic science and enabled the rapid and precise amplification of DNA molecules. Taq polymerase, derived from the thermophilic *Thermus aquaticus*, is commercially available and widely used due to its ability to withstand high temperatures required during DNA denaturation step of PCR.

Polymerases are integral enzymes for DNA sequencing technologies, such as Sanger sequencing and next-generation sequencing (NGS). These technologies have transformed genomics and allowed for the comprehensive analysis of genetic material, which is crucial for personalized medicine, genetic research, and evolutionary studies (Wright et al., 2022).

In genetic engineering, polymerases are frequently used to create recombinant DNA molecules. This involves the cloning of specific genes and their expression in host organisms to produce proteins, enzymes, or other biomolecules. This technology is pivotal in the production of pharmaceuticals, such as insulin and monoclonal antibodies.

Polymerases are essential in synthetic biology for the construction of artificial genetic circuits and the synthesis of entire genomes. This field aims to design and engineer new biological systems and organisms with novel functionalities, which has potential applications in medicine, biofuel production, and environmental remediation (Daher et al., 2016; Houlihan et al., 2017).

Nucleases hydrolyze the phosphodiester linkages within the nucleic acids. They are categorized into endonucleases, which cleave within the DNA

or RNA molecule, and exonucleases, which trim nucleotides from the ends. One of the most significant applications of nucleases is in gene editing (Badr & El-Shazly, 2024). Technologies like CRISPR-Cas9 employ nucleases to create targeted breaks in DNA and allow for precise modifications. This has revolutionized genetics by enabling the correction of genetic defects, the creation of genetically modified organisms (GMOs), and the development of gene therapies (Guha & Edgell, 2017; Schulze & Lammers, 2020). Nucleases are used in diagnosis to detect specific nucleic acid sequences. For example, restriction endonucleases are used in restriction fragment length polymorphism (RFLP) analysis to detect genetic variations (Abastabar et al., 2022). Exonucleases are used in techniques like allele-specific PCR to enhance the specificity of DNA amplification.

Nucleases are involved in RNAi, a biological process where RNA molecules inhibit gene expression by neutralizing targeted mRNA molecules. This technology is used in functional genomics studies and has therapeutic potential for silencing disease-causing genes. Nucleases can also be employed in bioremediation to degrade nucleic acids from microbial contaminants Sharma & Shukla, 2022).

Recent Advances and Emerging Technologies for Enzyme Production

Enzyme production and utilization faces several challenges in terms of sustainability, efficiency, and economic viability. Many strategies have been developed to overcome these challenges. In this context, emerging technologies such as enzyme engineering are one of the first steps in the improvement of enzymes. Enzyme engineering is employed to enhance the tolerance of biocatalysts to high temperatures, extreme pH levels, and various organic solvents. This field regularly modifies enzyme properties such as specific activity, substrate binding and specificity, and regio-, stereo-, and enantio-selectivity (Madhavan et al., 2021). There are two primary strategies used in enzyme and protein engineering: directed evolution and rational design. These strategies can be combined to achieve semi-rational design or focused directed evolution.

Directed Evolution

Directed evolution introduces random mutations in protein sequences using available tools like gene shuffling and error-prone PCR. Then, a library

of mutants is created, and mutants with the desired traits are identified by using appropriate selection or screening methods. Equivalent enzyme modifications can be created using *in vitro* techniques or engineering and the cell's biosynthetic machinery in heterologous or homologous host organisms (Madhavan et al., 2021; Chatterjee et al., 2023). The advantage of directed evolution is that it does not require structural information and can generate variations in unexpected positions away from the active site. However, the changes are typically small and require several rounds of evolution, and extensive screening of variants is needed, which can be labor-intensive and time-consuming (Steiner & Schwab, 2012). Additionally, it is challenging to predict which mutations are vital for creating a new enzyme. Advances in genetic engineering techniques and the development of high-throughput computational tools have revolutionized enzyme and protein engineering and enabled the creation of novel and improved biocatalysts for various industries. Recently, the advent of artificial intelligence and machine learning programs has allowed for the simulation and optimization of potential mutation combinations *in silico* (Xu et al., 2021; Orsi et al., 2024; Yang et al., 2024).

Li et al. (2017) conducted a directed enzyme evolution study on β-mannanase (Rm Man5A). This enzyme is crucial for the enzymatic degradation of hemicellulose in mannans and used in biofuel production and other industries. The mutant enzyme (m Rm Man5A) showed maximum activity at pH 4.5 and 65 °C, a shift of 2.5 units to a more acidic optimal pH and a 10 °C increase in optimal temperature compared to the wild type. The catalytic efficiencies (kcat/Km) of mRmMan5A against various mannan substrates increased more than threefold under acidic and thermophilic conditions. However, the high specific activity and hydrolysis properties of RmMan5A were maintained in the mutant mRmMan5A. Sequence analysis revealed three amino acid substitutions (Tyr233His, Lys264Met, and Asn343Ser) in mRmMan5A. Directed mutagenesis experiments changing Tyr233 and Lys264 positions were primarily responsible for the altered properties of mRmMan5A (Li et al., 2017).

Yoshida et al. (2021) employed a loop-walking method and machine learning to enhance the thermostability of *Burkholderia cepacia* lipase. They created enzyme libraries with random mutations at three consecutive positions in twelve loops and screened for thermostability-enhancing mutations. A model was developed using machine learning to assess the synergistic effects of these mutations. The triple mutant P233D/L234P/V235S showed the

highest thermostability, retaining 66% of its activity after 30 minutes at 60 °C, compared to the wild-type enzyme (5%) under the same conditions (Yoshida et al., 2021).

Yuan et al. (2023) aimed to increase the thermostability of *Bacillus amyloliquefaciens* alpha-amylase (BAA), widely used in food, textile, and detergent industries. They engineered a mutant version, MuBAA (D28E, V118A, S187D, K370N), which retained 70% of its activity at 50 °C and 20% at 60 °C after 60 minutes, while the wild-type BAA lost all activity under these conditions. MuBAA displayed a specific activity of 206.61 U/mg, 99.1% higher than that of the wild-type BAA (Yuan et al., 2023).

Rational Design

The increasing availability of protein structures, reliable models, biochemical data, and computational methods has facilitated a shift from random approaches in enzyme engineering (directed evolution) to semi-rational or rational (data-driven) design. These approaches leverage protein structures and molecular modeling data to identify mutations through site-directed mutagenesis.

One of the advantages of rational design is that it increases the likelihood of beneficial mutations while significantly reducing library size, thus decreasing the time and effort required for screening. This approach is particularly advantageous when high-throughput screening systems are unavailable. However, rational design requires a comprehensive understanding of the enzyme's active site, structural features, and their roles in biocatalysis. Based on prior knowledge of structural and functional characteristics, critical amino acids are selected for mutagenesis, and potential amino acids that can be mutated without disrupting catalytic activity are identified. Computational methods are used to analyze the enzyme's structure, align multiple sequences, and perform molecular dynamics simulations to identify the most promising amino acids for mutation (Chatterjee et al., 2023). One of the methods in rational design, *de novo* design, has become a popular research area in recent years. The goal of *de novo* enzyme design is to create new enzymes with desired functions from scratch, without relying on naturally occurring enzymes, by leveraging the physical principles of intra- and intermolecular interactions (Pan & Kortemme, 2021; Lou et al., 2023). Artificial intelligence-based programs, such as Rosetta, IntFOLD, I-TASSER, Swiss-Model, and the Protein Homology/AnalogY Recognition Engine, are

used to predict and optimize enzyme sequences for desired functions (Xie & Warshel, 2023; Orsi et al., 2024).

Li et al. (2020) studied a psychrophilic alpha-amylase (PHA) from *Pseudoalteromonas haloplanktis*. This enzyme is used in industries like food, detergent, and textiles at low temperatures but denatures with small temperature changes. They employed rational design, using molecular dynamics (MD) simulations to understand macromolecular conformations and simulate *in silico* mutations to enhance thermostability. The identified mutations were then experimentally tested, resulting in mutant enzymes with 1.6- to 2.7-fold increased catalytic activity and successful thermostability compared to the wild type (Li et al., 2020).

Yan et al. (2022) aimed to improve the acid resistance of a thermostable glucose oxidase (GoxM8) used in the production of gluconic acid (GA) from *Aspergillus niger*. They utilized rational design techniques and explored various microbial sources of Gox with known pH-activity optima. They used tools like ClustalW for multiple sequence alignments and Rosetta Supercharge to design proteins with high net charge to reduce protein aggregation and enhance folding efficiency, stability, and affinity. Visual analysis was conducted using PyMOL visualization software. Mutants Q241E, R499E, and Q241E/R499E were found to have shifted their optimal pH from 6.0 to 5.0, with specific activities 23%, 5%, and 17% higher than GoxM8 at pH 6.0, and 44%, 26%, and 37% higher at pH 5.0, respectively (Yan et al., 2022).

Conclusion

Enzymes, as versatile biological catalysts, play a crucial role in a wide range of industrial and scientific applications. This chapter has explored the classification of enzymes, with a particular focus on hydrolytic enzymes, highlighting their diverse functionalities and sources. The discussion on industrial enzyme production techniques provided insights into the various methods employed to harness these powerful biocatalysts, emphasizing the importance of optimizing production for cost-effectiveness and efficiency.

The applications of industrial enzymes span across numerous sectors, including food processing, pharmaceuticals, and biofuels, showcasing their indispensable role in modern industry. Notwithstanding the notable progress, issues with stability, activity, and yield in the manufacture of enzymes continue to require investigation and improvement. Enzyme engineering has

recently made strides toward addressing these issues, providing creative solutions that improve enzyme activity and increase their potential for use.

Driven by technical advancements and scientific discoveries, the ever-evolving field of enzyme technology keeps developing. The future of enzyme engineering holds great promise for developing more efficient, sustainable, and tailored enzymes, which will undoubtedly lead to new and improved applications in various industries. As we advance our understanding and capabilities in this area, enzymes will continue to be at the forefront of biotechnological innovation and contribute to a more sustainable and efficient industrial landscape.

Acknowledgements

This book chapter was written inspired by theses (Thesis No: 778096, 843508, and 848094) supported by the grant program of Atatürk University Scientific Research Projects Coordination Unit with project numbers FYL-2021-9280, FYL-2022-10654, and FYL-2023-12135.

REFERENCES

Abastabar, M., Shabanzadeh, S., Valadan, R., Mayahi, S., Haghani, I., Khojasteh, S., ... & Hedayati, M.T. (2022). Development of RFLP method for rapid differentiation of *Aspergillus flavus* and *Aspergillus oryzae*, two species with high importance in clinical and food microbiology. *Journal of Medical Mycology*, *32*(3), 101274.

Ahmed, S. A., Al-domany, R. A., El-Shayeb, N. M., Radwan, H. H., & Saleh, S. A. (2008). Optimization, immobilization of extracellular alkaline protease and characterization of its enzymatic properties. *Research Journal of Agricultural Science*, *4*(5), 434-446.

Aiyer, P. V. (2005). Amylases and their applications. *African Journal of Biotechnology*, *4*(13).

Akbaş, F. (2024). Statistical Optimization of Amylase Production by *Priestia megaterium* SFA Isolated from Potato Peels: Characterization of Amylase and Potential Applications in the Detergent Industry and Nanoparticle Synthesis Master's Thesis, Atatürk University, Institute of Natural and Applied Science, Erzurum

Al-Amri, A., Al-Ghamdi, M. A., Khan, J. A., Altayeb, H. N., Alsulami, H., Sajjad, M., ... & Nadeem, M. S. (2021). *Escherichia coli* expression and characterization of α-amylase from *Geobacillus thermodenitrificans* DSM-465. *Brazilian Journal of Biology*, *82*, e239449.

Aravindan, R., Anbumathi, P., & Viruthagiri, T. (2007). Lipase applications in food industry. *Indian Journal of Biotechnology, 6*,141–158.

Arya, D. B., Vincent, S. G. T., & Balagurusamy, N. (2022). Physiology of Ethanol Production by *Clostridium thermocellum*. In *Bioethanol* (pp. 43-52). Apple Academic Press.

Aschenbrenner, J., & Marx, A. (2017). DNA polymerases and biotechnological applications. *Current Opinion in Biotechnology, 48*, 187-195.

Badr, A., & El-Shazly, H. H. (2024). Genome editing by site-directed nucleases and its applications in producing climate change resilient crop plants. In Genome Editing and Global Food Security (pp. 17-41). Routledge.

Baker, R. A., & Wicker, L. (1996). Current and potential applications of enzyme infusion in the food industry. *Trends in Food Science & Technology*, *7*(9): 279-284.

Bhat, M. K. (2000). Cellulases and related enzymes in biotechnology. *Biotechnology Advances*, *18*(5), 355-383.

Brandam, C., Meyer, X. M., Proth, J., Strehaiano, P., & Pingaud, H. (2003). An original kinetic model for the enzymatic hydrolysis of starch during mashing. *Biochemical Engineering Journal*, *13*(1), 43-52.

Buehler, E. (2006). Bread Science: the chemistry and craft of making bread. Two Blue Books Publisher, Hillsbourough, NC, USA, 254 pp.

Çelik Algül, D. (2019). Topraktan izole edilen *Bacillus subtilis* A8'den proteaz üretimi ve karakterizasyonu. MS Thesis, Institute of Natural and Applied Science, Diyarbakır.

Chanalia, P., Gandhi, D., Jodha, D., & Singh, J. (2011). Applications of microbial proteases in pharmaceutical industry: an overview. *Reviews and Research in Medical Microbiology, 22*(4), 96-101.

Chatterjee, A., Puri, S., Sharma, P. K., Deepa, P. R., & Chowdhury, S. (2023). Nature-inspired Enzyme engineering and sustainable catalysis: biochemical clues from the world of plants and extremophiles. *Frontiers in Bioengineering and Biotechnology*, *11*, 1229300.

Chen, L., Huang, W., Hao, M., Yang, F., Shen, H., Yu, S., & Wang, L. (2023). Rapid and ultrasensitive activity detection of α-amylase based on γ-cyclodextrin crosslinked metal-organic framework nanozyme. *International Journal of Biological Macromolecules*, *242*, 124881.

Choudhury, P., & Bhunia, B. (2015). Industrial application of lipase: a review. *Biopharm Journal*, *1*(2), 41-47.

Contesini, F. J., Melo, R. R. D., & Sato, H. H. (2018). An overview of *Bacillus* proteases: from production to application. *Critical Reviews in Biotechnology*, *38*(3), 321-334.

Daher, R. K., Stewart, G., Boissinot, M., & Bergeron, M. G. (2016). Recombinase polymerase amplification for diagnostic applications. *Clinical Chemistry*, *62*(7), 947-958.

De Baere, H. (1999). Starch policy in the European community. *Starch-Stärke*, *51*(6), 189-193.

de Souza Vandenberghe, L. P., Karp, S. G., Pagnoncelli, M. G. B., von Linsingen Tavares, M., Junior, N. L., Diestra, K. V., ... & Soccol, C. R. (2020). Classification of enzymes and catalytic properties. In *Biomass, biofuels, biochemicals* (pp. 11-30). Elsevier.

de Souza, T. S., & Kawaguti, H. Y. (2021). Cellulases, hemicellulases, and pectinases: Applications in the food and beverage industry. *Food and Bioprocess Technology*, *14*(8): 1446-1477.

Demirkan, E. S., Mikami, B., Adachi, M., Higasa, T., & Utsumi, S. (2005). α-Amylase from *B. amyloliquefaciens*: purification, characterization, raw starch degradation and expression in *E. coli*. *Process Biochemistry*, *40*(8), 2629-2636.

Demuner, B. J., Pereira Junior, N., & Antunes, A. (2011). Technology prospecting on enzymes for the pulp and paper industry. *Journal of Technology Management & Innovation*, *6*(3), 148-158.

Doğan, M. (2015). The investigation of effect of protease enzyme produced by new isolate *Bacillus* sp. strain on woolen fabric physical properties, Master's Thesis, Uludag University, Institute of Natural and Applied Science, Bursa

Duman, Y. (2008). The investigation of kinetic and thermodynamic properties of *Bacillus clausii* alkaline protease in the presence of water misscible organic solvents. PhD Thesis, Institute of Natural and Applied Science, Kocaeli.

Ejaz, U., Sohail, M., & Ghanemi, A. (2021). Cellulases: From bioactivity to a variety of industrial applications. *Biomimetics*, *6*(3), 44.

Ellaiah, P., Srinivasulu, B., & Kunamneni, A. (2002). A review on microbial alkaline proteases, *Journal of Scientific and Industrial Research, 61*, 690-704.

Elyasi Far, B., Ahmadi, Y., Yari Khosroushahi, A., & Dilmaghani, A. (2020). Microbial Alpha-Amylase Production: Progress, Challenges and Perspectives. *Advanced Pharmaceutical Bulletin*, *2020*(3), 350–358.

Escaramboni, B., Fernández Núñez, E. G., Carvalho, A. F. A., & de Oliva Neto, P. (2018). Ethanol biosynthesis by fast hydrolysis of cassava bagasse using fungal amylases produced in optimized conditions. *Industrial Crops and Products*, *112*, 368–377.

Farooq, M. A., Ali, S., Hassan, A., Tahir, H.M., Mumtaz, S., & Mumtaz, S. (2021). Biosynthesis and industrial applications of α-amylase: A review. *Archives of Microbiology*, *203*, 1281-1292.

Gado, H. M., Metwally, H. M., Soliman, H., Basiony, A. Z. L., & El-Galil, E. R. (2007). Enzymatic treatments of bagasse by different sources of cellulase enzymes. In: *The 11th World Conference on Animal Nutrition*, *10*, 607-613.

Gonzalez-Rabade, N., Badillo-Corona, J. A., Aranda-Barradas, J. S., & del Carmen Oliver-Salvador, M. (2011). Production of plant proteases *in vivo* and *in vitro*-a review. *Biotechnology Advances*, *29*(6), 983-996.

Gouveia, I. C., Fiadeiro, J. M. & Queiroz, J. A. (2008). Combined bio-carbonization and dyeing of wool: a possibility using cell wall-degrading enzymes and 1:1 metal-complex dyes. *Engineering in Life Sciences*, *8*, 250-259.

Goyal, N., Gupta, J. K., & Soni, S. K. (2005). A novel raw starch digesting thermostable α-amylase from *Bacillus* sp. I-3 and its use in the direct hydrolysis of raw potato starch. *Enzyme and Microbial Technology*, *37*(7), 723-734.

Guha, T. K., & Edgell, D. R. (2017). Applications of alternative nucleases in the age of CRISPR/Cas9. *International Journal of Molecular Sciences*, *18*(12), 2565.

Gupta, R., Beg, Q., Khan, S., & Chauhan, B. (2002). An overview on fermentation, downstream processing and properties of microbial alkaline proteases. *Applied Microbiology and Biotechnology*, *60*(4), 381-395.

Gupta, R., Gigras, P., Mohapatra, H., Goswami, V. K., & Chauhan, B. (2003). Microbial α-amylases: a biotechnological perspective. *Process Biochemistry*, *38*(11), 1599-1616.

Gürkök, S. (2019). Microbial enzymes in detergents: a review. *International Journal of Science and Engineering Research 10*(9), 75-81.

Gurkok, S., & Ozdal, M., 2021. Purification and characterization of a novel extracellular, alkaline, thermoactive, and detergent-compatible lipase from *Aeromonas caviae* LipT51 for application in detergent industry. *Protein Expression and Purification*, *180*, 105819.

Gurung, N., Ray, S., Bose, S., & Rai, V. (2013). A broader view: microbial enzymes and their relevance in industries, medicine, and beyond. *BioMed Research International*, *2013*(1), 329121.

Hasan, F., Shah, A. A., & Hameed, A. (2006). Industrial applications of microbial lipases. *Enzyme and Microbial Technology*, *39*(2), 235-251.

Hebeish, A., & Ibrahim, N. A. (2007). The impact of frontier sciences on textile industry. *Colourage*, *54*(4), 41-55.

Hemşinli, A. (2022). Purification, Characterization and Biotechnological Applications of Alkali Protease Enzyme Produced by *Exiguobacterium alkaliphilum* VLP1. Master's Thesis, Atatürk University, Institute of Natural and Applied Science, Erzurum.

Hemsinli, A., & Gurkok, S. (2024). Evaluation of a novel purified and characterized alkaline protease from the extremophile *Exiguobacterium alkaliphilum* VLP1 as a detergent additive. *Biotechnology Journal*, *19*, 2300441.

Houlihan, G., Arangundy-Franklin, S., & Holliger, P. (2017). Engineering and application of polymerases for synthetic genetics. *Current Opinion in Biotechnology*, *48*, 168-179.

Hussain, I., Siddique, F., Mahmood, M. S., & Ahmed, S. I. (2013). A review of the microbiological aspect of α-amylase production. *International Journal of Agriculture & Biology*, *15*(5).

Jaeger, K. E., & Reetz, M. T. (1998). Microbial lipases form versatile tools for biotechnology. *Trends in Biotechnology*, *16*(9), 396-403.

Jaeger, K. E., Ransac, S., Dijkstra, B. W., Colson, C., van Heuvel, M., & Misset, O. (1994). Bacterial lipases. *FEMS Microbiology Reviews*, *15*(1), 29-63.

Kainoor, P. S., & Naik, G. (2010). Production and characterization of feather degrading keratinase from *Bacillus* sp. JB 99, *Indian Journal of Biotechnology 9*(4), 384-390.

Kara, M., Sivri, D., & Köksel, H. (2005). Effects of high protease-activity flours and commercial proteases on cookie quality. *Food Research International, 38*(5), 479-486.

Kondo, M., Fukao, T., Shinoda, S., Kawamoto, N., Kaneko, H., Kato, Z., Matsui, E., Teramoto, T., Nakano, T., & Kondo, N. (2007). Lymphocyte responses to chymotrypsin or trypsin v-digested beta-lactoglobulin in patients with cow's milk allergy. *Official Journal of the Canadian Society of Allergy and Clinical Immunology*, *3*(1), 1-9.

Kudrya, V. A., & Simonenko, I. A. (1994). Alkaline serine proteinase and lectin isolation from the culture fluid of *Bacillus subtilis*. *Applied Microbiology and Biotechnology*, *41*, 505-509.

Li, Q., Yan, Y., Liu, X., Zhang, Z., Tian, J., & Wu, N. (2020). Enhancing thermostability of a psychrophilic alpha-amylase by the structural energy optimization in the trajectories of molecular dynamics simulations. *International Journal of Biological Macromolecules*, *142*, 624–633.

Li, W.F., Zhou, X.X., & Lu, P. (2005). Structural features of thermozymes. *Biotechnology Advances*, *23*(4), 271-281.

Li, Y. X., Yi, P., Yan, Q. J., Qin, Z., Liu, X. Q., & Jiang, Z. Q. (2017). Directed evolution of a β-mannanase from Rhizomucor miehei to improve catalytic activity in acidic and thermophilic conditions. *Biotechnology for Biofuels*, *10*(1).

Lou, Y., Zhang, B., Ye, X., & Wang, Z. G. (2023). Self-assembly of the *de novo* designed peptides to produce supramolecular catalysts with built-in enzyme-like active sites: a review of structure–activity relationship. *Materials Today Nano*, *21*, 100302.

Madhavan, A., Arun, K. B., Binod, P., Sirohi, R., Tarafdar, A., ... & Sindhu, R. (2021). Design of novel enzyme biocatalysts for industrial bioprocess: Harnessing the power of protein engineering, high throughput screening and synthetic biology. *Bioresource Technology*, *325*, 124617.

McDonald, A. G., & Tipton, K. F. (2023). Enzyme nomenclature and classification: The state of the art. *The FEBS Journal*, *290*(9), 2214-2231.

Mojsov, K. (2012). Microbial α-amylases and their industrial applications: a review. *International Journal of Management, IT and Engineering*, *2*(10), 583-609.

Mota, D. S., Marques, J. M., Guimarães, J. M., & Mariúba, L. A. M. (2020). CRISPR/Cas Class 2 systems and their applications in biotechnological processes. *Genetics and Molecular Research, 20*, 1-10.

Murad, H. A., & Azzaz, H. H. (2010). Cellulase and dairy animal feeding. *Biotechnology*, *9*(3), 238-256.

Nadeem, F., Mehmood, T., Naveed, M., Shamas, S., Saman, T., & Anwar, Z. (2020). Protease production from *Cheotomium globusum* through central composite design using agricultural wastes and its immobilization for industrial exploitation. *Waste and Biomass Valorization, 11*(12), 6529-6539.

Nakiboğlu, N., Toscali, D., & Yaşa, İ. (2001). Silver recovery from waste photographic films by using enzymatic method. *Turkish Journal of Chemistry*, *25*(3), 349-353.

Ni, H., Chen, Q., Chen, F., Fu, M., Dong, Y., & Cai, H. (2011). Improved keratinase production for feather degradation by *Bacillus licheniformis* ZJUEL31410 in submerged cultivation. *African Journal of Biotechnology, 10*(37), 7236-7244.

Orsi, E., Schada von Borzyskowski, L., Noack, S., Nikel, P. I., & Lindner, S. N. (2024). Automated *in vivo* enzyme engineering accelerates biocatalyst optimization. *Nature Communications*, *15*(1).

Pan, X., & Kortemme, T. (2021). Recent advances in *de novo* protein design: Principles, methods, and applications. *Journal of Biological Chemistry*, *296*.

Parker, K., Salas, M., & Nwosu, V. C. (2010). High fructose corn syrup: production, uses and public health concerns. *Biotechnology and Molecular Biology Reviews*, *5*(5), 71-78.

Rani, K., Rana, R., & Datt, S. (2012). Review on latest overview of proteases. *The International Journal of Current Life Sciences*, *2*(1), 12-18.

Rao, M. B., Tanksale, A. M., Ghatge, M. S., & Deshpande, V. V. (1998). Molecular and biotechnological aspects of microbial proteases. *Microbiology and Molecular Biology Reviews*, *62*(3), 597-635.

Ratnayake, R., & Jayasekara, S. (2019). Microbial cellulases: an overview and applications. *Cellulose*, *22*, 92.

Ray, A. (2012). Application of lipase in industry. *Asian Journal of Pharmacy and Technology*, *2*(2), 33-37.

Saini, R., Saini, H. S., & Dahiya, A. (2017). Amylases: Characteristics and industrial applications. *Journal of Pharmacognosy and Phytochemistry*, *6*(4), 1865-1871.

Sangeetha, R., Arulpandi, I., & Geetha, A. (2011). Bacterial lipases as potential industrial biocatalysts: An overview. *Research Journal of Microbiology*, *6*(1), 1.

Schafer, T., Borchert, T. W., Nielsen, V. S., Skagerlind, P., Gibson, K., …, & Xu H. (2007). Industrial enzymes. *White Biotechnology, 105*, 59-131.

Schulze, S., & Lammers, M. (2020). The development of genome editing tools as powerful techniques with versatile applications in biotechnology and medicine: CRISPR/Cas9, ZnF and TALE nucleases, RNA interference, and Cre/loxP. *ChemTexts*, *7*(1), 3.

Shankar, S., More, S. V., & Laxman, R. S. (2010). Recovery of silver from waste X-ray film by alkaline protease from *Conidiobolus coronatus*. *Kathmandu University Journal of Science, Engineering and Technology*, *6*(1), 60-69.

Sharada, R., Venkateswarlu, G., Venkateswar, S., & Rao, M. A. (2014). Applications of Cellulases-Review. *International Journal of Pharmaceutical, Chemical & Biological Sciences*, *4*(2).

Sharma, B., & Shukla, P. (2022). Futuristic avenues of metabolic engineering techniques in bioremediation. *Biotechnology and Applied Biochemistry*, *69*(1), 51-60.

Sharma, H., Batra, N., & Singh, J. (2022). Purification, characterization and potential detergent industry application of a thermostable α-amylase from *Bacillus licheniformis* RA31. *Indian Journal of Experimental Biology*, *60*(05), 331-342.

Sharma, M., Gat, Y., Arya, S., Kumar, V., Panghal, A., & Kumar, A. (2019). A review on microbial alkaline protease: an essential tool for various industrial approaches. *Industrial Biotechnology, 15*(2), 69-78.

Sharma, R., Chisti, Y., & Banerjee, U. C. (2001). Production, purification, characterization, and applications of lipases. *Biotechnology Advances*, *19*(8), 627-662.

Silva, R. R. (2017). Bacterial and fungal proteolytic enzymes: production, catalysis and potential applications. *Applied Biochemistry and Biotechnology*, *183*(1), 1-19.

Singh, R., Mittal, A., Kumar, M., & Mehta, P. K. (2016). Microbial proteases in commercial applications. *Journal of Pharmaceutical, Chemical and Biological Sciences, 4*(3), 365-374.

Souza, P. M. & Magalhães P. O. (2010). Application of microbial-amylase in industry-a review. *Brazilian Journal of Microbiology, 41*, 850–861.

Steiner, K., & Schwab, H. (2012). Recent advances in rational approaches for enzyme engineering. *Computational and Structural Biotechnology Journal*, *2*(3), e201209010.

Sukumaran R. K., Singhania R.R., & Pandey A. (2005). Microbial cellulases production, applications and challenges. *Journal of Scientific and Industrial Research, 64,* 832-844.

Tapia, D. M., & Simões, M. L. G. (2008). Production and partial characterization of keratinase produced by a microorganism isolated from poultry processing plant wastewater. *African Journal of Biotechnology, 7*(3).

Tiwari, S. P., Srivastava, R., Singh, C. S., Shukla, K., Singh, R. K., Singh, P., ... & Sharma, R. (2015). Amylases: an overview with special reference to alpha amylase. *Journal of Global Bioscience*, *4*(1), 1886-1901.

Tran, P. L., Lee, J. S., & Park, K. H. (2014). Experimental evidence for a 9-binding subsite of *Bacillus licheniformis* thermostable α-amylase. *FEBS Letters*, *588*(4), 620-624.

Vaidya, S., Srivastava, P.K., Rathore, P., & Pandey, A.K. (2015). Amylases: a prospective enzyme in the field of biotechnology. *Journal of Applied Bioscience, 41*(1), 1-18.

Wahab, W. A. A., & Ahmed, S. A. (2018). Response surface methodology for production, characterization and application of solvent, salt and alkali-tolerant alkaline protease from isolated fungal strain *Aspergillus niger* WA 2017. *International Journal of Biological Macromolecules*, *115*, 447-458.

Wang, S., Jeyaseelan, J., Liu, Y., & Qin, W. (2016). Characterization and optimization of amylase production in WangLB, a high amylase-producing strain of *Bacillus*. *Applied Biochemistry and Biotechnology*, *180*, 136-151.

Wright, W. F., Simner, P. J., Carroll, K. C., & Auwaerter, P. G. (2022). Progress report: next-generation sequencing, multiplex polymerase chain reaction, and broad-range molecular assays as diagnostic tools for fever of unknown origin investigations in adults. *Clinical Infectious Diseases*, *74*(5), 924-932.

Xie, W. J., & Warshel, A. (2023). Harnessing generative AI to decode enzyme catalysis and evolution for enhanced engineering. *National Science Review*, *10*(12), nwad331.

Xu, Y., Wu, Y., Lv, X., Sun, G., Zhang, H., Chen, T., ... & Liu, L. (2021). Design and construction of novel biocatalyst for bioprocessing: Recent advances and future outlook. *Bioresource Technology*, *332*, 125071.

Yan, Y., Liu, X., Jiang, X., Zhang, W., Wang, Y., ... & Tu, T. (2022). Surface charge modifications modulate glucose oxidase pH-activity profiles for efficient gluconic acid production. *Journal of Cleaner Production*, *372*, 133817.

Yang, J., Li, F. Z., & Arnold, F. H. (2024). Opportunities and challenges for machine learning-assisted enzyme engineering. *ACS Central Science*, *10*(2), 226-241.

Yılmaz, A. A (2023). Cellulase production with *Bacillus pumilus* VLC7 Isolated from Lake Van, optimization of production conditions, purification, characterization and biotechnological applications of cellulase. Master's Thesis, Atatürk University, Institute of Natural and Applied Science, Erzurum.

Yuan, S., Yan, R., Lin, B., Li, R., & Ye, X. (2023). Improving thermostability of *Bacillus amyloliquefaciens* alpha-amylase by multipoint mutations. *Biochemical and Biophysical Research Communications*, *653*, 69-75.

Zhu, M., Zhai, W., Jiang, H., Lin, L., Wei, W., & Wei, D. (2023). Exploiting loop-grafting strategy resorting on computer-aided design to improve the thermostability of alpha-amylase from *Geobacillus stearothermophilus. Process Biochemistry*, *133*, 28-38.

Zoppas, F. M., Meneguzzi, A., & Tramontina, F. (2013). Alternatives for cellulase production in submerged fermentation with agroindustrial wastes. *International Journal of Modern 3*, 2374-2381.

REVOLUTIONIZING INDUSTRIAL PROCESSES IN HARSH CONDITIONS WITH THERMOPHILIC ENZYMES

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Abstract

Microorganisms have a variety of adaptations to withstand and thrive in extreme conditions regarding many factors temperature, osmolality, pH. Among these factors, temperature is a significant parameter that classifies microorganisms as psychrophiles, mesophiles, and thermophiles. Due to the miracle adaptations, they have been living in nearly all environments on Earth from the permanently frozen polar regions or the arctic permafrost to the scalding deep-sea hydrothermal vents, hot springs, or geysers between the temperatures of -20 °C and 122 °C. Recently, thermophiles, organisms with optimal growth above 50 °C, have attracted researchers' attention. Since, thermophiles have enzymes, that catalyze biochemical reactions in very harsh conditions. The enzymes of thermophiles called thermozymes work optimally in high temperatures and are more stable against different values of pH, temperature, and protein-denaturating solvents. Performing biotechnological processes under high temperatures improves substrate and reaction mixture solubility, and increases reaction yield. In addition, the use of thermophilic enzymes decreases the microbial contamination caused by mesophiles. Therefore, this chapter aims to briefly overview the extremophiles, and emphasize the importance and advantages of using thermophilic enzymes in harsh conditions for biotechnological industrial processes.

Introduction

According to the Report of the United States, Department of Economic and Social Affairs about World Population Prospects (2024), the global population is estimated to reach approximately 10.3 billion people in 2080, up from 8.2 billion in 2024 (https://population.un.org/wpp/). Although a growing population fosters economic expansion and sustainable development through a larger labor force, environmental conservation and securing natural resources must go hand in hand with economic progress. Because, the uncontrolled growing global population causes overconsumption, misuse of resources, over-wastage, excessive environmental pollution, and threats to the capacity of the earth to sustain life. For example, overconsumption can contribute to climate change by increasing greenhouse gas emissions and causing shortages of essential resources such as food, energy, and water. Rapid urbanization driven by population growth also leads to deforestation and severe environmental pollution, which threaten biodiversity and ecosystems (Mittal and Mittal, 2013). The global approach to addressing these

challenges, becoming increasingly integrated into our daily lives, involves adopting biotechnological solutions in production processes and environmental management. To put it more clearly, the widespread adoption of biotechnological methods in production and environmental management is emerging as a key strategy for tackling these issues on a global scale (Sheldon et al., 2018). Biotechnology is a branch of science that merges biology with technology, utilizing organisms such as microorganisms, plants, algae, and animals or their metabolites to perform industrial applications and manage environmental challenges. Its applications involve agriculture (encompassing areas like animal husbandry, cropping systems, soil science and conservation, plant physiology, seed technology, and crop management), and industries such as food, pharmaceuticals, chemicals, byproducts, and textiles. It also plays a crucial role in medicine, nutrition, environmental conservation, and cell biology, making biotechnology one of the fastest-growing fields. Biotechnology involves modifying the genetic structure of animals and plants to enhance them in ways that yield beneficial products (Greenwood, 2014). Before the widespread adoption of biotechnological applications, most industrial reactions were primarily catalyzed by chemical catalysts. Biologically mediated chemical reactions, commonly known as bioconversion, have largely replaced chemical conversions traditionally driven by chemical catalysts (Sheldon et al., 2018). Bioconversion methods are preferred in industrial processes for several key reasons: they significantly reduce waste, often exhibit low toxicity due to safer processes conducted under mild and safe conditions, and use safer solvents like water. Additionally, these methods involve less hazardous synthesis, rely on renewable feedstocks such as enzymes, and allow for real-time analysis in biocatalytic processes. They are also energy-efficient and minimize the need for protection and deprotection steps (Report of the World Commission on Environment and Development, 1987).

The scope of bioconversion is vast, making it useful to classify bioconversions into two different types. The first type of bioconversion, commonly termed fermentation, is carried out by growth-associated whole-cell bioconversion. Bioconversion through fermentation is a process in which the whole cell acts as a biocatalyst during growth due to its producing metabolites that induce chemical changes in the substrates (Woodley et al., 2013). For example, the production of lactic acid, 3-hydroxy propionic acid, succinic acid, amino acid, etc. is carried out by fermentation (Chen and

Nielsen, 2016). The second type of bioconversion is defined as those being performed by biocatalysts, especially enzymes.

Biocatalytic methods are popular due to their economic and environmental advantages. Enzymes, which are biodegradable and non-toxic, are derived from renewable resources, avoiding the need for precious metals and reducing waste. These reactions typically occur under mild conditions, making them more sustainable and cost-effective than traditional organic syntheses. Additionally, biocatalytic processes can be easily integrated into existing systems without requiring significant investments in specialized equipment, allowing for the efficient combination of multiple reactions (Sheldon and Woodley, 2017).

Enzymes are biocatalysts that accelerate chemical reactions without being consumed or permanently altered in the process. They do not get used up in the reaction and do not appear in the final reaction products. As a result of scientific developments, the enzymes used in the production processes of biotechnological products, now integral to almost all areas of our lives, were first named using a Greek word meaning 'in leaven' (Thapa et al., 2019). The term 'enzyme' was first introduced as scientific terminology by Kühne in 1877, even though the first enzyme, diastase was discovered in 1833. The enzymes have been utilized for millennia in various food production processes, such as baking bread, brewing alcoholic beverages, and making cheese, without mankind's knowledge of the underlying biochemical mechanisms (Gurung et al., 2013; Singh et al., 2016). In 1947, James B. Sumner earned the Nobel Prize for isolating, purifying, and crystallizing the urease for the first time (Thapa et al., 2019). Sumner likened enzymes to biological chemists, emphasizing their superior properties, such as the ability to synthesize various substances, in contrast to organic chemists. The discovery that enzymes could function both *in vivo* and *in vitro* marked the beginning of a new biotechnological era for humanity (Heckman and Paradise, 2020). In 1960, NOVO became the first to commercially produce protease using *Bacillus lichnformis*. Nowadays, the market size of industrial enzymes is estimated to reach 16.29 Billion by 2027 (https://www.bccresearch.com/partners/verified-market-research/global-enzymes-market report.html). The enzymes have been utilized in an extensive range of biotechnological processes. Some examples of the industrial applications are given below. The enzymes used in the process of dairy products are proteinase for milk coagulation (Artifon et al., 2023), neutral

proteinase, lipase, aminopeptidase, transglutaminase, and catalase for cheese ripening, processing, and debittering (Wilkinson and Kilcawley, 2005; Qureshi et al., 2015; Khan and Selamoglu, 2020; Xiang et al., 2023), β-galactosidase for removing lactose content of milk and whey products to prevent lactose-intolerant humans from severe health problems (Pivarnik et al., 1995; Qureshi et al., 2015). Some of the enzymes utilized beverage industry are pectinase, cellulase, xylanase, α-amylase, and β-amylase for fruit liquefaction, restricting haze formation, increasing product yield and shelf-life (Pal et al., 2011; Singh et al., 2016; Gaspar et al., 2019; Cosme et al., 2023), pullulanase for starch saccharification to improve taste (Naik et al., 2023). Amylase, xylanase, lipase, and glucose oxidase are the enzymes to improve dough quality in the baking industry (Fernandes, 2010; Kim and Yoo, 2020; Seema et al., 2020). In the detergent industry, specific enzymes are employed for targeted stain removal: amylase for carbohydrate stains, lipase for fat-based stains, protease for protein stains, cellulase for cellulose-based stains, and mannanase for mannan stains (Galanta and Formantici, 2003; Hasan et al., 2010). In the pulp and paper industry, various enzymes are used for specific processes: protease for biofilm removal, amylase and cellulase for deinking and drainage improvement, xylanase for bleach boosting, and laccase for non-chlorine bleaching and delignification (Singh et al., 2016; Imran et al., 2019). Enzymes are not only used to produce industrial products but also play a crucial role in waste management. For example, amylase, amyloglucosidase, xylanase, and pectinase are used in biomass hydrolysis for bioremediation; amidase and nitrile hydratase break down nitrile-containing waste; laccase degrades waste with olefin units, polyurethane, and phenolic compounds; lipase is employed for the degradation of crude oil hydrocarbons; manganese peroxidase and lignin peroxidase target phenolic compounds; oxygenase breaks down halogenated contaminants; and cutinase is used for plastic degradation (Verma et al., 2017; Vidmar et al., 2018; Mitra et al., 2024; Janeeshma et al., 2024). Today, enzymes are employed in a broad range of biotechnological applications across nearly all industrial sectors, both to produce countless products and to solve complex problems.

In this chapter, we summarize, that the sources of preferred enzymes for industrial applications and their advantages in applications. In this context, we overview the extremophiles and emphasize the importance and advantages of using thermophilic enzymes in harsh conditions for biotechnological industrial processes.

The Preferred Sources of Industrial Enzymes

Microbes are ubiquitously found and are often preferred as the primary source of various enzymes because they supply many advantages compared to animals and plants. Microorganisms are considered ubiquitous because of their ability to disperse easily, their metabolic adaptability, their smaller size, and their capacity to thrive in hostile environments using a wide range of nutrients. As a result, they make up a significant portion of most ecosystems (Uluçay, 2023). They make up approximately 60 % of the Earth's total biomass and they can thrive in every extreme environment including different types of soils, the gut of ruminants, rumens of different animals, fermented foods, fried oil, glacier ice, hot springs, etc. (Gurung et al., 2013; Tamang et al., 2016; Uluçay, 2023; Xu et al., 2023). Because of their great adaptations, compared to enzymes derived from plants and animals, microbial enzymes are considered more stable, active, cost-effective, and sustainable offering superior performance under a wide range of physicochemical conditions (Adrio and Demain, 2014; Thapa et al., 2019). Accordingly, yeasts and molds account for over 50 % of industrial enzymes, while bacteria contribute about 30 %. In comparison, animals provide 8 % of these enzymes, and plants contribute roughly 4 % (Thapa et al., 2019). Although enzymes were first discovered in microorganisms in the 20th century, research on their isolation, property characterization, bench-scale to pilot-scale production, and application in the bio-industry has steadily advanced, with knowledge being continuously updated. Today, many microbial enzymes are already used in various commercial processes. Also, researchers have been searching in highly extreme environments for new microbial sources of enzymes with valuable properties (Singh et al., 2016).

Microbial life can survive in extreme environments, such as hot springs (60-110 °C), cold polar regions (-2-15 °C), high salt concentrations (2-5 M NaCl or 5 %- 30 %), acidic (pH (< 4), or alkaline conditions (> 9), and deep ocean vents. The microorganisms surviving under extreme conditions are called 'extremophiles' belonging to the Archae and Bacteria and are used as reservoirs of enzymes with superior novel properties and potential applications in industrial processes. Extremozymes have significant industrial potential because of their stability, altered specificity, and ability to function under extreme conditions where enzymes from mesophilic enzymes fail. The extremozymes, which can thrive in multiple extreme environmental conditions that combine extremes, such as high temperature, high salinity, and

low pH, all at once, are defined as polyextremophiles. This remarkable adaptability makes polyextremophiles of particular interest in studies of extremophiles and their potential applications in biotechnology. Therefore, exploring microbial enzymes in extreme environments is a promising strategy for discovering robust extremophiles with enhanced tolerance to harsh conditions. (Gomes and Steiner, 2004; Thapa et al., 2019; Kochhar et al., 2022).

Extremophiles have adapted to thrive in these harsh conditions and are classified into various types; *i*) alkalophiles (alkaline-adapted), *ii*) acidophiles (acid-adapted), *iii*) halophiles (salt-adapted), *iv*) radiophiles (radiation-adapted), *v*) psychrophiles (cold-adapted), *vi*) thermophiles (heat-adapted) (Karan and DasSarma, 2012).

A Brief Overview of Extremophiles

Microorganisms, especially procaryotes, have been evolving over millions of years regarding environmental factors to which they are exposed. Due to their great adaptations, microorganisms can be found in every kind of media, even the valcon mouth or bottom of the ocean, they can cope with every stress factor such as radiation, drought, extreme temperatures, and pH. The microorganisms dealing with extreme conditions and thriving in them are defined as 'extremophiles' (Van den Burg, 2003). Brief definitions of various extremophiles are provided below.

i) Alkalophiles; While many microorganisms can exhibit multiple pH optima for growth depending on factors such as nutrients, metal ions, and temperature, the term 'alkaliphile' specifically refers to microorganisms that grow optimally, or at least well, at pH values above 9-typically between 10 and 12-but struggle to grow or grow only slowly at near-neutral pH levels around 6.5 (Horikoshi, 1999). The enzymes of alkaliphiles, especially alkaliphilic *Bacillus* strains, are mostly used in mainly detergent industry, and detergent enzymes make up approximately 30 % of the global enzyme production (Verma et al., 2014). The other usage of alkaliphilic enzymes is dehairing treatment in the leather industry. Conventional dehairing methods in the leather industry rely on harmful and polluting chemicals, such as sodium sulfide, and produce toxic wastes. Keratinolytic enzymes are proteolytic enzymes activating at pH values between 8.0 and 10.0 and are extensively used in the dehairing process instead of harmful chemicals, as they can break down keratin-derived substances. So that, they lead to producing higher

quality hides and reducing the environmental pollutants (Kumar and Takagi, 1999; Chinnathambi, 2015). Additionally, alkaliphilic enzymes hold significant potential for degrading xenobiotics and play a crucial role in the biogeochemical cycling of key inorganic compounds (Chinnathambi, 2015). Thermophilic and alkalophilic enzymes are of significant commercial value due to their resistance to denaturants and their superior thermal and pH stability compared to mesophilic enzymes (Verma et al., 2014; Chinnathambi, 2015).

ii) Acidophiles; Acidophiles are organisms that thrive in acidic environments with a pH below 4.0. This diverse group includes members from the domains of archaea, bacteria, fungi, algae, and protozoa, and they are found in both natural environments, such as sulfuric pools, solfataric fields, hydrothermal marine vents, and geysers, as well as in artificial environments associated with human activities, like coal and metal ore mining. Acidophiles are both ecologically and economically significant, as they adapt to and flourish in these acidic conditions, whether naturally occurring or man-made (Bertoldo et al., 2004; Sharma et al., 2012). The economically significant acid-stable enzymes like proteases, amylases, xylanases, cellulases, ligases, glucosidases, glucanases, and esterases stable at low pH are known from various acidophiles. The acidophilic enzymes have the potential to degrade polymeric carbon sources such as starch, xylan, cellulose, pullulan, etc. in biomass. Therefore, they are used in fruit juice and biofuel production, and the beverage and textile industry (Bertoldo et al., 2002; Serour and Antranikian, 2002). The most preferred acidophilic enzymes are obtained from thermoacidophiles, which are active at low pH and high temperature. Thermoacidophilic enzymes are particularly promising for use in harsh conditions (Burkhardt et al., 2024).

iii) Halophiles; Halophiles thrive in environments with salinity ranging from seawater levels (0.6 M) to saturation salinity (>5 M NaCl) due to their ability to maintain the osmotic and survive in hypersaline habitats and are found across the domains of Archaea, Bacteria, and Eukarya. These microorganisms have been isolated from various environments, including artificial solar salterns, submarine pools, and natural brines in coastal and deep salt mines. Common examples of halophiles are those found in salterns used for salt production, in fish and meat preserved with solar salt, and in certain protein-rich fermented foods (DasSarma and DasSarma, 2012; DasSarma and DasSarma, 2015). Halophilic enzymes must withstand extremely high salt

concentrations, such as 4 M KCl and over 5 M NaCl. These enzymes have adapted to such environmental pressures by incorporating a relatively high number of negatively charged amino acid residues on their surfaces, which helps prevent precipitation. As a result, their solubility in lower salt concentrations is often poor, potentially limiting their applicability. However, this characteristic has been leveraged to use in aqueous/organic and nonaqueous media, where halophilic enzymes can be particularly effective. Halophilic enzymes possess application to degrade various polymers through their amylases, proteases, lipases, DNases, and pullulanases (Kim and Dordick, 1997; Madern et al., 2000; de Lourdes Moreno et al., 2013). Along with alkalophilic industrial enzymes, thermo-halophilic enzymes are more advantageous in industrial processes due to their superior stability (Febriani et al., 2020).

iv) Radiophiles; This group of extremophiles represents a unique class of microbes capable of surviving harmful radiation. Primarily belonging to the Deinococcaceae family, these microbes can endure intense doses of gamma radiation up to 3.000 times higher than what is lethal to humans (Basu, 2022). The nuclear waste contains radionuclides such as plutonium, uranium, cesium, strontium, and technetium, heavy metals such as chromium, mercury, and lead along with organic solvents like toluene and trichloroethylene at toxic concentrations (Daly, 2000). In addition, some of the *Deinococcus* strains are naturally resistant to metalloids such as sodium arsenate and arsenic trioxide. *Deinococcus* strains are the best-suited bacteria to remediate radioactive wastes and metalloids because they are non-pathogenic, non-spore-forming, and radio-resistant (Xu et al., 2018). The radiophiles produce various natural compounds including antioxidants used in cosmetics, the food industry, and pharma (Basu, 2022). Especially, The use of natural antioxidants as radioprotectors is an upcoming field with far-reaching health implications for using radiation in healthcare without the damage it causes. Additionally, deinococcal enzymes are tapped as biocatalysts for industrial applications owing to their better thermostability, higher optimal temperatures, etc. (Basu, 2022).

v) Psychrophiles; Psychrophiles, the most abundant extremophiles known for their high biomass, diversity, and extensive distribution, thrive in permanently cold environments, including subzero temperatures found in supercooled liquid water (Deming, 2002; Cary et al., 2010). Psychrophiles have developed remarkable adaptations to low temperatures at the genomic,

proteomic, and transcriptomic levels, enabling them to maintain efficient translation and proper protein folding under cold conditions (Siddiqui et al., 2013). Psychrophilic enzymes hold significant biotechnological potential due to their high catalytic activity at low temperatures, albeit often with reduced substrate affinity. This adaptation lowers the free energy barrier of the transition state, enhancing reaction rates in cold environments. Additionally, their weak temperature dependence ensures only a moderate decrease in catalytic activity at low temperatures, making them highly efficient under cold conditions. In addition, these enzymes perform in tap water or at room temperature, therefore the heating process is unnecessary and decreases cost. At the end of the industrial process, psychrophilic enzymes can be easily inactivated, providing a convenient advantage for process control (Feller, 2013). The most used psychrophilic enzyme is protease as an additive in detergents (Narinx et al., 1997).

vi) Thermophiles: The term 'thermophile' is commonly used to describe microorganisms that grow at temperatures exceeding the mesophilic range (20-40 °C). Thermophiles can withstand or even thrive at extreme temperatures ranging from 50 °C to 121 °C, typically above 60 °C. Thermophilic microorganisms are adapted to high-temperature environments and can survive and grow under these conditions (Laemthong et al., 2022). Although thermophiles are phylogenetically related to mesophiles, their habitats show extreme differences from mesophiles' such as hot springs, deep-sea hydrothermal vents, coastal thermal springs, volcano vents, and geysers due to their morphological, biochemical, physiological, and genetic adaptations (Panda et al., 2013; Miroshnichenko and Bonch-Osmolovskaya, 2006; Zelenkina et al., 2009; Mangrola et al., 2015; Gaisin et al., 2017; Mangrola et al., 2022). They are divided into three categories regarding their optimum growth temperature; moderate thermophiles (50 -60 °C), extreme thermophiles (60-80 °C), and hyperthermophiles (80-110 °C) (Gupta et al., 2014). Thermophilic bacteria are highly advantageous in almost all biotechnological industrial applications because of their ability to withstand harsh conditions. Therefore, many researchers pay great attention to thermophiles and their metabolites such as enzymes across the world (Gallo et al., 2021; Sanka et al., 2023). Although the researches on thermophiles began 40 years ago, according to the bibliometric analysis performed by Che Hussian and Leong (2023), there have been over 1090 articles about thermophiles in the literature. Because the thermophilic enzymes are the most commercially used in industrial processes. Thermophilic enzymes such as

amylases (Ullah et al., 2021), cellulases (Ajeje et al., 2021), pectinases (Flores-Fernández et al., 2022), xylanases (Akpinar and Karaoglu, 2024), lipases (Hamdan et al., 2021), proteases (Xu et al., 2023), laccases (Sharma and Leung, 2021), glucose isomerases (Neifar et al., 2019), peroxidases (Twala et al., 2020), and DNA polymerases (Wang et al., 2022) have essential biotechnological applications, including the hydrolysis of biological polymers, bioethanol production, waste management, bioleaching, food processing, baking, paper bleaching, beverage production, pharmaceutical and cosmetic manufacturing, genetic engineering, and scientific research (Indriati and Megahati, 2023; Zuliani et al. 2021).

How do Thermophiles Survive in Harsh Conditions?

Temperature plays a crucial role in shaping the evolution, distribution of biodiversity, and the structure of microbial communities within an ecosystem. Research on the genome, transcriptome, and proteome is essential to understanding how thermophiles survive under extreme thermal conditions. Temperature is one of the most significant forces for the evolutionary changes of microorganisms, which can be achieved mainly through gene loss, horizontal gene transfer (HGT), or gene mutations.

As known, the main reason for the great adaptation ability of microorganisms is their compact, circular, and little inter-genic genome compared to eukaryotic organisms. Due to their genomic structure, they reduce the energy for nucleotide synthesis and can reproduce in a short time. Interestingly, thermophiles (smaller than 4 Mb) have mostly smaller genome sizes than mesophiles (larger than 6 Mb) (Wang et al., 2015). As a result, the number of protein family members of thermophiles and their protein lengths are less than non-thermophiles due to the small size of the genomes of thermophiles. Also as a hypothesis, thermophiles have eliminated the genes encoding proteins with low thermo-stability during evolution (Sabath et al., 2013).

According to recent studies, horizontal gene transfer services for bacterial genome evolution and adaptation to extreme conditions. Horizontal gene transfer, which is the exchange of DNA between organisms of different species, occurs by the replacement of a homologous sequence with a new sequence and gene integration such as transduction, conjugation, and transformation (van Wolferen et al., 2013). Many horizontally transferred

genes contribute to thermophilic traits crucial for survival in extreme conditions. A key example is reverse gyrase, a DNA topoisomerase that introduces positive supercoiling to increase the melting temperature of double-stranded DNA, enhancing thermo-adaptation. This gene was transferred from archaea to bacteria. Genome comparisons reveal that about 20 % of bacterial genes and 40 % of archaeal genomes have been acquired through HGT (Vettone et al., 2014).

The final driving force of genome evolution is mutation, which can enhance thermal adaptability. Studies have shown that amino acid substitutions, such as lysine to arginine, are linked to thermophily (Wang et al., 2015). However, in general, amino acid substitutions resulting from gene mutations tend to be more harmful in thermophiles compared to non-thermophiles. This increased risk is partly because DNA is more unstable at extreme temperatures, necessitating a more robust DNA repair system in thermophiles to maintain genomic stability (Drake, 2009). The high content of guanine (G) and cytosine (C) in the genome supplies genomic stability for thermophiles and genome thermostability and correlates with the organism's optimal growth temperature. The GC content in some thermophiles differs from that of mesophiles, as seen in examples like *Thermus thermophilus* with 69.41 % GC content (Jiang et al., 2013), and *Thermus* sp. strain with 68.6 % (Teh et al., 2012). However, there are exceptions in the correlation between purine content and optimum growth temperature. These exceptions should be evaluated by considering additional factors, such as the growth environment and the Gram-positive or Gram-negative characteristics of the bacteria, rather than solely focusing on purine content (Wang et al., 2015). In addition, tRNAs, rRNAs, and mRNAs have high GC contents (Wang et al. 2006). In addition, the ribosomal protein components of the gene expression system with tRNAs and rRNAs must be efficient and stable at extreme temperatures. According to the literature, the abundance of some ribosomal proteins such as S1, L17, L14, etc. increased at more elevated temperatures than optimum growth temperatures in various bacteria (Wang et al., 2015).

The study found that synonymous codon usage is directly related to the optimal growth temperature of the enzyme activity encoded by thermophilic species. This codon usage pattern is a critical feature influenced by the selective pressure of high temperatures, differentiating thermophiles from mesophiles. Specifically, thermophiles favor AGG, ATA, and AGA codons for arginine and isoleucine, while avoiding CGT and CGA. Frequent A and G

nucleotide pairs are observed in thermophile genomes. Because this codon bias may help maintain optimal codon-anticodon interaction energy, facilitating efficient translation (Liu et al. 2012; Wang et al., 2015).

The unique amino acid usage in thermophilic species is considered a strategy for adaptation to high temperatures. This specific usage helps stabilize proteins, enhancing their structural integrity and function under extreme thermal conditions, which is crucial for the survival and optimal performance of thermophilic organisms. In thermophiles, the frequency of charged residues (Glu, Arg, and Lys) and Glu-Lys)/(Gln-His) ratio increased, as the frequency of polar uncharged residues (Asn, Gln, Ser, and Thr) and thermo-labile amino acids (His, Gln, and Thr) decreased (Farias and Bonato, 2003; Kreil and Ouzounis, 2001).

In thermophiles, nearly half of the genes are organized within operon structures, allowing for multigene expression that plays a crucial role in adaptation to temperature fluctuations. This organization enables efficient and coordinated regulation of gene expression, as operon-encoded genes can be economically co-regulated in response to environmental stimuli. Operons in thermophiles are often subject to global regulatory systems, facilitating a rapid and synchronized response to environmental changes, including temperature stress. Some global regulators specifically control gene clusters in response to temperature variations. Evidence indicates that operon structures are more stable in thermophiles than non-thermophiles, and their gene expression is closely linked to thermophily (Pysz et al., 2004; Wilson et al., 2013).

Temperature-sensitive genes, such as heat shock proteins (HSPs), are upregulated in thermophiles under extreme temperatures at the transcriptional and proteomics levels. HSPs are chaperones, that play a crucial role in responding to heat stress and maintaining protein homeostasis by facilitating the proper folding of proteins that fail to achieve their required conformation at elevated temperatures (Mangrola et al., 2022). They are classified according to the molecular mass of their subunits as small Heat-Shock Proteins (sHSPs) and prefoldin. Prefoldin is only found in archaea and eukaryotes, however, small heat-shock proteins are the universal chaperone proteins. Some of the sHSPs are Hsp10 (GroES), Hsp27 (sHsp), Hsp40 (DnaJ), Hsp60 (Chaperonin, GroEL), Hsp70 (DnaK), Hsp90 (HtpG), and Hsp104 (ClpB). sHSPs are dormant and exist in their natural form as oligomeric structures under normal physiological conditions. On the other hand, the bacteria are exposed to extreme temperatures, and the oligomeric structures of sHsps separate into

small oligomers (possibly dimers). In the presence of denatured proteins, separated sHsps produce a bulky stable complex to shield the visitor protein from mass formation (Wang et al., 2013; Mangrola et al., 2022).

Why Thermophilic Enzymes are the Preferred Choice for Industrial Applications?

Thermophilic enzymes, also known as thermozymes, exhibit exceptional stability not only at extreme temperatures but also in the presence of denaturants, extreme pH levels, and high concentrations of heavy metals. Recent studies have identified key factors contributing to this enhanced stability. A significant feature of thermozymes is their higher number of disulfide bridges than mesophilic enzymes, which provides additional structural stabilization (Bezsudnova et al. 2012). In thermophilic organisms, the enzyme disulfide oxidoreductase plays a crucial role in the formation and maintenance of these disulfide linkages within intracellular proteins, acting as a primary molecular mechanism for protein stabilization. Disulfide bridges are thought to stabilize proteins primarily through entropic effects by reducing the entropy of the protein's unfolded state. Recent characterizations of disulfide bridge-containing proteins that remain optimally active and stable at temperatures exceeding 100 °C indicate that disulfide bridges can serve as a stabilization strategy under extreme conditions. The conformational environment and solvent accessibility of the disulfide bridges are key factors in protecting them from degradation, further enhancing protein stability (Vieille and Zeikus, 2001).

The differences in amino acid composition between mesophilic and thermophilic proteins reflect adaptations that help thermophilic proteins maintain stability and functionality at higher temperatures. The thermophilic enzymes have high levels of arginine, aspartate, and glutamate. Arginine forms multiple hydrogen bonds and salt bridges, enhancing protein stability. Aspartate and glutamate, being negatively charged, contribute to electrostatic interactions that help maintain the protein's structure in high-temperature environments. In addition, the thermophilic enzymes prefer Alanine (Ala), Threonine (Thr), Arginine (Arg), and Glutamate (Glu) over Glycine (Gly), Serine (Ser), Lysine (Lys), and Aspartate (Asp), respectively. These amino acid preferences enhance the structural rigidity, hydrogen bonding, and ionic interactions within thermophilic proteins, providing them with the necessary stability to function in high-temperature environments. Additionally, these enzymes contain a high concentration of acid-stable and hydrophobic amino

acids, which contribute to their stability in acidic conditions. Theoretically, the thermostability of enzymes can be assessed by calculating the free energy difference between their folded and unfolded states. Extremozymes exhibit a lower rate of unfolding and a higher rate of refolding, which increases their melting temperature (Tm) and reduces the thermal inactivation rate. As a result, these enzymes are both thermodynamically and kinetically stabilized at high temperatures (Kumar et al., 2000; Szilagyi and Zavodszky, 2000; Vieille and Zeikus, 2001)

Thermophilic enzymes exhibit several structural features that confer enhanced thermal stability, rigidity, and resistance to chemical denaturation. These features include a high frequency of salt bridges, a compact hydrophobic core, increased surface ion pairs, additional hydrogen bonding, and shorter loop regions. Due to these characteristics, the enzymes have greater thermal stability, rigidity, and robustness, making them resistant and active even in harsh environments such as denaturating solvents and surfactants. Moreover, molecular packing plays a significant role in the thermostability of these enzymes, often achieved through the presence of additional α-helices in their structures. This increased structural density further contributes to their ability to maintain function under extreme conditions (Charron et al., 2000).

Consequently, thermozymes offer immense potential thanks to their exceptional activity and stability across a broad range of temperatures and pH levels. They are highly resistant to extreme conditions, including high concentrations of organic solvents, surfactants, and salts, making them particularly well-suited for challenging industrial processes. Conducting industrial reactions at elevated temperatures provides numerous advantages, such as increased solubility of polymeric substrates, reduced viscosity, enhanced bioavailability, faster reaction rates, and a lower risk of microbial contamination. Additionally, these enzymes serve as valuable models for studying thermostability and thermal activity, providing insights that are instrumental in protein engineering (Atalah et al., 2019).

Conclusion

The use of enzymes as catalysts offers a greener alternative to traditional chemical catalysts by minimizing the generation of harmful byproducts, thereby significantly reducing the environmental impact of industrial processes. The preference for enzymes derived from microorganisms possesses many advantages compared to those derived from

animals and plants. One advantage is that microbial enzymes can be produced more rapidly in bacteria due to their fast cell division rates. This allows for easier scalability of enzyme production in large quantities, significantly reducing the time required for enzyme synthesis. Among microbial enzymes, enzymes from thermophiles have a great position because of their resistivity to denaturing agents, organic solvents, extreme pH and temperature, high reaction rate, and convenience for purification processes. With the ongoing discovery and development of enzymes from thermophiles that can operate under diverse conditions, the potential for biocatalysts in various industrial applications continues to grow. Studying thermophiles and their enzymes is crucial as it holds the promise of transforming mankind's environmental footprint, paving the way for a more sustainable model of industrial production, because it allows performing almost all industrial processes in harsh conditions.

Acknowledgements

This study was supported by Giresun University, Scientific Research Projects Commission Presidency due to the Project coded as 'FEN-BAP-A-270220-37'.

REFERENCES

Adrio, J.L., Demain, A.L. (2014). Microbial enzymes: Tools for biotechnological processes. *Biomolecules*, 4(1), 117-139.

Ajeje, S.B., Hu, Y., Song, G., Peter, S.B., Afful, R.G., Sun, F., Asadollahi, M.A., Amiri, H., Abdulkhani, A., Sun, H. (2021) Thermostable cellulases / xylanases from thermophilic and hyperthermophilic microorganisms: current perspective. *Frontier Bioengineering and Biotechnology*, 9, 794304.

Akpinar, Z., Karaoglu, H. (2024). Characterization of a highly thermostable recombinant xylanase from *Anoxybacillus ayderensis*. *Protein Expression and Purification*, 219,106478.

Artifon, S.E.S., Sumny, E.H., Fabris, T., de Jesus, B.A.P., Magalhães, M.D.B., et al. (2023). Biosynthesis of the acid protease produced by *Lacticaseibacillus casei* LBC 237 and *Limosilactobacillus fermentum* LBF 433 and their potential application in the bovine milk clotting. *Food Bioscience*, 54, 102879.

Atalah, J., Cáceres-Moreno, P., Espina, G., Blamey, J.M. (2019). Thermophiles and the applications of their enzymes as new biocatalysts. *Bioresource Technology*. 280, 478-488.

Basu, B. (2022). The radiophiles of Deinococcaceae family: Resourceful microbes for innovative biotechnological applications. *Current Research in Microbial Sciences*, 3(3), 100153.

Bertoldo, C., Antranikian, G. (2002). Starch-hydrolyzing enzymes from thermophilic Archaea and bacteria. *Current Opinion of Chemical Biology*, 6, 151-160.

Bertoldo, C., Dock, C., Antranikian, G. (2004) Thermoacidophilic microorganisms and their novel biocatalysts. *Engineering of Life Sciences*, 4, 521-531.

Bezsudnova, E. Y., Boyko, K. M., Polyakov, K. M., Dorovatovskiy, P. V., Stekhanova, T. N. et al. (2012). Structural insight into the molecular basis of polyextremophilicity of short-chain alcohol dehydrogenase from the hyperthermophilic archaeon *Thermococcus sibiricus*. *Biochimie*, 94(12), 2628-2638.

Burkhardt, C., Baruth, L., Neele Meyer-Heydecke et al. (2024). Mining thermophiles for biotechnologically relevant enzymes: evaluating the potential of European and Caucasian hot springs. *Extremophiles*, 28(5).

Cary, S.C., McDonald, I.R., Barrett, J.E., Cowan, D.A. (2010).On the rocks: The microbiology of Antarctic Dry Valley soils, *Nature Reviews Microbiology*, 8(2), 129-138.

Charron, C., Talfournier, F., Isupov, M.N., Littlechild, J.A., Branlant, G., Vitoux, B., Aubry, A. (2000). The crystal structure of d-glyceraldehyde-3-phosphate dehydrogenase from the hyperthermophilic Archaeon *Methanothermus fervidus* in the presence of $NADP^+$ at 2.1 A°, *Journal of Molecular Biology*, 297, 481-500.

Che Hussian, C.H.A., Leong, W.Y. (2023). Thermostable enzyme research advances: a bibliometric analysis. *Journal of Genetic Engineering and Biotechnology*, 27, 21(1), 37.

Chen, Y., Nielsen, J. (2016). Biobased organic acids production by metabolically engineered microorganisms. *Current Opinion Biotechnology*, 37, 165-172.

Chinnathambi, A. (2015). Industrial important enzymes from alkaliphiles - An overview. *Biosciences Biotechnology Research Asia*, 12(3).
Cosme, F., Inês, A., Vilela, A. (2023). Microbial and commercial enzymes applied in the beverage production process. *Fermentation*. 9(4):38.
Daly, M.J. (2000). Engineering radiation-resistant bacteria for environmental biotechnology. *Current Opinion of Biotechnology*, 11(3), 280-285.
DasSarma, S., DasSarma, P. (2012). Halophiles. *Encyclopedia of life sciences (London: Wiley*).
DasSarma, S., DasSarma, P. (2015). Halophiles and their enzymes: negativity put to good use. *Current Opinion in Microbiology*, 25, 120-126.
de Lourdes Moreno, M., Pérez, D., García, M.T., Mellado, E. (2013). Halophilic bacteria as a source of novel hydrolytic enzymes. *Life (Basel)*, 3(1), 38-51.
Deming, J.W. (2002). Psychrophiles and polar regions. *Current Opinion in Microbiology*, 5(3), 301-309.
Drake, J.W. (2009). Avoiding dangerous missense: thermophiles display especially low mutation rates. *PLOS Genetics*, 5(6):e1000520.
Farias, S.T., Bonato, M.C. (2003). Preferred amino acids and thermostability. *Genetic and Molecular Research*, 2, 383-393.
Febriani, Aura, N., Kemala, P., Saidi, N., Iqbalsyah, T.M. (2020). Novel thermostable lipase produced by a thermo-halophilic bacterium that catalyses hydrolytic and transesterification reactions, *Heliyon*, 6(7).
Feller, G. (2013). Psychrophilic enzymes: from folding to function and biotechnology. *Scientifica (Cairo)*, 2013, 512840.
Fernandes, P. (2010) Enzymes in food processing: a condensed overview on strategies for better biocatalysis. *Enzyme Research*, 2010 (862537), 19.
Flores-Fernández, C.N., Cárdenas-Fernández, M., Lye, G.J., Ward, J.M. (2022). Synergistic action of thermophilic pectinases for pectin bioconversion into D-galacturonic acid. *Enzyme and Microbial Technology*, 160, 110071.
Gaisin, V.A., Kalashnikov, A.M., Grouzdev, D.S., Sukhacheva, M.V., Kuznetsov, B. B., Gorlenko, V.M. (2017). *Chloroflexus islandicus* sp. nov., a thermophilic filamentous anoxygenic phototrophic bacterium from a geyser. *International Journal of Systematic and Evolutionary Microbiology*, 67(5), 1381-1386.
Galante, Y.M., Formantici, C. (2003) Enzyme applications in detergency and in manufacturing ındustries. *Current Organic Chemistry*, 7, 1399-1422.
Gallo, G., Aulitto, M., Contursi, P., Limauro, D., Bartolucci, S., Fiorentino, G. (2021). Bioprospecting of extremophilic microorganisms to address environmental pollution. *Journal of Visualized Experiments* (178), e63453,
Gaspar, L.M., Machado, A., Coutinho, R., Sousa, S., Santos, R., Xavier, A., et al. (2019). Development of potential yeast protein extracts for red wine clarification and stabilization. *Frontier Microbiology*, 10, 2310.
Gomes, J., Steiner, W. (2004). The biocatalytic potential of extremophiles and extremozymes. *Food technology and Biotechnology*, 42(4), 223-235.
Greenwood, J.C. (2014). Unleashing the promise of biotechnology to help heal, fuel, and feed the world, Editor(s): Craig Shimasaki, *Biotechnology Entrepreneurship, Academic Press*, Chapter 1, 3-13.
Gupta, G., Srivastava, S., Khare, S. K., & Prakash, V. (2014). Extremophiles: An overview of microorganism from extreme environment. *International Journal of Agriculture Environment and Biotechnology*, 7(2), 371-380.

Gurung, N., Ray, S., Bose, S., Rai, V. (2013). A broader view: Microbial enzymes and their relevance in industries, medicine, and beyond. *BioMed Research International*, 1-18.

Hamdan, S.H., Maiangwa, J., Ali, M.S.M., Normi, Y.M., Sabri, S., Leow, T.C. (2021). Thermostable lipases and their dynamics of improved enzymatic properties. *Applied Microbiology and Biotechnology*, 105(19), 7069-7094.

Hasan, F., Shah, A.A., Javed, S., Hameed, A. (2010) Enzymes used in detergents: Lipases. *African Journal of Biotechnology*, 9(31), 4836-4844.

Heckmann, C.M., Paradisi, F.(2020). Looking Back: A short history of the discovery of enzymes and how they became powerful chemical tools. *ChemCatChem*, 16, 12(24), 6082-6102.

Horikoshi, K. (1999). Alkaliphiles: some applications of their products for biotechnology. *Microbiology and Molecular Biology Reviews*, 63(4), 735-50,

Imran, M., Bano, S., Nazir, S., Javid, A., Asad, M.J., Yaseen, A. (2019). Cellulases production and application of cellulases and accessory enzymes in pulp and paper industry: A Review. *PSM Biological Research*, 4(1), 29-39.

Indriati, G., Megahati S., R. R. P. (2023). Exploration of thermophilic bacteria: Systematic literature review. *Jurnal Penelitian Pendidikan IPA*, 9(8), 411-416.

Janeeshma, E., Habeeb, H., Sinha, S., Arora, P., Chattaraj, S., et al. (2024).Enzymes-mediated solid waste management: A sustainable practice for recycling, *Waste Management Bulletin*, 1(4), 104-113.

Jiang, L., Lin, M., Li, X., Cui, H., Xu, X., Li, S., Huang, H. (2013). Genome sequence of *Thermus thermophilus* ATCC 33923, a thermostable trehalose-producing strain. *Genome Announces*, 1e00493-e00513.

Karan, R., Capes, M. D., DasSarma, S. (2012). Function and biotechnology of extremophilic enzymes in low water activity. *Aquatic Biosystems*, 8(1), 4.

Khan, U., Selamoglu, Z. (2020). Use of Enzymes in Dairy Industry: A review of current progress. *Archives of Razi Institute*, 75(1), 131-136.

Kim, H.J., Yoo, S.H. (2020). Effects of combined α-amylase and endo-xylanase treatments on the properties of fresh and frozen doughs and final breads. *Polymers (Basel)*, 15,12(6),1349.

Kochhar, N., Shrivastava I.K.K., Ghosh, A., Rawat, V.S., Sodhi, K.K., Kumar, M. (2022). Perspectives on the microorganism of extreme environments and their applications. *Current Researches in Microbial Sciences*, 21(3), 100134.

Kreil, D.P., Ouzounis, C.A. (2001). Identification of thermophilic species by the amino acid compositions deduced from their genomes. *Nucleic Acids Research*, 29, 1608-1615.

Kumar, C.G., Takagi, H. (1999). Microbial alkaline proteases: from a bioindustrial viewpoint. *Biotechnology advances*; 17, 561-594.

Kumar, S., Tsai, C.H., Nussirov, R. (2000). Factor enhancing protein thermostability. *Protein Engineering*, 13(3), 179-181.

Kim, J., Dordick, J.S. (1997). Unusual salt and solvent dependence of a protease from an extreme halophile. *Biotechnology and Bioengineering*, 55, 471-479.

Laemthong, T., Lewis, A.M., Crosby, J.R., Bing, R.G., Schneider, W.H., et al. (2022). Enzymes from extremely thermophilic bacteria and Archaea: current status and future prospects, Editor(s): Arora, N.K., Agnihotri, S., Mishra, J., Extremozymes and Their Industrial Applications. *Academic Press*, Chapter 2, 41-84.

Liu, L., Wang, L., Zhang, Z., Wang, S., Chen, H. (2012). Effect of codon message on xylanase thermal activity. *Journal of Biological Chemistry*, 287, 27183-27188.

Madern, D., Ebel, C., Zaccai, G. (2000). Halophilic adaptation of enzymes. *Extremophiles*, 4, 91-98.

Mangrola, A., Dudhagara, P., Koringa, P., Joshi, C. G., Parmar, M., Patel, R. (2015). Deciphering the microbiota of Tuwa hot spring, India using shotgun metagenomic sequencing approach. *Genomics Data*, 4, 153-155.

Mangrola, A., Patel, R.K., Dudhagara, P., Gandhi, H., Ghelani, A., et al. (2022). Thermophiles: Physiology, metabolism, enzymology, and adaptation mechanisms. *IGI Global*, Chapter 4, 65-93. Mittal, R., Mittal, C.G., (2013). Impact of population explosion on environment. *The National* Journal, 1(1), 5.

Miroshnichenko, M.L., Bonch-Osmolovskaya, E. A. (2006). Recent developments in the thermophilic microbiology of deep-sea hydrothermal vents. *Extremophiles*, 10(2), 85-96.

Mitra, D., Panneerselvam, P., Mohapatra, P.K.D., Pellegrini, M., Selvakumar, G. (2024). Emerging frontiers in microbial-mediated utilization of crop residues for economically valuable biomaterials. *Current Research in Microbial Sciences*, 9(6), 100225.

Naik, B., Kumar, V., Goyal, S.K, Dutt, T.A., Mishra, S., Joakim, S.P.E., et al. (2023). Pullulanase: unleashing the power of enzymes with a promising future in the food industry. *Frontier Bioengineering and Biotechnology*, 28(11), 1139611.

Narinx, E., Baise, E., Gerday, C. (1997). Subtilisin from psychrophilic antarctic bacteria: characterization and site-directed mutagenesis of residues possibly involved in the adaptation to cold. *Protein Engineering*, 10(11), 1271-1279.

Neifar, S., Hlima, H.B., Mhiri, S., Mezghani, M., Bouacem, K., Ibrahim, A.H., et al. (2019). A novel thermostable and efficient Class II glucose isomerase from the thermophilic *Caldicoprobacter algeriensis*: Biochemical characterization, molecular investigation, and application in high fructose syrup production, *International Journal of Biological Macromolecules*, 129, 31-40.

Pal, A., Khanum, F. (2011). Efficacy of xylanase purified from *Aspergillus niger* DFR-5 alone and in combination with pectinase and cellulase to improve yield and clarity of pineapple juice. *Journal of Food Science and Technology*, 48(5), 560-8.

Panda, M.K., Sahu, M.K., Tayung, K. (2013). Isolation and characterization of a thermophilic *Bacillus* sp. with protease activity isolated from hot spring of Tarabalo, Odisha, India. *Iranian Journal of Microbiology*, 5(2), 159-165.

Pivarnik, L.F., Senegal, A.G., Rand, A.G. (1995). Hydrolytic and transgalactosil activities of commercial b-galactosidase (lactase) in food processing. *Advances in Food and Nutrition Research*, 38, 1-102.

Pysz, M.A., Ward, D.E., Shockley, K.R., Montero, C.I., Conners, S.B., Johnson, M.R., Kelly, R.M. (2004). Transcriptional analysis of dynamic heat-shock response by the hyperthermophilic bacterium *Thermotoga maritima*. *Extremophiles*, 8, 209-217.

Qureshi, M.A., Khare, A.K., Pervez, A. (2015). Enzymes used in dairy industries. *International Journal of Applied Researches*, 1(10), 523-527.

Report of the World Commission on Environment and Development (1987). Our Common Future. *Oxford University Press*, Oxford, U.K.

Sabath, N., Ferrada, E., Barve, A., Wagner, A. (2013). Growth temperature and genome size in bacteria are negatively correlated, suggesting genomic

streamlining during thermal adaptation. *Genome Biology and Evolution*, 5, 966-977.

Sanka, I., Kusuma, A. B., Martha, F., Hendrawan, A., Pramanda, I. T., Wicaksono, A., Jati, A. P., Mazaya, M., Dwijayanti, A., Izzati, N., Maulana, M. F., Widyaningrum, A. R. (2023). Synthetic biology in Indonesia: Potential and projection in a country with mega biodiversity. *Biotechnology Notes*, 4, 41-48.

Seema, D., Bijender, K.B., Kumar, A., Tiwari, S.K., Singh, B. (2020). A review on biotechnological potential of multifarious enzymes in bread making, *Process Biochemistry*, 99, 290-306.

Serour, E., Antranikian, G. (2002). Novel thermoactive glucoamylases from the thermoacidophilic Archaea *Thermoplasma acidophilum*, *Picrophylus torridus* and *Picrophilus oshhimae*. *Antonie Van Leeuwenhoek*, 81, 73-83.

Sharma, A., Kawarabayasi, Y., Satyanarayana, T. (2012). Acidophilic bacteria and archaea: acid stable biocatalysts and their potential applications. *Extremophiles*, 16(1),1-19.

Sharma, N., Leung, I.K.H. (2021). Novel Thermophilic Bacterial Laccase for the Degradation of Aromatic Organic Pollutants. *Frontier Chemistry*, 9, 711345.

Sheldon, R.A., Woodley, J.M. (2017). Role of biocatalysis in sustainable chemistry, Chemical Reviews, 118(2).

Sheldon, R.A., Woodley, J.M., (2018). Role of biocatalysis in sustainable chemistry. *Chemical Reviews*, 118, 801-838.

Siddiqui, K.S., Williams, T.J., Wilkins, D., Yau, S., Allen, M. A., et al. (2013). Psychrophiles. *Annual Review of Earth and Planetary Sciences*, 41(1), 87-115.

Singh, G., Kaur, N.C., K. Puri, S., Sharma, P. (2016). Chapter 7 - Enzymes: applications in pulp and paper industry, Editor(s): Gurpreet Singh Dhillon, Surinder Kaur, Agro-Industrial Wastes as Feedstock for Enzyme Production, *Academic Press*, 2016, 157-172.

Singh, R., Kumar, M., Mittal, A., Mehta, P.K. (2016). Microbial enzymes: industrial progress in 21st century. *3 Biotech*, 6(2), 174. Szilagyi, A., Zavodszky, P. (2000). Structural differences between mesophilic, moderately thermophilic and extremely thermophilic protein subunits: results of a comprehensive survey. *Structure*, 8(5), 493-504.

Tamang, J.P., Watanabe, K., Holzapfel, W.H. (2016). Review: Diversity of microorganisms in global fermented foods and beverages. *Frontiers in Microbiology*, 7, 181961.

Teh, B.S., Abdul Rahman, A.Y., Saito, J.A., Hou, S., Alam, M. (2012). Complete genome sequence of the thermophilic bacterium *Thermus* sp. strain CCB-US3-UF1. *Journal of Bacteriology*, 194, 1240.

Thapa, S., Li, H., OHair, J., Bhatti, S., Chen, F.C., Nasr, K.A., Johnson, T., Zhou, S. (2019). Biochemical characteristics of microbial enzymes and their significance from industrial perspectives. *Molecular Biotechnology*, 61(8), 579-601.

Twala, P.P., Mitema, A., Baburam, C., Feto, N.A. (2020). Breakthroughs in the discovery and use of different peroxidase isoforms of microbial origin. *AIMS Microbiology*, 6(3), 330-349.

Ullah, I., Khan, M.S., Khan, S.S., Ahmad, W., Zheng, L., Shah, S.U.A., Ullah, M., Iqbal, A. (2021). Identification and characterization of thermophilic amylase

producing bacterial isolates from the brick kiln soil. *Saudi Journal of Biological Sciences*, 28(1), 970-979.
Uluçay, O. (2023). Microorganisms and diversity of bacteria. In: Akpınar, A. (ed.), *Research on Mathematics and Science*. Özgür Publications.
Van den Burg, B. (2003). Extremophiles as a source for novel enzymes. *Current Opinion in Microbiology*, 6(3), 213-218.
van Wolferen, M., Ajon, M., Driessen, A.J., Albers, S.V. How hyperthermophiles adapt to change their lives: DNA exchange in extreme conditions. *Extremophiles*, 17, 545-563, 2013.
Verma, A., M.W., Ansari, M.S. Anwar, R. Agrawal, Agrawal. S. (2014). Alkaline protease from *Thermoactinomyces* sp. RS1 mitigates industrial pollution. *Protoplasma*, 251, 711-718.
Verma, A., Singh, H., Anwar, S., Chattopadhyay, A., Tiwari, K.K., et al. (2017). Microbial keratinases: industrial enzymes with waste management potential. *Critical Reviews in Biotechnology*, 37(4),476-491,
Vettone, A., Perugino, G., Rossi, M., Valenti, A., Ciaramella, M. (2014). Genome stability: recent insights in the topoisomerase reverse gyrase and thermophilic DNA alkyltransferase. *Extremophiles*, 18, 895-904.
Vidmar, B., Vodovnik, M. (2018). Microbial keratinases: Enzymes with promising biotechnological applications. *Food Technology and Biotechnology*, 56(3), 312-328.
Vieille, C., Zeikus, G.J. (2001). Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability. *Microbiology and Molecular Biology Reviews*, 65(1):1-43.
Wang, G., Du, Y., Ma, X., Ye, F., Qin, Y., Wang, Y., Xiang, Y., Tao, R., Chen, T. (2022). Thermophilic nucleic acid polymerases and their application in xenobiology. *International Journal of Molecular Sciences*, 23(23), 14969.
Wang, H.C., Xia, X., Hickey, D. (2006). Thermal adaptation of the small subunit ribosomal RNA gene: a comparative study. *Journal of Molecular Evolution*, 63, 120-126.
Wang, Q., Cen, Z., Zhao, J. (2015). The survival mechanisms of thermophiles at high temperatures: An angle of omics. *Physiology*, 30, 97-106.
Wilkinson, M.G., Kilcawley, K.N. (2005). Mechanisms of incorporation and release of enzymes into cheese during ripening, *International Dairy Journal*, 15(6), 817-830.
Wilson, C.M., Yang, S., Rodriguez, M., Ma, Q., Johnson, C.M., Dice, L., Xu, Y., Brown, S.D. (2013). *Clostridium thermocellum* transcriptomic profiles after exposure to furfural or heat stress. *Biotechnology and Biofuels*, 6, 131.
Woodley, J.M., Breuer, M., Mink, D. (2013). A future perspective on the role of bioprocesses for chemical production. *Chemical Engineering Research and Design*, 91, 2029-2036.
Xiang, Q., Xia, Y., Fang, S., Zhong, F. (2024). Enzymatic debittering of cheese flavoring and bitterness characterization of peptide mixture using sensory and peptidomics approach. *Food Chemistry*, 15, 440:138229.
Xu, R., Wu, K., Han, H., Ling, Z., Chen, Z., Liu, P., et al. (2018). Co-expression of YieF and PhoN in *Deinococcus radiodurans* R1 improves uranium bioprecipitation by reducing chromium interference. *Chemosphere* 211, 1156-1165.

Xu, Y., Xuan, X., Gao, R., Xie, G. (2023). Increased expression levels of thermophilic serine protease TTHA0724 through signal peptide screening in *Bacillus subtilis* and applications of the enzyme. *International Journal of Molecular Sciences*, 3, 24(21), 15950.

Xu, Y., Wang, J., Liu, Q., Zhang, Q., Wu, J., Zhou, M., Nie, Y., Wu, X.L. (2023). pH and nitrate drive bacterial diversity in oil reservoirs at a localized geographic scale. *Microorganisms*, 11(1), 151.

Zelenkina, T., Eshinimayev, B.T., Dagurova, O., Suzina, N., Namsarayev, B.B., Trotsenko, Y. (2009). Aerobic methanotrophs from the coastal thermal springs of Lake Baikal. *Microbiology*, 78(4), 492-497.

Zuliani, L., Serpico, A., De Simone, M., Frison, N., Fusco, S. (2021). Biorefinery gets hot: thermophilic enzymes and microorganisms for second-generation bioethanol production. *Processes*, 9, 1583.

ANTIOXIDANT ENZYMES IN BIOTECHNOLOGY

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Abstract

Live organisms generate unstable and very reactive compounds known as free radicals as part of their regular metabolic activities. Free radicals can react with biological molecules and denature them. This can result in the depletion of crucial cellular structures and functions. Antioxidant enzymes provide in the body a safeguarding function against the possible harm induced by free radicals. Key antioxidant enzymes widely employed in industry are catalases, superoxide dismutases, and ascorbate peroxidases. These enzymes, which can be easily obtained from renewable natural resources, have become biological and environmentally friendly alternatives to chemical catalysts in industrial products and processes thanks to biotechnological developments. The present chapter provides a comprehensive examination of many antioxidant enzymes, namely catalases, superoxide dismutases, ascorbate peroxidases, and glutathione peroxidases, together with their respective industrial uses.

Introduction

Enzymes are an essential part of the metabolic processes of organisms; they catalyze and regulate the countless reactions that are essential for the functioning of cells. They are biological catalysts, so-called biocatalysts, which accelerate biological processes in organisms. Additionally, it is possible to isolate enzymes from cells that can catalyze several significant commercial processes (Robinson, 2015). The food, animal feed, agricultural, paper, leather, and textile industries provide advantageous prospects for the use of enzyme technology due to the presence of biomolecules in both products and raw materials that can be generated, destroyed, or altered by enzymatic processes. Enzymes are used in many different industries, *e.g.* in engineering, food production, animal nutrition, cosmetics, pharmaceuticals and as research and development tools. Currently, about 4000 enzymes are known, of which about 200 are of microbial origin and are used in industry. However, only about 20 enzymes are produced industrially (Li et al., 2012). Biological, enzymatic and environmentally friendly alternatives to chemical catalysts have become possible thanks to biotechnological progress. Such advancements have facilitated the application of enzymes in several sectors (Beilen and Li, 2002). Commercial enzymes are widely available and about 90 % of them are biotechnologically produced. Enzyme stability, purity, and

reusability have seen significant improvement mostly due to biotechnological methods (Adrio and Demain, 2014). Advancements in enzyme bioengineering employing biotechnology have led to a rise in the number of industrial enzymes, as well as significant improvements in efficiency and production (Fasim et al., 2021).

During regular metabolic processes, the organism produces highly reactive substances known as free radicals. These molecules have a lone pair of electrons, which makes them inherently unstable and extremely reactive. They can denature biological molecules (proteins, carbohydrates and lipids) by reacting with them. This leads to the loss of essential cellular structures and functions, which in turn causes a range of clinical diseases. Enzymes with antioxidant properties can either stabilize or remove free radicals prior to their detrimental effects on biological components.

To make free radicals more stable, antioxidant enzymes either lower their energy or donate some of their electrons to them. Furthermore, they can mitigate the injury induced by free radicals by halting the oxidative cascade (Krishnamurthy and Wadhwani, 2012). Minimizing the interaction with free radicals and augmenting the intake of foods rich in antioxidant enzymes or supplements containing antioxidant enzymes are crucial strategies to mitigate the adverse effects of free radicals. Therefore, antioxidant enzymes are crucial for maintaining the best possible cellular condition and are being studied by many researchers.

This chapter presents a comprehensive account of significant antioxidant enzymes that participate in the process of free radical scavenging and its practical uses in industry. Catalases, superoxide dismutase, and ascorbate peroxidases are the most often employed commercial antioxidant enzymes.

Catalases

The enzyme catalase (EC 1.11.1.1.6) is a member of the oxidoreductase family. The conversion of hydrogen peroxide (H_2O_2) into oxygen and water is of crucial importance for the cellular defense mechanisms against oxidative stress (reaction 1). Furthermore, catalase plays a role in the antioxidant reaction system and maintains the cellular redox equilibrium. In addition to its ubiquitous presence in both anaerobic and aerobic species, this enzyme finds

application in several sectors such as food processing, paper and textile, medicines, and bioremediation (Hussein, 2012).

$$2H_2O_2 \rightarrow H_2O + O_2 \qquad (1)$$

Catalases are divided into four types according to their physical and biochemical properties. Single-function heme catalases are found in many different animals, plants and microorganisms. A prosthetic heme group is present in the catalytic center of these catalases, which have a molecular mass ranging from 200 to 340 kDa (Lee et al., 2007). Less prevalent and mostly present in aerobic bacteria, the second category of enzymes, known as catalase peroxidases, has a molecular weight ranging from 120 to 340 kDa (Chelikani et al., 2004). These enzymes are similar to the peroxidases of plants and fungi but are not present in animals and plants. According to Zamocky et al. (2008), these bifunctional catalases exhibit both catalytic and peroxidative activities. The third category consists of a small family of bacterial proteins, the so-called non-heme catalases, which have an active di-manganese center instead of heme. Manganese catalases or pseudocatalases refer to enzymes defined as having a molecular weight ranging from 170 to 210 kDa (Barynin et al., 2001). The fourth group contains several proteins characterized by low catalytic activity and insufficient heme content. Bifunctional enzymes, including catalase-phenoloxidases, bromoperoxidases, and chloroperoxidases, are classified within this category (Vetrano et al., 2005). Typically, catalases are located in the cytoplasm of prokaryotes and in the peroxisomes of eukaryotes. Numerous higher plants and animals contain this enzyme in various forms (Yang et al., 2013; Zhang et al., 2013). Given their benefits of fast growth, simple processing, and straightforward genetic modification for specific product characteristics, microorganisms are gaining popularity as a source for enzyme synthesis (Puri et al., 2012).

Catalases are important enzymes on the global enzyme market and are used in many industrial processes. They are widely used in the production of biosensors, food processing and packaging, environmental remediation and bioremediation, biotransformation, biomedical applications and textiles (Dana and Bauder, 2011).

Electrochemical biosensors for a number of applications have been fabricated using biosensor technology and catalase (Prakash et al., 2009; Vatsyayan et al., 2010). Yıldız et al. (2004) used gelatin to immobilize catalase

on a dissolved oxygen membrane to create a biosensor system for quantifying hydrogen peroxide degradation in milk samples. Akbayırlı and Akyılmaz (2007) devised an amperometric biosensor to detect glucose by selectively activating catalase with glucose. This method enabled a reaction time of 2 minutes and a detection range spanning from 0.5 to 5.0 mM.

Within the paper, pulp, and textile sectors, hydrogen peroxide is a frequently employed bleaching agent that necessitates removal following bleaching due to its interference with the subsequent dyeing process (Poulsen et al., 2000). The widely used herbicide atrazine occurs in the environment and is often found in groundwater, ponds, rivers and streams. The variation in catalase activity in fish organs serves as a valuable biomarker for identifying atrazine in water bodies, since it perturbs catalase activity in different organs of aquatic species (Nwani et al., 2010).

The enzyme catalase-peroxidase from purified *Bacillus pumilus* was employed in a separate investigation to convert pharmacophores, including β-lactams, into their respective sulfoxides. Accordingly, a biocatalytic method for selective oxidation was devised with the potential to be employed in the treatment of bacterial diseases (Sangar et al., 2012). Furthermore, the targeted administration of catalase through galactosylation, pegylation, mannosylation, and succinylation has demonstrated significant efficacy in inhibiting the proliferation of metastatic cancers (Nishikawa et al., 2005).

Catalase is also used in the food industry for a number of tasks, e.g. in cheese production, food packaging and milk quality assessment. According to Geciova et al. (2002), catalase is particularly useful in preventing oxidative damage to food and improving product quality. In addition to food packaging, active packaging, also known as intelligent packaging, is used for the packaging of pharmaceuticals and numerous other products that require a longer shelf life. This type of packaging preserves the quality and freshness of products by protecting them from harmful influences such as oxidation, moisture, temperature fluctuations and weather conditions (Nikola and Marco, 2015). Active packaging protects food and many other products from unfavorable conditions. This type of packaging uses oxygen absorption technology in food packaging or packaging materials based on glucose oxidase catalysts. The highly specialized enzyme glucose oxidase is found in the fungi *Penicillium* and *Aspergillus niger*. By the translocation of two hydrogen atoms from the CHOH group of glucose to the oxygen atom, it forms

glucono-1,5-lactone. During the spontaneous hydrolysis of the lactone, hydrogen peroxide is released and gluconic acid is formed. As the most dangerous by-product of this reaction is hydrogen peroxide, the enzyme catalase is used in food packaging to protect food from reactive oxygen (Grigoras, 2017).

Superoxide dismutases

The antioxidant enzyme superoxide dismutase (SOD, EC 1.15.1.1) harnesses the oxidation/reduction processes to remove oxygen radicals. Its response time is astoundingly rapid. The active center of the system consists of transition metal ions that facilitate these cycles. Singlet oxygen dismutase (SOD) transforms molecular oxygen into hydrogen peroxide (reaction 2). Such enzyme is ubiquitously present in microbes, plants, and mammals. Typically, it comprises metallic ions including zinc (Zn), copper (Cu), and manganese (Mn) (Younus, 2018).

$$2O_2^- + 2\,H^+ \rightarrow H_2O_2 + O_2 \qquad (2)$$

SODs have been detected in nearly all tissues of mammals and other species. Cu/Zn-SOD, a homo-dimeric isoform identified by Okado-Matsumoto and Fridovich (2001), is present in either extracellular areas (SOD3) or the intracellular cytoplasm (SOD1). Furthermore, SOD1 is present in the periplasmic pocket of bacteria (SOD-C), within the chloroplasts of plants, and in other cellular compartments including the nucleus, cytosol, and lysosome. Extracellular Superoxide Dismutase 3 (SOD3) enzymes with homotopy-forming properties and a high affinity for heparin have been detected in human plasma, cerebrospinal fluid, and the lymphatic system. Cu/Zn-SOD enzymes are present in plants in two distinct biochemical forms: cytosolic and chloroplastic.

All antioxidant enzymes can rapidly neutralize reactive oxygen species (ROS) (Culotta et al., 2006). Cu/Zn-SODs are essential for the dormant and aerobic growth stages of fungi and bacteria. The molecular weight separating SOD1 and SOD3 and their evolutionary history in animals are the two main differences between them. Since SOD is distributed in different locations, it is important to understand that O_2 is not membrane permeable. Detoxification of these superoxide radicals at the source of their generation is therefore crucial (Owsiak et al., 2010). The ability of SOD to inhibit several signaling pathways

responsible for protein degradation via peroxidation enables it to contribute to the delay of diseases such as diabetes, cancer, heart disease, and neurological disorders. Additionally, it helps to prevent premature tissue aging and maintain the quality of food (Lu et al., 2018). Environmental stressors such as drought, high temperatures, flooding, heavy metal pollution and macronutrient deficiencies often cause plants to produce more ROS and have less oxygen available.

SOD is often obtained by biochemical methods from non-plant sources such as pig, chicken and bovine serum and liver. It is also extracted from a variety of plant materials such as grains, fruits, seeds and vegetables. Large quantities have already been produced for commercial production (Bafana et al., 2011).

Several research papers have examined the application of SODs in the medical domain. SODs are commonly employed in the management of diabetes, cancer, cerebral injury, and various other medical conditions (Alateyah et al., 2022). Oxidative stress is a crucial mechanism that contributes significantly to diabetes problems. There exists a direct correlation between insulin resistance and diabetes complications and oxidative damage. Continual high blood sugar levels in diabetes stimulate the generation of ROS from many origins and weaken the antioxidant defense mechanism. SOD also demonstrates effectiveness in this condition (Du et al., 2003). Given that free radicals are a primary factor in the process of aging, SOD is regarded as an enzyme that counteracts aging phenomena. In this case, biological molecules such as the mitochondria, which are the actual target of the attack, are oxidatively damaged. This damage leads to aging (Hekimi and Guarente, 2003). Changes in the gut microbiota and muscle mitochondrial dysfunction are thought to be due to causes such as oxidative stress. Antioxidants are able to maintain sperm motility. This improves sperm quality by preventing sperm apoptosis. In a study in which the sperm of a rooster was mixed with a 200 U/mL SOD diluent, sperm motility increased. In addition, the SOD-treated group showed improved plasma membrane integrity and a lower rate of sperm apoptosis after thawing (Partyka et al., 2013).

Human skin ages and is damaged when exposed to direct oxygen. SOD has protective properties against radiation and sun exposure and is also a powerful scavenger of free oxygen radicals. SOD is an enzyme that also has significant anti-inflammatory properties. The toxicity of superoxide dismutase

has been studied in detail by scientists. It has been shown to have no negative effects in many medical applications (Na et al., 2007). Studies on cancer have shown that SOD levels are increased and CuZn and Mn-SOD activities are decreased in cancer cells, which supports the phenotypic regression of cancer cells (Oberley, 2004). Furthermore, experiments have demonstrated that CuZn-SOD plays a crucial role in the therapy of multiple myeloma (Salem et al., 2015). Prostate cancer, a disease in which SOD2 expression is reduced, has been shown to respond well to SOD treatment (Thomas and Sharifi, 2012). Cystic fibrosis can also be effectively treated with SOD. Persistent inflammation and activated neutrophils are characteristic of this disease (De Rose, 2002).

Cystic fibrosis patients have been shown to have decreased plasma levels of SOD compared to unaffected persons (Madarasi et al., 2000). Rheumatoid arthritis, an autoimmune disease, can develop as a result of reduced antioxidant levels (Mahajan and Tandon, 2004). Studies on test animals with arthritis have demonstrated the anti-inflammatory effects of SOD and other antioxidants, including vitamin E. Furthermore, multiple case studies have demonstrated that the activity of superoxide dismutase (SOD) slows down the development of rheumatoid arthritis by decreasing the breakdown of lipids in the mitochondria (Karatas et al., 2003). This study by Ugur et al. (2004) shown that the delivery of SOD by liposomes effectively postponed the development of arthritis in experimental animals. Cu/Zn-SODs are used worldwide in wine technology for the detoxification of products. (Gamero-Sandemetrio et al., 2013).

Many functional foods, including mayonnaise and milk chocolate, have been enriched with SOD (Stephenie et al., 2020). However, during food production and transportation, the freshness retention effect of SOD can generate significant amounts of O_2^-, which readily interacts with food nutrients such as ascorbic acid and tocopherol. SOD acts as an antioxidant that effectively protects against the deterioration of food quality caused by peroxidation. Therefore, SOD is also referred to as a food preservative (Islam et al., 2022). SOD efficiency has emerged as a reliable metric for evaluating the quality of yogurt fermentation. With a relatively high SOD value, *Lactobacillus plantarum subsp. plantarum* F8 is used as a probiotic for yogurt fermentation (Bhatti et al., 2022). The dairy industry aims to improve dairy products by adding SOD (Selamoglu, 2020). Similarly, SODs in packaged

dairy products have been shown to be powerful antioxidants; their effectiveness is further enhanced when paired with CAT. These enzymes have been used by many multinational manufacturers, including Nestle, SmithKline Beecham and Amul, for the biotechnological improvement of dairy products (Zaheer and Gupta, 2019).

Ascorbate Peroxidases

The ascorbate peroxidases (APX) are classified as class I peroxidases within the heme peroxidase subfamily of non-animal peroxidases (Shigeoka et al., 2002). Ascorbate peroxidase (APX; EC 1.11.1.11) catalyzes the hydrolysis of H_2O_2 by utilizing ascorbate as an electron donor (reaction 3).

$$H_2O_2 + 2\ \text{L-ascorbate} \rightarrow H_2O + 2\ \text{monodehydroascorbate} \quad (3)$$

APXs are crucial enzymes that facilitate the detoxification of hydrogen peroxide in the cytosol and chloroplasts of plant cells, therefore safeguarding plants from oxidative stress (Asada, 1992). APXs facilitate the production of the monodehydroascorbate radical when hydrogen peroxide oxidizes ascorbate.

Depending on where they are in the cell, APXs can be classified into four different forms: soluble in the chloroplast stroma (sAPX), thylakoid-bound in the chloroplast (tAPX), cytosolic in the cytosol (cAPX) and membrane-bound in the glyoxysome (gmAPX) (Bunkelmann and Trelease, 1995).

Most plant species contain these enzymes, which have similar structures and functions. When ascorbate is used as an electron donor in the reduction of H_2O_2, the APXs exhibit a high degree of specificity. The ascorbate concentration within the cell has a major influence on APX activity. Furthermore, under certain circumstances, the cAPX isoform shows an affinity for additional electron donors such as guaiacol or pyrogallol, indicating the presence of a kinetically compatible binding site within this isoform (Lad et al., 2002).

H_2O_2 can be neutralized in plants by a variety of antioxidant enzymes, including catalases (CATs), ascorbate peroxidases (APXs), glutathione S-transferases (GSTs) and peroxiredoxins (PRXs). Each of these enzyme's functions according to a different mechanism (Foyer and Noctor, 2011). For

example, the primary enzyme catalase is responsible for the degradation of H_2O_2 in the peroxisomal matrix. Plant catalases have a low affinity for H_2O_2, with a Km value in the millimolar range, while APX has a much stronger affinity, as indicated by its lower Km value in the micromolar range (Ray et al., 2012).

The regulatory control of ROS levels in cells is mostly mediated via the Halliwell-Asada pathway, sometimes referred to as the ascorbate-glutathione pathway, which is mostly governed by APX enzymes. This process occurs in a variety of subcellular organelles but is particularly important in chloroplasts and mitochondria because photosynthesis and respiration produce ROS in these organelles (Jimenez et al., 1997).

The enzymes implicated in the ascorbate-glutathione cycle provide protection to plants against oxidative stress. An ascorbate peroxidase enzyme facilitates the oxidation of ascorbate to produce monodehydroascorbate (MDA). The regeneration of ascorbate from MDA is necessary to sustain the absorption of hydrogen peroxide in the chloroplasts. Stem cell MDA is converted by monodehydroascorbate reductase (MDAR). If reduced ferredoxin or MDAR are unable to convert MDA to ascorbate, it becomes dehydroascorbate (DHA). Dehydroascorbate reductase (DHAR) is the enzyme that produces ascorbate from DHA, although it plays a lesser role in ascorbate production than MDAR (Minkov et al., 1997).

APX, a crucial enzyme in the ascorbate-glutathione cycle (ASC-GSH), has been shown to be involved in and essential for numerous plant growth and development processes, including lateral root formation (Correa-Aragunde et al., 2013), nodule development (Keyster et al., 2011), leaf senescence (Ribeiro et al., 2017), seed germination (Huang et al., 2018) and programmed cell death (Caverzan et al., 2012; de Pinto et al., 2013). Zhang et al. (2022) demonstrated that transgenic maize plants that overexpress ZmAPX1 have enhanced resistance to *Bipolaris maydis* through the reduction of H_2O_2 levels and activation of the jasmonic acid signaling system. A study by Murgia et al. (2004) found that *Arabidopsis* plants that overexpress AtAPX exhibit enhanced tolerance to paraquat stress. The investigations underscore the significance of APX in preserving H_2O_2 equilibrium throughout extreme environmental circumstances. For instance, *Arabidopsis* mutants lacking APX2 have low heat stress tolerance during the seedling stage but elevated thermotolerance during the reproductive stage (Suzuki et al., 2013).

Previous research has demonstrated that plants have increased expression of APX in reaction to abiotic stresses including salt, strong light, drought, SO_2 and O_3 (Noctor and Foyer, 1998). Lopez et al. (1996) reported an elevation in APX activity in Raphanus sativus plants subjected to salt stress. Furthermore, a separate investigation revealed that the upregulation of APX in the chloroplasts of tobacco plants resulted in an improved capacity to endure both inadequate salt and water supply (Badawi et al., 2004).

Glutathione Peroxidases

An important antioxidant, glutathione peroxidase (E.C. 1.11.1.9), decomposes hydrogen peroxide in the mitochondria and sometimes in the cytosol to generate water. The primary determinant of its activity is selenium. It is classified into two groups: non-selenium-dependent species (GPX) and selenium-dependent species (GPX). The primary function of GPX is to safeguard the cell against oxidative stress (Cnubben et al., 2001).

Mammalian Glutathione Peroxidases

GPX is a crucial constituent of the antioxidant metabolism in animals. It is essential for the repair of biological membranes. The compounds GPX1-4, 7, and 8 exist as monomers, whereas GPX1-3, 5, and 6 are homotetramers (Imai and Nakagawa, 2003).

At least eight GPX enzymes are known, and the corresponding chromosomes for each of the GPX genes are 3, 14, 5, 19, 6, 6, 1 and 5. GPX1 is present in all tissues and is dependent on selenium. While GPX2 is predominantly present in the gastrointestinal tract, GPX3 is mostly expressed in the kidneys, and GPX4 has the ability to decrease phospholipid hydroperoxides. In response to oxidative stress, GPX has the capacity to modulate the apoptotic response. Both GPX5 and GPX6 lack selenium, while the human genome harbors the precisely characterized gene GPX6. Selenium is not required for GPX7 and GPX8 to scavenge hydrogen peroxide (Sanyal et al., 2009). Studies suggest that low GPX levels can cause problems for the antioxidant defense system. The presence of oxidative damage to membrane fatty acids and functional proteins suggests a compromised antioxidant defense mechanism, which can result in neurotoxic damage, neurodegeneration, and irreversible deficits (Chabory et al., 2009). Reduced GPX1 activity in individuals with coronary heart disease may be associated

with cardiovascular disease according to a 2003 study by Blankenberg et al. In addition, studies have shown that sperm DNA is damaged, sperm motility and sperm count decrease and GPX levels decrease in testicular tissue exposed to electric fields (Gandhi et al., 2012).

Plant Glutathione Peroxidases

Plant glutathione peroxidases (GPXs) are ubiquitously present in many plant tissues and ecological niches. Glutathione transferase (GST) isoenzymes in plants are commonly linked to the function of glutathione-dependent peroxidases. In different species and under different stress situations, these enzymes play a crucial role in detoxifying lipid hydroperoxides and other reactive compounds (Dixon et al., 2009). The plant GPX genes extracted from several plant taxa have a significant level of sequence similarity to animal phospholipid hydroperoxide glutathione peroxidases. With the exception of *Populus trichocarpa* GPX5, which interacts with Cd^{2+} ions, all plant GPXs that have been described are monomeric (Navrot et al., 2006; San Koh et al., 2007). The reduced enzymatic activity of plant GPX relative to mammalian GPX may be attributed to the substitution of selenocysteine with cysteine in the active regions. Furthermore, plant GPXs typically employ thioredoxin (Trx) as the reducing agent, rather than glutathione.

Hence, they are alternatively referred to as thioredoxin peroxidases. Moreover, it has been demonstrated that plant GPXs directly utilize Trx for the purpose of reducing hydroperoxides (Herbette et al., 2007). Some plant GPXs exhibit similar enzyme activity to thioredoxin and glutathione peroxidase, although the thioredoxin regeneration mechanism is far more efficient than the glutathione system (Herbette et al., 2002). Milla et al. (2003) reported that there is data indicating that plant gene product Xs (GPXs) can effectively decrease peroxides other than H_2O_2. Illustrative instances include lipid peroxides and organic hydroperoxides.

Conclusion

Advancements in protein engineering and a more precise discovery of enzymes, facilitated by modern biotechnology, have enabled the application of enzymes in many industries. A crucial function of antioxidant enzymes is to detoxify free radicals in organisms. These characteristics have captured the interest of scholars. By virtue of extensive research, these enzymes have been

integrated into diverse sectors. The three predominant antioxidant enzymes employed in industrial applications are catalase, superoxide dismutase, and ascorbate peroxidase. Numerous studies have demonstrated the significant relevance of these enzymes to a diverse array of businesses. Progressions in biotechnology have facilitated the large-scale production of these enzymes, therefore giving rise to novel domains of application.

REFERENCES

Adrio, J. L., & Demain, A. L. (2014). Microbial enzymes: tools for biotechnological processes. *Biomolecules*, 4(1), 117–139.

Akbayirli P., & Akylimaz E. (2007). Activation based catalase enzyme electrode and its usage for glucose determination. *Analytical Letters*, 40(18), 3360–3372.

Alateyah, N., Gupta, I., Rusyniak, R. S., & Ouhtit, A. (2022). SOD2, a potential transcriptional target underpinning CD44-promoted breast cancer progression. *Molecules*, *27*(3), 811.

Asada, K. (1992). Ascorbate peroxidase–a hydrogen peroxide-scavenging enzyme in plants. *Physiologia Plantarum*, 85(2), 235–241.

Badawi, G. H., Yamauchi, Y., Shimada, E., et al. (2004). Enhanced tolerance to salt stress and water deficit by overexpressing superoxide dismutase in tobacco (*Nicotiana tabacum*) chloroplasts. *Plant Science*, 166(4), 919–928.

Bafana, A., Dutt, S., Kumar, S., & Ahuja, P. S. (2011). Superoxide dismutase: an industrial perspective. *Critical Reviews in Biotechnology*, 31(1), 65–76.

Barynin V. V., Whittaker M. M., Antonyuk S.V. et al. (2001). Crystal structure of manganese catalase from *Lactobacillus plantarum*. *Structure*, 9(725), 38.

Beilen, J. B., & Li, Z. (2002). Enzyme technology: an overview. *Current Opinion in Biotechnology*, 13(4), 338–344.

Bhatti, J. S., Sehrawat, A., Mishra, J., et al. (2022). Oxidative stress in the pathophysiology of type 2 diabetes and related complications: Current therapeutics strategies and future perspectives. *Free Radical Biology and Medicine*, 184, 114–134.

Blankenberg, S., Rupprecht, H. J., Bickel, C., et al. (2003). Glutathione peroxidase 1 activity and cardiovascular events in patients with coronary artery disease. *New England Journal of Medicine*, 349(17), 1605–1613.

Bunkelmann J. R, & Trelease R. N. (1995). Molecular cloning and characterization of ascorbate peroxidase localized to the glyoxysome membranes of cotton cotyledons. *Plant Physiology*, 108, 8–67.

Caverzan, A., Passaia, G., Rosa, S. B., Ribeiro, C. W., Lazzarotto, F., & Margis-Pinheiro, M. (2012). Plant responses to stresses: role of ascorbate peroxidase in the antioxidant protection. *Genetics and Molecular Biology*, 35, 1011–1019.

Chabory, E., Damon, C., Lenoir, A., et al. (2009). Epididymis seleno-independent glutathione peroxidase 5 maintains sperm DNA integrity in mice. *The Journal of Clinical Investigation*, 119(7), 2074–2085.

Chelikani P., Fita I., & Loewen P. C. (2004). Diversity of structures and properties among catalases. *Cellular and Molecular Life Sciences*, 61, 192–208.

Cnubben, N. H., Rietjens, I. M., Wortelboer, H., van Zanden, J., & van Bladeren, P. J. (2001). The interplay of glutathione-related processes in antioxidant defense. *Environmental Toxicology and Pharmacology*, 10(4), 141–152.

Correa-Aragunde, N., Foresi, N., Delledonne, M., & Lamattina, L. (2013). Auxin induces redox regulation of ascorbate peroxidase 1 activity by S-nitrosylation/denitrosylation balance resulting in changes of root growth pattern in Arabidopsis. *Journal of Experimental Botany*, 64(11), 3339–3349.

Culotta, V. C., Yang, M., & O'Halloran, T. V. (2006). Activation of superoxide dismutases: putting the metal to the pedal. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 1763(7), 747–758.

Dana, L. D., & Bauder, J. W. (2011). A general essay on bioremediation of contaminated soil, *Montana State University, Bozeman, Mont, USA*.

de Pinto, M. C., Locato, V., Sgobba, A., Romero-Puertas, M. D. C., Gadaleta, C., Delledonne, M., & De Gara, L. (2013). S-nitrosylation of ascorbate peroxidase is part of programmed cell death signaling in tobacco Bright Yellow-2 cells. *Plant Physiology*, 163(4), 1766–1775.

De Rose, V. (2002). Mechanisms and markers of airway inflammation in cystic fibrosis. *European Respiratory Journal*, 19(2), 333–340.

Dixon, D. P., Hawkins, T., Hussey, P. J., & Edwards, R. (2009). Enzyme activities and subcellular localization of members of the *Arabidopsis* glutathione transferase superfamily. *Journal of Experimental Botany*, 60(4), 1207–1218.

Du, X., Matsumura, T., Edelstein, D., et al. (2003). Inhibition of GAPDH activity by poly (ADP-ribose) polymerase activates three major pathways of hyperglycemic damage in endothelial cells. *The Journal of Clinical Investigation*, 112(7), 1049–1057.

Fasim, A., More, V. S., & More S. S. (2021). Large-scale production of enzymes for biotechnology uses. *Current Opinion in Biotechnology*, 69, 68–76.

Foyer, C. H., & Noctor, G. (2011). Ascorbate and glutathione: the heart of the redox hub. *Plant Physiology*, 155(1), 2–18.

Gamero-Sandemetrio, E., Gómez-Pastor, R., & Matallana, E. (2013). Zymogram profiling of superoxide dismutase and catalase activities allows *Saccharomyces* and non-*Saccharomyces* species differentiation and correlates to their fermentation performance. *Applied Microbiology and Biotechnology*, 97, 4563–4576.

Gandhi, S., & Abramov, A. Y. (2012). Mechanism of oxidative stress in neurodegeneration. *Oxidative Medicine and Cellular Longevity*, 2012(1), 428010.

Geciova, J., Bury, D., & Jelen, P. (2002). Methods for disruption of microbial cells for potential use in the dairy industry-a review. *International Dairy Journal*, 12 (6), 541–553.

Grigoras, A. G. (2017). Catalase immobilization - A review. *Biochemical Engineering Journal*, 117, 1–20.

Hekimi, S., & Guarente, L. (2003). Genetics and the specificity of the aging process. Science, 299(5611), 1351–1354.

Herbette, S., Lenne, C., Leblanc, N., Julien, J. L., Drevet, J. R., & Roeckel-Drevet, P. (2002). Two GPX-like proteins from Lycopersicon esculentum and Helianthus annuus are antioxidant enzymes with phospholipid hydroperoxide glutathione peroxidase and thioredoxin peroxidase activities. *European Journal of Biochemistry*, 269(9), 2414–2420.

Herbette, S., Roeckel-Drevet, P., & Drevet, J. R., (2007). Seleno-independent glutathione peroxidases: More than simple antioxidant scavengers. *The FEBS journal*, 274(9), 2163–2180.

Huang, L., Jia, J., Zhao, X., et al. (2018). The ascorbate peroxidase APX1 is a direct target of a zinc finger transcription factor ZFP36 and a late embryogenesis abundant protein OsLEA5 interacts with ZFP36 to co-regulate OsAPX1 in seed germination in rice. *Biochemical and Biophysical Research Communications*, 495(1), 339–345.

Hussein, A. A. (2012). Purification and characterization of thermo-alkali stable catalase from *Bacillus sp*. *International Research Journal of Biotechnology*, 3(10), 207–214.

Imai, H., & Nakagawa, Y. (2003). Biological significance of phospholipid hydroperoxide glutathione peroxidase (PHGPx, GPx4) in mammalian cells. *Free Radical Biology and Medicine*, 34(2), 145–169.

Islam, M. N., Rauf, A., Fahad, F. I., et all. (2022). Superoxide dismutase: an updated review on its health benefits and industrial applications. *Critical Reviews in Food Science and Nutrition*, 62(26), 7282–7300.

Jimenez, A., Hernandez, J. A., del Rio, L. A., & Sevilla, F. (1997). Evidence for the presence of the ascorbate-glutathione cycle in mitochondria and peroxisomes of pea leaves. *Plant Physiology*, 114(1), 275–284.

Karatas, F., Ozates, I., Canatan, H., Halifeoglu, I., Karatepe, M., & Colak, R. (2003). Antioxidant status & lipid peroxidation in patients with rheumatoid arthritis. *Indian Journal of Medical Research*, 118, 178–181.

Keyster, M., Klein, A., Egbich, I., Jacobs, A., & Ludidi, N. (2011). Nitric oxide increases the enzymatic activity of three ascorbate peroxidase isoforms in soybean root nodules. *Plant Signaling & Behavior*, 6(7), 956–961.

Krishnamurthy, P., & Wadhwani, A. (2012). Antioxidant enzymes and human health. *Antioxidant Enzyme*, 1, 3–18.

Lad, L., Mewies, M., & Raven, E. L. (2002). Substrate binding and catalytic mechanism in ascorbate peroxidase: evidence for two ascorbate binding sites. *Biochemistry*, 41(46), 13774–13781.

Lee D. H., Oh D. C., Oh Y. S., Malinverni J. C., Kukor J. J., & Kahang, H. Y. (2007). Cloning and characterization of monofunctional catalase from photosynthetic bacterium *Rhodospirillum rubrum* S1. *Journal of Microbiology and Biotechnology*, 17(9), 1460–1468.

Li, S., Yang, X., Yang, S., Zhu, M., & Wang, X. (2012). Technology prospecting on enzymes: application, marketing and engineering. *Computational and Structural Biotechnology Journal*, 2(3), e201209017.

Lopez, F., Vansuyt, G., Casse-Delbart, F., & Fourcroy, P. (1996). Ascorbate peroxidase activity, not the mRNA level, is enhanced in salt-stressed *Raphanus sativus* plants. *Physiologia Plantarum*, 97(1), 13–20.

Lu, R., Zhang, T., Wu, D., He, Z., Jiang, L., Zhou, M., & Cheng, Y. (2018). Production of functional human CuZn-SOD and EC-SOD in bitransgenic cloned goat milk. *Transgenic Research*, 27, 343–354.

Madarasi, A., Lugassi, A., Greiner, E., Holics, K., Biró, L., & Mozsáry, E. (2000). Antioxidant status in patients with cystic fibrosis. *Annals of Nutrition and Metabolism*, 44(5-6), 207–211.

Mahajan, A., & Tandon, V. R. (2004). Antioxidants and rheumatoid arthritis. *Indian Journal of Rheumatology*, 12, 139–142.

Milla, M. A. R., Maurer, A., Huete, A. R., Gustafson, J. P. (2003). Glutathione peroxidase genes in *Arabidopsis* are ubiquitous and regulated by abiotic stresses through diverse signaling pathways. *The Plant Journal*, 36(5), 602–615.

Minkov, I. N., Jahoubjan, G. T., Denev, I. D., & Toneva, V. T. (1997). Photooxidative stress in higher plants. *Handbook of Plant and Crop Stress*, 2, 499–525.

Murgia, I., Tarantino, D., Vannini, C., Bracale, M., Carravieri, S., & Soave, C. (2004). *Arabidopsis thaliana* plants overexpressing thylakoidal ascorbate peroxidase show increased resistance to Paraquat-induced photooxidative stress and to nitric oxide-induced cell death. *The Plant Journal*, 38(6), 940–953.

Na, K., Kim, K. E., Park, S. T., & Kim, T. Y. (2007). EC-SOD suppresses contact hypersensitivity in mouse skin by impairing Langerhans cell migration. *Journal of Investigative Dermatology*, 127(8), 1930–1937.

Navrot, N., Collin, V., Gualberto, J., et al. (2006). Plant glutathione peroxidases are functional peroxiredoxins distributed in several subcellular compartments and regulated during biotic and abiotic stresses. *Plant Physiology*, 142(4), 1364–1379.

Nikola, L., & Marco, W. F. (2015). Catalases as biocatalysts in technical applications: current state and perspectives. *Applied Microbiology and Biotechnol*ogy, 99, 3351–3357.

Nishikawa M., Hyoudou K., Kobayashi Y., et al. (2005). Inhibition of metastatic tumor growth by targeted delivery of antioxidant enzymes. *Journal of Controlled Release*, 109(1–3), 101–107.

Noctor, G., & Foyer, C. H. (1998). Ascorbate and glutathione: keeping active oxygen under control. *Annual Review of Plant Biology*, 49(1), 249–279.

Nwani C. D., Lakra W. S., Nagpure N. S., Kumar R., Kushuwaha B., & Srivastava S. K. (2010). Toxicity of the herbicide atrazine: effects on lipid peroxidation and

activities of antioxidant enzymes in the freshwater fish *Channa punctatus*. *International Journal of Environmental Research and Public Health*, 7, 3298–3312.

Oberley, T. D. (2004). Mitochondria, manganese superoxide dismutase, and cancer. *Antioxidants and Redox Signaling*, 6(3), 483–487.

Okado-Matsumoto, A., & Fridovich, I. (2001). Subcellular distribution of superoxide dismutases (SOD) in rat liver: Cu, Zn-SOD in mitochondria. *Journal of Biological Chemistry*, 276(42), 38388–38393.

Owsiak, A., Bartosz, G., & Bilinski, T. (2010). Oxidative stress during aging of the yeast in a stationary culture and its attenuation by antioxidants. *Cell Biology International*, 34(7), 731–736.

Partyka, A., Niżański, W., Bajzert, J., Łukaszewicz, E., & Ochota, M. (2013). The effect of cysteine and superoxide dismutase on the quality of post-thawed chicken sperm. *Cryobiology*, 67(2), 132–136.

Poulsen, H. E., Jensen, B. R., Weimann, A. L. L. A. N., Jensen, S. A., Sorensen, M., & Loft, S. (2000). Antioxidants, DNA damage and gene expression. *Free Radical Research*, 33, S33–S40.

Prakash P. A., Yogeswaran U., & Chen S. M. (2009). A review on direct electrochemistry of catalase for electrochemical sensors. *Sensors* 9, 1821–1844.

Puri M., Sharma D., Barrow C., & Tiwary A. K. (2012). Optimization of novel method for the extraction of stevioside from *S. rebudiana* leaves. *Food Chem*istry, 132(3), 1113–1120.

Ray, M., Mishra, P., Das, P., Sabat, S. C. (2012). Expression and purification of soluble bio-active rice plant catalase-A from recombinant *Escherichia coli*. *Journal of Biotechnology*, 157(1), 12–19.

Ribeiro, C. W., Korbes, A. P., Garighan, J. A., Jardim-Messeder, D., Carvalho, F. E., Sousa, R. H., ... & Margis-Pinheiro, M. (2017). Rice peroxisomal ascorbate peroxidase knockdown affects ROS signaling and triggers early leaf senescence. *Plant Science*, 263, 55–65.

Robinson, P.K. (2015). Enzymes: principles and biotechnological applications. *Essays in Biochemistry*, 59, 1.

Salem, K., McCormick, M. L., Wendlandt, E., Zhan, F., & Goel, A. (2015). Copper–zinc superoxide dismutase-mediated redox regulation of bortezomib resistance in multiple myeloma. *Redox Biology*, 4, 23–33.

San Koh, C., Didierjean, C., Navrot, N., et al. (2007). Crystal structures of a poplar thioredoxin peroxidase that exhibits the structure of glutathione peroxidases: insights into redox-driven conformational changes. *Journal of Molecular Biology*, 370(3), 512–529.

Sangar, S., Pal, M., Moon L. S., & Jolly, R. S. (2012). A catalase–peroxidase for oxidation of β-lactams to their (R)-sulfoxides. *Bioresource Technology,* 115, 102–110.

Sanyal, J., Bandyopadhyay, S. K., Banerjee, T. K., et al. (2009). Plasma levels of lipid peroxides in patients with Parkinson's disease. *European Review for Medical & Pharmacological Sciences*, 13(2).

Selamoglu, Z. (2020). Use of enzymes in dairy industry: a review of current progress. *Archives of Razi Institute*, 75(1), 131.

Shigeoka, S., Ishikawa, T., Tamoi, M., et al. (2002). Regulation and function of ascorbate peroxidase isoenzymes. *Journal of Experimental Botany*, 53(372), 1305–1319.

Stephenie, S., Chang, Y. P., Gnanasekaran, A., Esa, N. M., & Gnanaraj, C. (2020). An insight on superoxide dismutase (SOD) from plants for mammalian health enhancement. *Journal of Functional Foods*, 68, 103917.

Suzuki, N., Miller, G., Sejima, H., Harper, J., & Mittler, R. (2013). Enhanced seed production under prolonged heat stress conditions in *Arabidopsis thaliana* plants deficient in cytosolic ascorbate peroxidase 2. *Journal of Experimental Botany*, 64(1), 253–263.

Thomas, R., & Sharifi, N. (2012). SOD mimetics: a novel class of androgen receptor inhibitors that suppresses castration-resistant growth of prostate cancer. *Molecular Cancer Therapeutics*, 11(1), 87–97.

Ugur, M., Yildirim, K., Kiziltunc, A., Erdal, A., Karatay, S., & Senel, K. (2004). Correlation between soluble intercellular adhesion molecule 1 level and extracellular superoxide dismutase activity in rheumatoid arthritis: a possible association with disease activity. *Scandinavian journal of rheumatology*, 33(4), 239–243.

Vatsyayan P., Bordoloi S., & Goswami P. (2010). Large catalase based bioelectrode for biosensor application. *Biophysical Chemistry*, 153, 36–42.

Vetrano A. M., Heck D. E., Mariano T. M., Mishin V., Laskin D. L., & Laskin J. D. (2005). Characterization of the oxidase activity in mammalian catalase. *Journal of Biological Chemistry*, 280(42), 35372–35381.

Yang J., Dong S., Jiang Q., Kuang T., Huang W., & Yang J. (2013). Changes in expression of manganese superoxide dismutase, copper and zinc superoxide dismutase and catalase in *Brachionus calyciflorus* during the aging process. *PLoS One*, 8(2), e57186.

Yildiz H., Akyilmaz E., & Dinckaya E. (2004). Catalase immobilization in cellulose acetate beads and determination of its hydrogen peroxide decomposition level by using a catalase biosensor. *Artificial Cells, Nanomedicine, and Biotechnology*, 32(3), 443–452.

Younus, H. (2018). Therapeutic potentials of superoxide dismutase. *International Journal of Health Sciences*, 12(3), 88.

Zaheer, M. R., & Gupta, A. (2019). Current development and future perspectives of microbial enzymes in the dairy industry, In *Enzymes in Food Biotechnology* (pp. 287–302). Academic Press.

Zamocky M., Furtmuller P. G., & Obinger C. (2008). Evolution of catalases from bacteria to humans. *Antioxidants & Redox Signaling*, 10(9), 1527–1548.

Zhang B., Liu K., Zheng Y., Wang Y., Wang J., & Liao H. (2013). Disruption of AtWNK8 enhances tolerance of *Arabidopsis* to salt and osmotic stresses via modulating proline content and activities of catalase and peroxidase. *International Journal of Molecular Sciences*, 14(4), 7032–7047.

Zhang, J., Jia, X., Wang, G. F., et al. (2022). Ascorbate peroxidase 1 confers resistance to southern corn leaf blight in maize. *Journal of Integrative Plant Biology*, 64(6), 1196–1211.

APPLICATION OF CHITIN IN BIOTECHNOLOGY

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Introduction

Chitin is a natural amino polysaccharide known for its distinctive structure, multifaceted properties, and exceptional functional capabilities. Chitin, recognized as one of the most prevalent biopolymers in nature, was first identified by Henri Braconnot in 1811. Braconnot named this polysaccharide isolated from fungi as "fungine". Afterward, in 1823, it was determined by Odier that the identical substance was present in the exoskeletons of insects and named it "chitin." The term "chitin" originates from the Greek word "chiton," meaning tunic or shell (Shahidi *et al.,* 1999; Kertmen & Ehrlich, 2022).

Despite its early discovery, ongoing research today continues to expand and enhance our knowledge of chitin. Chitin, ubiquitously distributed in nature, is the second most abundant renewable biopolymer after cellulose (Özdemir, 2014). From a chemical perspective, chitin comprises N-acetyl-D-glucosamine units connected by β-1,4-glycosidic bonds, resulting in a structural configuration analogous to cellulose (Figure 1). However, unlike cellulose, which consists of D-glucose units, chitin comprises D-glucosamine units (Figure 2), imparting distinct functional properties (Lv *et al.*, 2023).

Hydrogen bonds within the chitin structure facilitate the development of crystalline phases and support the formation of various derivatives. The molecular chain of chitin comprises hydroxyl groups at the C-3 and C-6 positions, acetamide groups, and a limited number of amino groups, which can engage in diverse chemical reactions (Prashanth & Tharanathan, 2007). Chitin can be categorized into α-chitin, β-chitin, and γ-chitin based on its crystalline form (Figure 3). In α-chitin, the polymeric chains are arranged in an anti-parallel configuration, promoting robust intermolecular hydrogen bonds. (Brigham, 2017). This structural arrangement imparts significant stability and diminishes the solubility of α-chitin in aqueous environments. α-chitin is the predominant form found in crustaceans, including species such as crabs (Jin *et al.*, 2021). β-chitin: Unlike α-chitin, the polymeric chains of β-chitin are aligned in a parallel orientation, which results in comparatively weaker hydrogen bonds. This arrangement grants β-chitin increased flexibility and excellent solubility in water, differentiating it from α-chitin. β-chitin is especially prevalent in species such as squid and cuttlefish (Jin *et al.*, 2021). γ-chitin**:** γ-chitin is a polymorph characterized by a combination of parallel

and anti-parallel chain arrangements, generally comprising two parallel chains alongside one anti-parallel chain. This hybrid structural arrangement imparts distinct physical properties to γ-chitin, distinguishing it from both α- and β-chitin. γ-chitin is primarily found in the cocoon fibers of insects (Hahn *et al.*, 2020). These structural differences markedly influence chitin's mechanical properties, solubility, and reactivity, expanding its potential applications across fields such as biomedicine, agriculture, and materials science (Iber *et al.*, 2022).

Chitin, particularly abundant in the exoskeletons of crustaceans and insects, is a structural support material insoluble in water. As the most prevalent natural amino polysaccharide, chitin is renowned for its non-toxic, antibacterial, and biocompatible properties. The unique structure of chitin polymers grants them multifaceted properties, high functional capabilities, and wide-ranging applications across biochemical and industrial domains (Singh *et al.*, 2021).

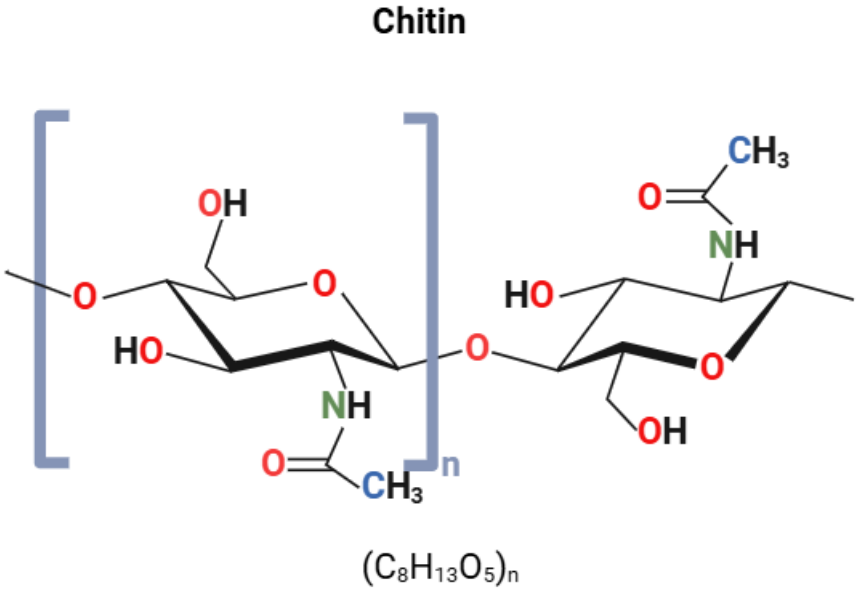

Figure 1. Chemical structure of chitin, depicting the N-acetyl-D-glucosamine units linked by β-1,4-glycosidic bonds, along with hydroxyl and acetamide groups (adapted from González *et al.*, 2000).

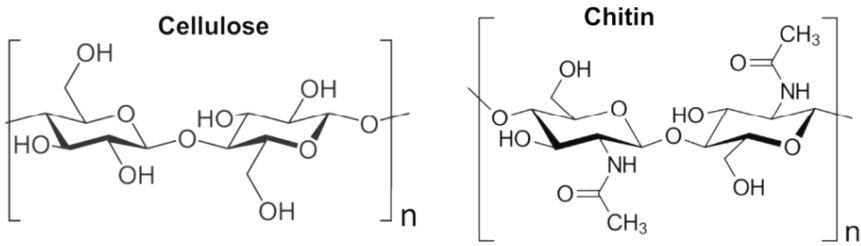

Figure 2. Structure of cellulose and chitin (Pellis *et al.*, 2022).

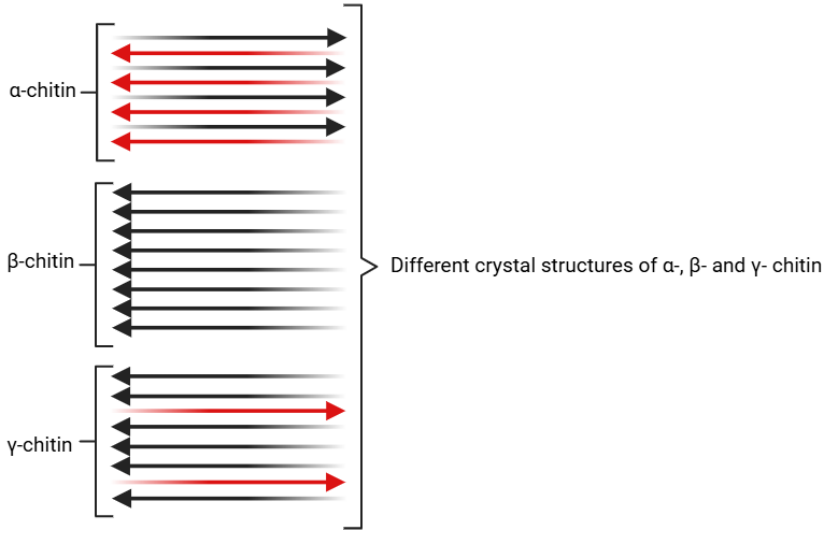

Figure 3. Different crystal structures of α-, β- and γ-chitin (adapted from Lv *et al.*, 2023).

Sources of Chitin

Chitin is found in crustacean and insect exoskeletons, fungal cell walls, and molluscan cartilage (Table 1). Crustaceans are the primary source of chitin, which generally comprises 20-40% chitin, 30-40% protein, 30-50% $CaCO_3$, and trace amounts of lipids, astaxanthin, and other mineral salts (Yadav *et al.*, 2019).

Today's principal source of large-scale chitin is crustacean exoskeletons, obtained from the post-consumer waste of marine creatures, notably shrimp and crabs. Crustaceans are now overconsumed, and this is predicted to rise in the future. Crustacean waste exoskeletons contain 15% to 40% chitin (Tan *et al.*, 2020). Crustaceans are expected to be the most crucial source of chitin today and in the future due to rising demand for crustacean consumption and the high chitin content of discarded exoskeletons (Khajavian *et al.*, 2022).

Fungi are becoming increasingly popular as an alternate source of chitin. Various scientific research has shown that chitin contained in fungi's cell walls might be an alternate source of chitin to the chitin commonly acquired from shellfish. Chitin, which accounts for 1% to 15% of the bulk of the fungal cell wall, suggests that these organisms can be regarded as a secondary source of chitin after crustaceans. These discoveries have sparked interest in fungi's economic potential and cleared the door for more study in this area (Lv *et al.*, 2023).

One of the principal factors contributing to the growing utilization of fungi as a chitin source is their superior resilience to seasonal fluctuations compared to shellfish (Arbia *et al.*, 2013). Additionally, the chitin extraction process from fungi is characterized by its relative simplicity, reduced cost, and more excellent procedural stability compared to animal-based sources. Research findings have demonstrated that while not all fungal species are chitinaceous, specific phyla, notably Basidiomycota, Ascomycota, and Zygomycota, exhibit elevated chitin concentrations (Hahn *et al.*, 2020).

Beyond crustaceans and fungi, insects are emerging as a promising and sustainable source of chitin despite limited prior research. As some of the most diverse and abundant organisms in the animal kingdom, insects possess

exoskeletons with chitin content exceeding 30%, offering a viable alternative source of this biopolymer. (Rameshthangam *et al.*, 2018).

A rigid and protective exoskeleton encases the internal soft tissues of insects. This exoskeleton performs several critical functions, including serving as a protective shield and facilitating the process of metamorphosis (Iber *et al.*, 2020). Within insects, chitin is naturally organized into microfibers embedded within a protein matrix. Typically, insects are composed of 30-60% protein, 25% lipids, 5-25% chitin, 5-10% catechols, and 2-10% minerals such as calcium, phosphorus, potassium, and magnesium salts (Singh *et al.*, 2021).

Insects are less susceptible to seasonal variations compared to crustaceans and can be readily cultivated due to their high fecundity and reproductive rates. The internal soft tissues of insects are encased by a hard, protective exoskeleton, notably rich in chitin (Synowiecki *et al.*, 2003). Additionally, various arthropods, including giant centipedes and woodlice, have been explored as sources of chitin. Consequently, chitin-rich by-products derived from insect farming present a novel and sustainable source for commercial chitin. Furthermore, chitin has also been gained from other organisms, such as poriferous, bryozoans, tardigrades, and the guano of insectivorous bats (Iber *et al.*, 2022; Lv *et al.*, 2023).

Table 1. Obtaining chitin from various sources (Iber *et al.*, 2022).

INSECTS	Chitin is found exoskeleton
Ant, Spider, Butterfly, Cockroach, Scorpions, Silkworm	
CRUSTACEAN	Chitin is found exoskeleton
Crabs, Crayfish, Lobsters, Clams, Shrimps, Prawns	
FUNGI	Chitin is found cell walls
Aspergillus niger, Penicillium crysogenum, Lactarius vellereus, Yeast	
MOLLUSK/OTHERS	Chitin is found cartilage
Oysters, Squids, Brown sea weed, Green algea, Fish scales	

Extraction of Chitin

Chitin extraction from designated sources typically involves a tripartite process: deproteinization, demineralization, and decolorization. During the deproteinization stage, the objective is to eliminate proteins by applying a solid base, such as sodium hydroxide (NaOH). In the subsequent demineralization phase, the goal is to extract calcium carbonates and calcium phosphates using a solid acid, such as hydrochloric acid (HCl). Lastly, if the chitin source, such as an insect exoskeleton, possesses coloration and colorless chitin is desired, the decolorization stage is implemented. This stage can be effectively achieved through the use of an alcohol, such as ethanol. Numerous methods exist for chitin extraction, yet these three stages remain consistent across all approaches (Islam *et al.*, 2017; Iber*et al.*, 2022)**.**

Typically, chitin extraction utilizes chemical or biological (microbial) methods, with efficiency often enhanced through the integration of physical techniques (Hamed *et al.,* 2016). However, the environmental and biological harm associated with chemical methods, alongside the protracted nature of biological processes, has led to the exploration of novel extraction techniques in recent years (Lv *et al.*, 2023). These include using ionic liquids (ILs) such as imidazoles, pyrroles, and morpholines, green solvents like deep eutectic solvents (DES), and electrochemical technologies. While these emerging methods may not yet match the efficiency of traditional chemical and biological approaches, they have proven effective in mitigating certain disadvantages (Morais *et al.*, 2020).

Despite their numerous disadvantages, newly developed methods have yet to surpass chemical methods in chitin extraction. The brevity of processing time in chemical methods has solidified their position as the most commercially prevalent approach. However, recent studies indicate that the adverse effects of chemical methods extend beyond environmental and biological harm, impacting the physico-chemical properties of the extracted chitin (Peter *et al.*, 2021). Although the proteins and minerals removed during chitin purification are valuable supplements for human food and animal feed, chemical methods render these byproducts unsuitable for such applications. Consequently, biological extraction methods are gaining increasing importance, with ongoing research focused on enhancing their sustainability (El-Shishtawy *et al.*, 2021; Kasprzak & Galiński, 2021).

Chemical Extraction Methods

Prior to employing the most commonly used chemical methods in chitin extraction, preliminary preparations are necessary to ready the chitin for purification. The raw chitin obtained from various sources undergoes washing, drying, and grinding to reduce its size and convert it into a powder. Following these preparatory steps, the standard three-step process for chemical purification is applied (Yadav *et al.*, 2019).

In the first step, deproteinization, various essential chemical reagents such as potassium hydroxide (KOH) and calcium hydroxide ($Ca(OH)_2$) have been utilized to remove proteins. However, sodium hydroxide (NaOH) remains the reagent of choice due to its high efficacy, even at low concentrations. For instance, a study reported achieving 97.88% purity in chitin using a 2% NaOH concentration during deproteinization, underscoring its effectiveness (Dayakar *et al.*, 2021; Dhanabalan *et al.*, 2021).

The second step, demineralization, involves the removal of minerals such as calcium carbonate and calcium phosphate through the application of acid. While hydrochloric acid (HCl) is predominantly used in demineralization, studies have shown that the choice of acid significantly influences chitin quality (Liao & Huang, 2020). A comparative study investigated the effectiveness of four different acids (HCl, sulfuric acid (H_2SO_4), acetic acid (CH_3COOH), and nitric acid (HNO_3)) on chitin quality. The findings indicated that the three inorganic acids (HCl, H_2SO_4, HNO_3) were more effective than acetic acid, although acetic acid was noted for being more environmentally friendly. Additionally, it was found that physical technologies, such as microwave-assisted processing during demineralization, contribute to optimizing chitin yield and quality (Lv *et al.*, 2023).

Decolorization is performed in the final stage of chitin purification to achieve colorless, pure chitin. Crustaceans and insects, in particular, contain substantial amounts of pigments, which impart a dark color to the extracted chitin and can detract from its application and commercial value. Therefore, decolorization is crucial for producing high-quality chitin. This process typically employs organic solvents such as ethanol and acetone, as well as potent oxidizing agents, including potassium permanganate ($KMnO_4$), hydrogen peroxide (H_2O_2), and sodium hypochlorite (NaClO). Additionally,

physical methods, such as ultrasound, may be used with organic solvents to enhance the efficiency of the decolorization process (Arbia *et al*., 2013; Lv *et al.*, 2023).

Biological Extraction Methods

While chemical methods are highly efficient and widely utilized, chitin extraction necessitates substantial quantities of acidic and alkaline solutions (Rahayu *et al.*, 2022). Given the environmental drawbacks of chemical methods, biological methods for chitin extraction offer a more eco-friendly alternative, with additional advantages such as enhanced resource utilization. Furthermore, biological methods preserve nutrients like proteins and mineral salts, which are removed during extraction, in a form that remains reusable for human food and animal feed (Hamed *et al.*, 2016).

However, in industrial production, the primary considerations are often the extraction time and the cost-effectiveness of the process. Since the purification phase in biological methods is typically longer and more expensive than chemical methods, integrating trace amounts of chemicals with biological techniques can enhance the process's stability and efficiency (Zhang *et al.*, 2021).

Chitin extraction using biological methods primarily involves the production of proteases and organic acids to remove proteins and mineral components, achieved by incorporating selected bacteria or fungi into the process. In the deproteinization stage, protease-producing bacteria degrade and eliminate proteins from the medium. During the demineralization stage, lactic acid-producing bacteria remove mineral components. This is accomplished through the reaction of lactic acid, produced by lactic acid bacteria, with calcium carbonate components, resulting in the formation of calcium lactate, which is subsequently removed from the medium (Hamdi *et al.*, 2017; Tan *et al.*, 2020).

For instance, one study demonstrated that 98% of proteins were successfully removed from shellfish waste through fermentation using halophilic archaea, with minimal chemical intervention. In another study, chitin was extracted from shrimp shells via co-fermentation using *Acetobacter pasteurianus* and *Bacillus subtilis*, achieving 90% deproteinization and

demineralization. In addition to bacterial use, a study was conducted using *Mucor circinelloides* to produce proteases for the deproteinization step. The findings from this study demonstrated the successful purification of exceptionally high-quality chitin (Hongkulsup *et al.*, 2016; Dhanabalan *et al.*, 2021).

Given these studies, biological extraction methods are reliable in minimizing environmental and health risks and facilitating the active reuse of extracted protein and mineral components within the food industry (Lv *et al.*, 2023).

Other Extraction Methods

In recent years, green solvent extraction methods have been developed to promote sustainability as alternatives to traditional chemical and biological extraction techniques. Among these methods, ionic liquids (ILs) represent a notable advancement. ILs are composed of predetermined organic cations paired with organic or inorganic anions, and they can effectively purify chitin without decomposing during the process (Morais *et al.*, 2020). Commonly used standard ILs include pyrroles, morpholines, and imidazoles. Research has demonstrated that imidazole-based ionic liquids can be highly effective for the purification and optimization of chitin. However, using ILs for chitin extraction poses significant challenges, including high costs and potential risks. Additionally, ILs may present toxic effects, which is particularly concerning for the food industry. Given these drawbacks, ILs still need to be considered the optimal choice for chitin extraction (Ma *et al.*, 2021).

An innovative green solvent extraction method involving deep eutectic solvents (DESs) has recently emerged. DESs consist of binary or ternary mixtures of hydrogen bond acceptors and donors in specific molar ratios, exhibiting properties akin to ionic liquids (ILs). However, a significant drawback of DESs is their high viscosity, which impedes efficient interaction between the solvent and chitin. This limitation renders DESs less optimal for chitin extraction (Sun *et al.*, 2022).

In addition to green solvents, electrochemical methods for chitin extraction represent a significant area of research (Nowacki *et al.*, 2020). One study involved treating shrimp shells with 1% acidic and alkaline electrolytic

solutions at 20 V for 6 hours. This process successfully separated minerals and proteins, achieving a 19.46% chitin extraction rate. Another study demonstrated chitin extraction from marine sponges using various electrolytes, including sodium sulfate (Na_2SO_4) and sodium chloride (NaCl). The physicochemical properties of chitin obtained through electrochemical methods are broadly comparable to those derived from traditional chemical methods. This approach offers a high-efficiency, environmentally friendly alternative with minimal reagent usage. However, the process remains in the developmental stage, and further research is necessary to refine the method, specifically to optimize the amount of chemical reagents required to enhance chitin purity and yield (Lv *et al.*, 2023).

Innovative Uses of Chitin in Biotechnology

Biomedical Applications of Chitin

Chitin and its derivatives, notably chitosan, are extensively utilized in various biomedical applications. These biomolecules are distinguished by their exceptional biological properties, including biocompatibility, immunological inertness, antibacterial efficacy, wound healing facilitation, and hemostatic activity. Their non-toxic nature and biodegradability within the human body further enhance their suitability for medical use (Hamed *et al.*, 2016). These compounds, validated as safe for daily consumption up to a dose of 17 g/kg, are widely utilized in contemporary cell culture, tissue engineering, and drug delivery systems (Crini *et al*., 2009). Furthermore, chitin is an excipient and drug carrier, particularly in applications demanding mucoadhesiveness, and available in film, gel, or powder forms due to its biocompatibility in physiological environments. These characteristics underscore chitin's versatility, significant value, and its derivatives within the biomedical field (Arbia *et al.*, 2013).

Chitin has achieved considerable advancements in the field of tissue engineering. Tissue engineering involves the strategic manipulation and control of living cells and their extracellular matrices to develop tissue substitutes for implantation into the body (Freier *et al.*, 2005). The primary objectives in this field include the repair, replacement, maintenance, or enhancement of the function of specific tissues or organs. Chitin-based materials have proven to be highly effective as scaffolds for cells in tissue

engineering, particularly for nerves and blood vessels, mainly when fabricated in tubular forms (Yang, 2011). Chitin-based scaffolds are highly versatile in regenerative medicine and can be tailored for various applications. In tissue engineering, chitin and its derivatives are extensively utilized in fabricating polymer scaffolds (Zhang *et al.*, 2006). The design of polymer scaffolds must adhere to essential criteria, including high porosity, biodegradability, structural integrity, non-toxicity, biocompatibility, and effective interaction with cells to facilitate cell adhesion. Additionally, these scaffolds should support cellular functions by promoting cell proliferation, migration, and differentiation (Elieh-Ali-Komi & Hamblin, 2016).

Chitin, particularly its derivative chitosan, can be subjected to various processing techniques in scaffold fabrication. These methodologies encompass phase separation and lyophilization, particle infiltration techniques, cryoablation, rapid prototyping technologies, and the formation of microparticles and microspheres. Such diverse techniques facilitate the optimization of chitin-based scaffolds for applications in tissue engineering, thereby extending their potential utility across a broad spectrum of biomedical fields (Ju *et al.*, 2008; Chesnutt *et al.*, 2009).

Another prominent application of chitin in the biomedical domain is in wound healing. The antimicrobial resistance exhibited by microorganisms can significantly impede the wound-healing process, which may lead to infections and suboptimal wound repair, ultimately resulting in unsightly scarring (Arbia *et al*., 2013). Research has demonstrated the efficacy of chitin-based dressings in safeguarding burns and wounds from bacterial infections. Chitin and its derivatives have the potential to serve as artificial skin matrices, thereby facilitating accelerated dermal regeneration (Madhumathi *et al.*, 2010). These materials are subject to degradation by endogenous enzymes after application to wounds, which helps minimize potential damage and trauma associated with dressing removal. Chitin and its derivatives maintain a moist environment at the wound interface and exhibit oxygen permeability, facilitating effective gas exchange. They establish a protective barrier against microbial invasion and can absorb excessive exudates, making them ideal for wound dressing materials (Elieh-Ali-Komi & Hamblin, 2016).

Madhumathi *et al.* (2010) developed composite scaffolds incorporating α-chitin and nanosilver for wound healing applications. These scaffolds

exhibited antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* and also demonstrated blood clotting promotion (Madhumathi *et al.*, 2010). Such properties make them valuable for wound healing applications. Similarly, Sudheesh Kumar and colleagues (2010) developed and characterized β-chitin and nanosilver composite scaffolds using β-chitin hydrogel embedded with silver nanoparticles. Evaluations with vero cells indicated that these chitin scaffolds with nanosilver are highly effective for wound healing applications. These studies highlight the potential and efficacy of chitin-based dressings in enhancing the wound healing process (Kumar *et al*., 2010).

Research has demonstrated that chitin and its derivatives possess anticancer properties. Studies have shown that chitin and chitosan conjugates, combined with 5-fluorouracil, serve as effective therapeutic agents against tumors. Notably, these conjugates exhibit significantly fewer side effects than 5-fluorouracil administered alone (Arbia *et al*., 2013). According to studies, chitin/chitosan-5-fluorouracil conjugates aggregate preferentially in tumor cells and have a potent anticancer effect. Chitin and chitosan conjugates outperformed 5-fluorouracil and other polysaccharide conjugates in terms of efficacy and toxicology. A new synthetic biological response modulator with strong immunopotentiation and anticancer activity has produced a hybrid compound of chitin and muramyl dipeptide immobilized D-glucose analog (Elieh-Ali-Komi & Hamblin, 2016).

Furthermore, carboxymethyl chitin coupled with the cell adhesive sequence Arg-Gly-Asp-Ser (RGDS) was discovered to inhibit lung metastatic B16-BL6 melanoma and liver metastatic L51784-ML25 lymphoma cells in mice. This suggested that RGDS-polymer conjugation might result in a long-term anti-metastatic action of the RGDS peptide in vivo (Elieh-Ali-Komi & Hamblin, 2016). So far, studies on the utilization of chitin and its derivatives have produced promising findings in terms of anticancer effects. However, further research and variety in the literature are required.

Chitin's exceptional biocompatibility in physiological conditions makes it a promising candidate for controlled drug delivery applications. Studies have verified chitin's efficacy in medication delivery (Arbia *et al*., 2013). Research done using carboxymethyl chitin, in particular, yielded satisfactory results in the controlled and sustained release of methamphetamine via subcutaneous injections. It was discovered that the injected substance could

produce an efficient biological reaction for the necessary duration. Chitin's water solubility and biodegradability contribute to its effectiveness in this field. The findings of this investigation demonstrated carboxymethyl chitin's potential as an excellent carrier for long-term and regulated drug release (Elieh-Ali-Komi & Hamblin, 2016).

Furthermore, tablets composed of chitin and its derivatives have been investigated for the sustained release of oxytetracycline, yielding positive outcomes. Specifically, tablets formulated from dihydroxypropyl chitin and partially deacetylated chitin derivatives have demonstrated effectiveness in this application. Additionally, another study reported the use of hydroxypropyl chitosan acetate, a biodegradable chitin derivative, for the controlled release of fibroblast growth factor encapsulated in polytetrafluoroethylene, achieving successful results (Jayakumar, 2010).

Recently, chitin and its derivatives have gained significant attention in the field of ophthalmology. These materials offer distinctive properties such as immunocompatibility, optical clarity, transparency, gas permeability, wettability, mechanical stability, and flexibility, making them suitable for producing contact lenses. Due to their safety and comfort, chitin and its derivatives increasingly replace synthetic polymers in ophthalmological applications. Additionally, their antimicrobial and wound-healing properties make them well-suited for developing eye bandage lenses for treating traumatic injuries (Sietsma *et al*., 1996; Elieh-Ali-Komi & Hamblin, 2016).

Cosmetic and Dermatological Applications of Chitin

Chitin is increasingly gaining recognition in cosmetic and dermatological applications. Research into using chitin as an active carrier in cosmetic formulations is advancing rapidly (Hamed *et al.*, 2016). In a study by Morganti *et al.* (2008), chitin nanofibrils were combined with antioxidant components such as melatonin, lutein, and ectoine. This combination facilitated the enhanced penetration of these active ingredients into the skin layers and contributed to improved skin protection. (Morganti *et al.,* 2008).

Chitin biopolymers possess a high water retention capacity and can form a permeability barrier on the skin's surface. These attributes make chitin

polymers highly suitable for various skincare products, including creams, lotions, nail polishes, and makeup. Additionally, they are employed in hair care products such as shampoos, hair dyes, and hair sprays, as well as in oral care products, including toothpaste, mouthwashes, and chewing gum (Gautier *et al.*, 2008).

In a separate study, block copolymer nanoparticles composed of phosphatidylcholine and linoleic acid were nanocomplexed with hyaluronan and chitin nanofibrils. These nanoconstructs encapsulated various active components, including cholesterol, creatine, caffeine, melatonin, vitamins E and C, and the amino acids glycine and arginine. The objective of these nanocarriers was to rejuvenate the skin through the encapsulated ingredients. Observations indicated that after one month of application, the skin exhibited increased softness, vibrancy, and moisture. Nevertheless, further research is necessary to achieve more definitive results in this area (Gautier *et al.*, 2008; Hamed *et al.*, 2016).

Textile Applications of Chitin

Chitinous compounds can interact with natural fibers, such as cotton and wool, and synthetic fibers, such as polyester. Their properties, including water solubility, bioadhesiveness, positive charge, biocompatibility, biodegradability, non-toxicity, and antimicrobial activity, suggest that chitin and its derivatives could be effectively utilized as antimicrobial agents in the textile industry (Pillai *et al.*, 2009).

The proliferation of microorganisms such as bacteria and fungi in textile products can compromise the mechanical strength of the fabric and lead to unpleasant odors, affecting both the textile and its user. Chitin's antimicrobial properties inhibit the growth of these microorganisms, thereby extending the lifespan of the fabrics and ensuring hygienic use (Hassan *et al.*, 2009).

Additionally, chitin fibers are distinguished by their non-allergenic properties. These attributes make chitin fibers particularly suitable for garments designed for babies and elderly individuals with sensitive skin. Such clothing, with its comfortable and protective features, ensures a worry-free experience without causing skin irritation (Hamed *et al.*, 2016). As a result,

chitin and its derivatives are increasingly recognized in the textile industry as crucial components that enhance both the durability and hygiene of textile products.

Nanotechnology Applications of Chitin

Nano-sized chitin particles demonstrate exceptional dispersion in aqueous environments due to their high specific surface area, low density, and superior forming properties. The primary categories of chitin nanoparticles include chitin nanocrystals (ChNCs) and chitin nanofibers (ChNFs). These nanoparticles are characterized by distinct external morphologies and internal structures, integral to advancing multifunctional nanocomposites and hybrid nanomaterials (Lv *et al*., 2023). Recent advancements have involved the extraction of ChNCs and ChNFs from various chitin-rich sources using both 'top-down' and 'bottom-up' methodologies. These techniques highlight Chitin's considerable promise for groundbreaking applications within the realm of nanotechnology. The remarkable attributes of Chitin nanoparticles facilitate their use across diverse fields, ranging from biomedical innovations to environmental remediation (Ngasotter *et al.*, 2022).

Chitin nanocrystals and nanofibers, in particular, hold the potential to provide high-performance and sustainable solutions in nanotechnology, engineering materials, biomaterials, and energy storage systems. Chitin-based nanocomposites present significant opportunities for innovation and enhanced performance across these domains (Lv *et al.*, 2023).

Agricultural Applications of Chitin

Chitin and its derivatives are extensively utilized in the agricultural sector, serving multiple functions to enhance soil health, manage plant diseases, and boost agricultural productivity. Chitin aids nutrient retention within the soil and is crucial in the nitrogen cycle, optimizing plant nutrient uptake (Hamed*et al.*, 2016).

Traditionally, chemical pesticides have been employed to combat plant pests. However, the high toxicity of these pesticides and the potential development of resistance in pests have driven the search for alternative solutions. In this context, chitin and its derivatives emerge as environmentally

friendly and effective alternatives. Chitin and chitosan are particularly noteworthy as plant protectors because they shield against fungi, viruses, bacterial diseases, and nematodes. These biopolymers prevent plant diseases and effectively mitigate soil-borne infections (Zeng *et al.*, 2012; Hamed *et al.*, 2016).

In a study by Mahdavi and Rahimi (2013), chitin derivatives were utilized as seed-coating agents to combat soil pests and enhance plant defenses. The study's results were promising, demonstrating the potential of chitin derivatives for agricultural applications. Nevertheless, further research is required to fully explore and validate their effectiveness in this field (Mahvadi & Rahimi, 2013).

Additionally, the beneficial effects of chitosan on plant growth are noteworthy. For instance, a study on the germination and growth of *Trachyspermum ammi* revealed that chitosan enhanced germination and growth by mitigating the adverse effects of abiotic stress conditions, such as high salinity. These findings underscore chitosan's potential to improve stress tolerance in agricultural applications (Hamed *et al.*, 2016).

Food and Nutrition Applications of Chitin

Obesity is recognized as a significant global health issue that diminishes quality of life and increases the risk of cancer and various metabolic disorders. As a natural polymer, chitin possesses a positive charge and exhibits a specific binding affinity for negatively charged bile acids and salts in the human body. This characteristic suggests that chitin has the potential to inhibit lipase enzyme activity and prevent the differentiation of adipocytes. Consequently, incorporating chitin into foods could reduce lipid levels and limit cholesterol and fat synthesis, offering protective effects against obesity (Tzoumaki *et al.*, 2013).

In a study by Huang, Wu *et al.* (2020), obese rats were administered solutions containing chitin at various concentrations. The results demonstrated that chitin markedly decreased body weight, blood lipid levels, and the incidence of aortic atherosclerosis in the rats. Additionally, chitin enhanced liver function and alleviated the severity of obesity-induced hepatic steatosis (Huang *et al.*, 2020). In contrast, Saeb and colleagues (2022) observed

significant modifications in the intestinal microbiota of obese rats fed with insect cuticles, a rich chitin source. This study revealed a substantial increase in Lactobacillaceae and Actinobacteria populations, coupled with a notable reduction in lipid accumulation, in rats consuming a diet comprising 3% insect cuticles. These findings suggest that chitin-containing cuticles could be considered a beneficial functional food additive for individuals afflicted with obesity or metabolic disorders (Saeb *et al.,* 2022; Lv et al., 2023)

Furthermore, findings indicating that carboxymethyl-chitin inhibits the differentiation and lipid accumulation of 3T3-L1 adipocytes by modulating molecular targets associated with adipogenesis are particularly noteworthy. These studies provide compelling evidence supporting the anti-obesity effects of carboxymethyl-chitin (Kong *et al.,* 2011).

Chitin nanomaterials are deliberately incorporated into specific food products to improve their functional properties. The impact of chitin nanoparticles on the gastrointestinal tract has been extensively investigated using in vitro digestion models. Polysaccharides and other macromolecules are integral to daily dietary habits. Chitin neutralizes gastric acids by releasing glucosamine, reducing gastric acid irritation and preventing gastric ulcers (Anandan *et al.,* 2004). Moreover, the potential of chitin to improve gastrointestinal health and mitigate the adverse effects of metabolic syndrome related to diabetes and cardiovascular disease has been repeatedly substantiated through scientific research (Wang *et al.,* 2021). The dextran-chitin complex has been reported to ameliorate metabolic disorders and enhance the proliferation of *Bifidobacterium* and its metabolites in rat models (Rodriguez *et al.,* 2020). These findings suggest that chitin may be incorporated into healthy foods as a probiotic ingredient, underscoring its potential to support digestive health and resolve intestinal disorders (Lv *et al.,* 2023).

The global phenomenon of aging is increasingly recognized as a significant issue. Individuals are routinely exposed to environmental and endogenous free radicals, contributing to oxidative stress. Chitin has been identified as an effective antioxidant, offering protection against oxidative damage through its capacity to scavenge free radicals (Sharma *et al.,* 2021). Given chitin's beneficial effects on the immune system and overall health, its

incorporation as a dietary supplement or therapeutic agent to mitigate the aging process is advocated (Lv *et al.*, 2023).

Toxic metabolites produced by moldy grains can have severe health implications, including carcinogenesis or organ failure in advanced stages. As a principal component of fungal cell walls, chitin is present in high concentrations in fungi but is scarce in plants and vertebrates. This characteristic enables the detection of food contamination by quantifying chitin levels in processed food products. For example, fungal contamination in processed tomatoes can be effectively assessed using chitin-specific lectins labeled with fluorescein isothiocyanate (FITC), which offers superior sensitivity compared to conventional methods such as Howard mold counting and visual inspection (Potts *et al.,* 2002). Moreover, individuals exposed to prolonged humid conditions or those with compromised immune systems or inadequate hygiene are at heightened risk for fungal infections. In vitro studies have shown that chitin-binding proteins labeled with technetium-99m (99mTc) can selectively bind to chitin, enabling precise differentiation between bacterial and fungal infections (Siaens *et al.,* 2006). Similarly, gold nanoparticles interact with the chitin layer on fungal surfaces, facilitating highly accurate screening and detection within a 30-minute timeframe (Xu *et al.,* 2017)

Applications of Chitin on Environmental Protection

Heavy metals and organic pollutants in wastewater, such as dyes and microplastics, pose severe threats to environmental safety and can harm human health when they enter the food chain (Peter *et al.,* 2021). Chitin has attracted significant interest for its potential in environmental remediation due to its exceptional adsorption capabilities and cost-efficiency. Chitin-based nanocomposite microspheres have been shown to remove methylene blue from wastewater effectively, thanks to their intricate layered structure, high stability throughout the adsorption process, and economical production costs (Xu *et al.,* 2018). Additionally, amphoteric chitin can efficiently decolorize both anionic and cationic dyes in wastewater, providing a sustainable and eco-friendly treatment alternative (El-Shishtawy *et al.,* 2021). Moreover, chitin nanomaterials demonstrate high efficacy in the adsorption of heavy metals,

underscoring their significant contribution to environmental protection (Lv *et al.*, 2023).

Plastic waste exacerbates environmental issues, with approximately 350 million tons of plastic packaging waste generated annually from the food, cosmetics, and industrial sectors (Ye *et al.*, 2020). A substantial portion of this waste is converted into microplastics (<5 mm), which are pervasive in aquatic systems and pose severe threats to ecological and human health. Research on chitin-based sponges has demonstrated their effectiveness in removing 71.6-92.1% of various styrene microplastics modified in an alkaline medium (Sun *et al.*, 2021). These chitin-based sponges exhibit a high capacity for removing microplastics with diverse functional groups, are reusable, and can be degraded by soil microorganisms. These characteristics highlight chitin-based sponges' economic and environmental sustainability as a significant solution for plastic waste management (Lv *et al.*, 2023).

Applications of Chitin to Produce Green Energy Alternatives

The rapid depletion of global fossil fuel reserves underscores the potential of biofuels as a viable alternative to bridge the energy gap. Conventional biofuels are typically derived from energy crops, biomass straws, and forest residues. However, these production methods encounter challenges such as extensive land requirements, high costs, protracted production times, and elevated feedstock values. In this context, chitin is increasingly recognized as a promising candidate for biofuel production from both economic and biological standpoints (Lv *et al.*, 2023).

While numerous microorganisms can utilize carbon sources like N-acetylglucosamine for ethanol production, only a few organisms can directly convert unprocessed chitin into ethanol (Suryawanshi & Eswari, 2022). For instance, *Mucor circinelloides* NBRC 6746 generates 0.11 g/L of ethanol using chitin powder as a substrate; this yield can be enhanced to 1.32 g/L with colloidal chitin (Inokuma *et al.*, 2013). Chitin waste can be transformed into high-value compounds such as 5-hydroxymethylfurfural and other organic nitrogen-containing chemicals, contributing significantly to industrial sustainability (Ma *et al.*, 2020).

Microbial fuel cell (MFC) systems present a promising green technology for energy recovery through biomass decomposition. Li *et al.* (2017) demonstrated that a microbial fuel cell developed using chitin degradation by *Shewanella oneidensis* MR-1 exhibited superior chitin degradation capacity compared to traditional fermentation methods, offering both bioelectric and chemical recovery potentials (Li *et al.*, 2017). Furthermore, chitin can be utilized as a piezoelectric material in sensor devices, serving as an alternative to fluorine-based polymers. This application provides notable advantages in terms of energy efficiency and environmental protection (Kim *et al.*, 2018).

Conclusion

Chitin, nature's most prevalent biopolymer after cellulose, has a distinct chemical structure. This unusual chemical structure has given it a diverse set of features. Chitin and its derivatives have been the subject of intense research in recent years, and their contribution to biotechnology has piqued the scientific and industrial community's attention. This study compiles and emphasizes information from the literature on chitin's rising popularity and application in biotechnology disciplines such as biomedicine, cosmetics, textiles, agriculture, nanotechnology, food, environmental protection, and green energy. As a result of this research, it is anticipated that chitin and its derivatives will influence additional application areas in the following years, generating enthusiasm in the scientific community.

REFERENCES

Anandan, R., Nair, P. V., & Mathew, S. (2004). Anti-ulcerogenic effect of chitin and chitosan on mucosal antioxidant defence system in HCl-ethanol-induced ulcer in rats. *Journal of Pharmacy and Pharmacology*, *56*(2), 265-269.

Arbia, W., Arbia, L., Adour, L., & Amrane, A. (2013). Chitin extraction from crustacean shells using biological methods–a review. *Food Technology and Biotechnology*, *51*(1), 12-25.

Brigham, C. J. (2017). Chitin and chitosan: sustainable, medically relevant biomaterials. *International Journal of Biotechnology for Wellness Industries*, *6*(2), 41-47.

Chesnutt, B. M., Viano, A. M., Yuan, Y., *et al.* (2009). Design and characterization of a novel chitosan/nanocrystalline calcium phosphate composite scaffold for bone regeneration. *Journal of Biomedical Materials Research Part A: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials*, *88*(2), 491-502.

Crini, G., Guibal, E., Morcellet, M., Torri, G., & Badot, P. M. (2009). Chitine et Chitosane–synthèse, propriétés et principales applications. Chitine et Chitosane-Du biopolymère à l'application (19-54). Presses Universitaires de Franche-Comté chapitre-1.

Dayakar, B., Xavier, K. M., Das, O., Porayil, L., Balange, A. K. and Nayak, B. B. (2021). Application of extreme halophilic archaea as biocatalyst for chitin isolation from shrimp shell waste. *Carbohydrate Polymer Technologies and Applications*, *2*, 93-100.

Dhanabalan, V., Xavier, K. M., Eppen, S., Joy, A., Balange, A., Asha, K. K. and Nayak, B. B. (2021). Characterization of chitin extracted from enzymatically deproteinized Acetes shell residue with varying degree of hydrolysis. *Carbohydrate Polymers*, *253*, 117-203.

Elieh-Ali-Komi, D., & Hamblin, M. R. (2016). Chitin and chitosan: production and application of versatile biomedical nanomaterials. *International Journal of Advanced Research*, *4*(3), 411.

El-Shishtawy, R. M., Asiri, A. M. and Ahmed, N. S. (2021). Removal of toxic dyestuffs from aqueous solution by amphoteric bioadsorbent. *Current Analytical Chemistry*, *17*(7), 957-974.

Freier, T., Montenegro, R., Koh, H. S., & Shoichet, M. S. (2005). Chitin-based tubes for tissue engineering in the nervous system. *Biomaterials*, *26*(22), 4624-4632.

Gautier, S., Xhauflaire-Uhoda, E., Gonry, P., & Piérard, G. E. (2008). Chitin–glucan, a natural cell scaffold for skin moisturization and rejuvenation. *International Journal of Cosmetic Science*, *30*(6), 459-469.

González, V., Guerrero, C., & Ortiz, U. (2000). Chemical structure and compatibility of polyamide–chitin and chitosan blends. *Journal of Applied Polymer Science*, *78*(4), 850-857.

Hahn, T., Tafi, E., Paul, A., Salvia, R., Falabella, P., & Zibek, S. (2020). Current state of chitin purification and chitosan production from insects. *Journal of Chemical Technology & Biotechnology*, *95*(11), 2775-2795.

Hamdi, M., Hammami, A., Hajji, S., Jridi, M., Nasri, M., & Nasri, R. (2017). Chitin extraction from blue crab (*Portunus segnis*) and shrimp (*Penaeus kerathurus*) shells using digestive alkaline proteases from *P. segnis* viscera. *International Journal of Biological Macromolecules*, *101*, 455-463.

Hamed, I., Özogul, F., & Regenstein, J. M. (2016). Industrial applications of crustacean by-products (chitin, chitosan, and chitooligosaccharides): A review. *Trends in Food Science & Technology*, *48*, 40-50.

Hassan, M. A., Li, T. P., & Noor, Z. Z. (2009). Coagulation and flocculation treatment of wastewater in textile industry using chitosan. *Journal of Chemical and Natural Resources Engineering*, *4*(1), 43-53.

Hongkulsup, C., Khutoryanskiy, V. V., & Niranjan, K. (2016). Enzyme assisted extraction of chitin from shrimp shells (*Litopenaeus vannamei*). *Journal of Chemical Technology & Biotechnology*, *91*(5), 1250-1256.

Huang, J., Wu, Q., Lin, Z., Liu, S., Su, Q., & Pan, Y. (2020). Therapeutic effects of chitin from *Pleurotus eryngii* on high-fat diet induced obesity in rats. *Acta Scientiar Polonorum Technologia Alimentaria*, *19*(3), 279-289.

Iber, B. T., Kasan, N. A., Torsabo, D., & Omuwa, J. W. (2022). A review of various sources of chitin and chitosan in nature. *Journal of Renewable Materials*, *10*(4), 1097-1123.

Inokuma, K., Takano, M., & Hoshino, K. (2013). Direct ethanol production from N-acetylglucosamine and chitin substrates by *Mucor* species. *Biochemical Engineering Journal*, *72*, 24-32.

Islam, S., Bhuiyan, M. A. R., & Islam, M. N. (2017). Chitin and chitosan: Structure, properties and applications inbiomedical engineering. *Journal of Polymers and the Environment*, *25*(3), 854–866.

Jayakumar, R., Menon, D., Manzoor, K., Nair, S. V., & Tamura, H. (2010). Biomedical applications of chitin and chitosan based nanomaterials. A short review. *Carbohydrate Polymers*, *82*(2), 227-232.

Ju, Y. M., Park, K., Son, J. S., Kim, J. J., Rhie, J. W., & Han, D. K. (2008). Beneficial effect of hydrophilized porous polymer scaffolds in tissue-engineered cartilage formation. *Journal of Biomedical Materials Research Part B: Applied Biomaterials: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials*, *85*(1), 252-260.

Kasprzak, D., & Galiński, M. (2021). Chitin and chitin-cellulose composite hydrogels prepared by ionic liquid-based process as the novel electrolytes for electrochemical capacitors. *Journal of Solid State Electrochemistry*, *25*(10), 2549-2563.

Kertmen, A., & Ehrlich, H. (2022). Patentology of chitinous biomaterials. Part I: Chitin. *Carbohydrate Polymers*, *282*, 1-37.

Khajavian, M., Vatanpour, V., Castro-Muñoz, R., & Boczkaj, G. (2022). Chitin and derivative chitosan-based structures—Preparation strategies aided by deep eutectic solvents: A review. *Carbohydrate Polymers*, *275*, 1-22.

Kim, K., Ha, M., Choi, B., *et al.* (2018). Biodegradable, electro-active chitin nanofiber films for flexible piezoelectric transducers. *Nano Energy*, *48*, 275-283.

Kumar, P. S., Abhilash, S., Manzoor, K., Nair, S. V., Tamura, H., & Jayakumar, R. (2010). Preparation and characterization of novel β-chitin/nanosilver

composite scaffolds for wound dressing applications. *Carbohydrate Polymers*, *80*(3), 761-767.

Li, S. W., Zeng, R. J., & Sheng, G. P. (2017). An excellent anaerobic respiration mode for chitin degradation by Shewanella oneidensis MR-1 in microbial fuel cells. *Biochemical Engineering Journal*, *118*, 20-24.

Liao, J., & Huang, H. (2020). Extraction of a novel fungal chitin from Hericium erinaceus residue using multistep mild procedures. *International Journal of Biological Macromolecules*, *156*, 1279-1286.

Lv, J., Lv, X., Ma, M., Oh, D. H., Jiang, Z., & Fu, X. (2023). Chitin and chitin-based biomaterials: A review of advances in processing and food applications. *Carbohydrate Polymers*, *299*, 1-13.

Ma, Q., Gao, X., Bi, X., Xia, M., Han, Q., Peng, M., & Wang, M. (2021). Combination of steam explosion and ionic liquid pretreatments for efficient utilization of fungal chitin from citric acid fermentation residue. *Biomass and Bioenergy*, *145*, 1-8.

Ma, X., Gözaydın, G., Yang, H., *et al.* (2020). Upcycling chitin-containing waste into organonitrogen chemicals via an integrated process. *Proceedings of the National Academy of Sciences*, *117*(14), 7719-7728.

Madhumathi, K., Sudheesh Kumar, P. T., Abhilash, S., *et al.* (2010). Development of novel chitin/nanosilver composite scaffolds for wound dressing applications. *Journal of Materials Science: Materials in Medicine*, *21*, 807-813.

Mahdavi, B., & Rahimi, A. (2013). Seed priming with chitosan improves the germination and growth performance of ajowan (*Carum copticum*) under salt stress. *Eurasian Journal of Biosciences*, *7*, 69-76.

Morais, E. S., Lopes, A. M. D. C., Freire, M. G., Freire, C. S., Coutinho, J. A., & Silvestre, A. J. (2020). Use of ionic liquids and deep eutectic solvents in polysaccharides dissolution and extraction processes towards sustainable biomass valorization. *Molecules*, *25*(16), 36-52.

Morganti, P., Fabrizi, G., Palombo, P., Palombo, M., Ruocco, E., Cardillo, A., & Morganti, G. (2008). Chitin-nanofibrils: a new active cosmetic carrier. *Journal of Applied Cosmetology*, *26*(3), 113-128.

Ngasotter, S., Sampath, L., & Xavier, K. M. (2022). Nanochitin: An update review on advances in preparation methods and food applications. *Carbohydrate Polymers*, *291*, 1-27.

Nowacki, K., Stępniak, I., Langer, E., Tsurkan, M., Wysokowski, M., Petrenko, I., & Ehrlich, H. (2020). Electrochemical approach for isolation of chitin from the skeleton of the black coral *Cirrhipathes sp*. (Antipatharia). *Marine Drugs*, *18*(6), 297.

Özdemir, Z. (2014). Kitin, kitosanın fonksiyonel özellikleri ve kullanım alanları. *Türkiye Kimya Derneği*, (October 2014), 104, 117.

Peter, S., Lyczko, N., Gopakumar, D., Maria, H. J., Nzihou, A., & Thomas, S. (2021). Chitin and chitosan based composites for energy and environmental applications: a review. *Waste and Biomass Valorization*, *12*, 4777-4804.

Pillai, C. K., Paul, W., & Sharma, C. P. (2009). Chitin and chitosan polymers: Chemistry, solubility and fiber formation. *Progress in Polymer Science*, *34*(7), 641-678.

Potts, S. J., Slaughter, D. C., & Thompson, J. F. (2002). Measuring mold infestation in raw tomato juice. *Journal of Food Science*, *67*(1), 321-325.

Prashanth, K. H., & Tharanathan, R. N. (2007). Chitin/chitosan: modifications and their unlimited application potential—an overview. *Trends in Food Science & Technology*, *18*(3), 117-131.

Rahayu, A. P., Islami, A. F., Saputra, E., Sulmartiwi, L., Rahmah, A. U., & Kurnia, K. A. (2022). The impact of the different types of acid solution on the extraction and adsorption performance of chitin from shrimp shell waste. *International Journal of Biological Macromolecules*, *194*, 843-850.

Rameshthangam, P., Solairaj, D., Arunachalam, G., & Ramasamy, P. (2018). Chitin and Chitinases: biomedical and environmental applications of chitin and its derivatives. *Journal of Enzymes*, *1*(1), 20-43.

Rodriguez, J., Neyrinck, A. M., Zhang, Z., *et al.* (2020). Metabolite profiling reveals the interaction of chitin-glucan with the gut microbiota. *Gut Microbes*, 12(1), 1-15.

Saeb, A., Grundmann, S. M., Gessner, D. K., *et al.* (2022). Feeding of cuticles from *Tenebrio molitor* larvae modulates the gut microbiota and attenuates hepatic steatosis in obese *Zucker rats*. *Food & Function*, *13*(3), 1421-1436.

Shahidi, F., Arachchi, J. K. V., & Jeon, Y. J. (1999). Food applications of chitin and chitosans. *Trends in Food Science & Technology*, *10*(2), 37-51.

Sharma, S., Kaur, N., Kaur, R., & Kaur, R. (2021). A review on valorization of chitinous waste. *Journal of Polymer Research*, *28*, 1-20.

Siaens, R., Eijsink, V. G. H., Vaaje-Kolstad, G., & Vandenbulcke, K. (2006). Synthesis and evaluation of a^ sup 99m^ Technetium labeled chitin-binding protein as potential specific radioligand for the detection of fungal infections in mice. *The Quarterly Journal of Nuclear Medicine and Molecular Imaging*, *50*(3), 155-166.

Sietsma, J. H., Din, A. B., Ziv, V., Sjollema, K. A., & Yarden, O. (1996). The localization of chitin synthase in membranous vesicles (chitosomes) in *Neurospora crassa*. *Microbiology*, *142*(7), 1591-1596.

Singh, R., Upadhyay, S. K., Singh, M., Sharma, I., Sharma, P., Kamboj, P., & Khan, F. (2021). Chitin, chitinases and chitin derivatives in biopharmaceutical, agricultural and environmental perspective. *Biointerface Research in Application Chemical*, *11*(3), 9985-10005.

Sun, C., Wang, Z., Zheng, H., Chen, L., & Li, F. (2021). Biodegradable and re-usable sponge materials made from chitin for efficient removal of microplastics. *Journal of Hazardous Materials*, *420*, 1-12.

Sun, X., Wei, Q., Yang, Y., Xiao, Z., & Ren, X. (2022). In-depth study on the extraction and mechanism of high-purity chitin based on NADESs method. *Journal of Environmental Chemical Engineering*, *10*(1), 1-8.

Suryawanshi, N., & Eswari, J. S. (2022). Shrimp shell waste as a potential raw material for biorefinery—a revisit. *Biomass Conversion and Biorefinery*, *12*(5), 1977-1984.

Synowiecki, J., & Al-Khateeb, N. A. (2003). Production, properties, and some new applications of chitin and its derivatives. *Critical Reviews in Food Science and Nutrition*, *43*(2), 145–171.

Tan, Y. N., Lee, P. P., & Chen, W. N. (2020). Dual extraction of crustacean and fungal chitosan from a single *Mucor circinelloides* fermentation. *Fermentation*, *6*(2), 40-51.

Tzoumaki, M. V., Moschakis, T., Scholten, E., & Biliaderis, C. G. (2013). In vitro lipid digestion of chitin nanocrystal stabilized o/w emulsions. *Food & Function*, *4*(1), 121-129.

Wang, M., Zhou, J., Selma-Royo, M., Simal-Gandara, J., Collado, M. C., & Barba, F. J. (2021). Potential benefits of high-added-value compounds from aquaculture and fish side streams on human gut microbiota. *Trends in Food Science & Technology*, *112*, 484-494.

Xu, K., Bui, M. P. N., Fang, A., & Abbas, A. (2017). Plasmonic cell nanocoating: a new concept for rapid microbial screening. *Analytical and Bioanalytical Chemistry*, *409*, 6305-6314.

Xu, R., Mao, J., Peng, N., Luo, X., & Chang, C. (2018). Chitin/clay microspheres with hierarchical architecture for highly efficient removal of organic dyes. *Carbohydrate Polymers*, *188*, 143-150.

Yadav, M., Goswami, P., Paritosh, K., Kumar, M., Pareek, N., & Vivekanand, V. (2019). Seafood waste: a source for preparation of commercially employable chitin/chitosan materials. *Bioresources and Bioprocessing*, *6*(1), 1-20.

Yang, T. L. (2011). Chitin-based materials in tissue engineering: applications in soft tissue and epithelial organ. *International Journal of Molecular Sciences*, *12*(3), 1936-1963.

Ye, S., Cheng, M., Zeng, G., *et al.* (2020). Insights into catalytic removal and separation of attached metals from natural-aged microplastics by magnetic biochar activating oxidation process. *Water Research*, *179*, 1-10.

Zeng, D., Luo, X., & Tu, R. (2012). Application of bioactive coatings based on chitosan for soybean seed protection. *International Journal of Carbohydrate Chemistry*, *2012*(1), 1-5.

Zhang, L., Ao, Q., Wang, A., *et al.* (2006). A sandwich tubular scaffold derived from chitosan for blood vessel tissue engineering. *Journal of Biomedical Materials Research Part A: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials*, *77*(2), 277-284.

Zhang, Q., Wang, L., Liu, S., & Li, Y. (2021). Establishment of successive co-fermentation by *Bacillus subtilis* and *Acetobacter pasteurianus* for extracting chitin from shrimp shells. *Carbohydrate Polymers*, *258*, 1-8.

CELL-TARGETED DRUG DELIVERY SYSTEMS

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Abstract

In the treatment of diseases, it is very valuable that drugs can be transported to the target area without being degraded or retained by the immune system and that they can maintain their effect in the area for as long as it takes to create the treatment effectiveness. Various carriers are used to transport drugs to the environment where they are expected to affect. These drug carriers allow drugs with low solubility, bioavailability, and stability to remain in the bloodstream for longer periods. Drug-loaded carriers should not be toxic to healthy cells, should not be captured by the reticuloendothelial system, which is the body's defense system, should be able to successfully deliver the loaded drug to the desired area, and should be removed from the body by metabolism when the effect ends. In recent years, nanoformulations have been used due to their bioactivity and as drug carriers. However, the immunogenic effect of nano-sized formulations, the reduction of their effectiveness with presystemic elimination before reaching the target area, various difficulties in their dosing, and the potential toxicity risks due to their accumulation in unwanted areas limit their use. When cells are used as drug carriers, the risk of immunogenicity is reduced due to their presence in the body's natural physiology. In addition, they have a low risk of creating toxicity in unwanted areas with their natural targeting abilities in the body. Stem cells, erythrocytes, platelets, leukocytes, and lymphocytes are among the cell types used for drug delivery. Although there are advantages and disadvantages, cell-based drug delivery systems show promising results in treatment. In this chapter, cell types used as carriers in cell-based drug delivery systems, cell targeting mechanisms, drug loading methods into carrier cells, clinical applications, advantages and disadvantages of cell-based drug delivery systems are evaluated.

Introduction

The development of low-toxicity carriers that enable drugs to reach the target area at the desired concentration for effective treatment is very valuable. Problems such as the retention of drugs by the reticuloendothelial system (RES) before they reach the target area and low solubility, and bioavailability cause the desired effect of the drugs to not be achieved. This results in toxicity due to the need for higher doses of medication (Yang et al., 2022). In recent years, various nanoformulations such as liposomes, micelles, and solid lipid nanoparticles have been developed to increase drug efficacy through targeting and solubility. Factors such as low stability of nanoformulations, immunogenic effects and various toxicities limit the use of nanomaterials (Wu et al., 2022). In addition, presystemic elimination of nanoformulations and prevention of uptake by organ/tissue barriers reduce drug efficacy. In this context, there is a need to develop biomaterials that are biocompatible, have high targeting ability, can remain in the bloodstream for a long time, and have low toxicity (Yang et al., 2022).

Cell-based drug delivery systems are systems that use cells as carriers to effectively and safely deliver drugs or drug-loaded nanoparticles to the target area. Cells used as carriers include erythrocytes, platelets, neutrophils, monocytes, macrophages, natural killer (NK) cells, T cells, and stem cells (Wu et al., 2022; Choi et al., 2023). When cells are used as carriers, they can bind specifically to the target tissue and prevent the drug from being perceived as an antigen due to their biocompatibility. After reaching the target area and releasing the drug, the carrier is biodegradable, which reduces the risk of toxicity for healthy cells. Thus, drugs loaded into cells reach the target area with low immunogenicity and cytotoxicity in healthy cells (Choi et al., 2023). In addition, some of these cells can cross the blood-brain barrier (BBB) with their chemotactic and natural targeting ability and can exhibit intracranial activity. This situation offers a new alternative to the treatment of brain tumors, encephalitis, Parkinson's disease, Alzheimer's disease, and other neurodegenerative diseases by overcoming the BBB barrier, which creates great difficulty for drugs to reach the central nervous system (CNS) (Li et al., 2021).

In peripheral diseases as well as in the CNS, drugs tend to be rapidly removed from the circulation by phagocytosis, clearance, and other mechanisms. This situation leads to the elimination of drugs before they reach

the targeted location, and even to the toxicity of drug metabolites that are degraded outside the target tissue in healthy cells. On the other hand, carrier cells that exhibit regenerative properties can escape from the mononuclear phagocytic system with "Don't eat me" signals due to the cluster of differentiation 47 (CD47) receptor on their surface, and this situation prolongs their circulation time in the blood. Cells with advantageous surface properties in targeting therapeutic agents to the desired tissue are a promising strategy in achieving effective treatment with fewer side effects in the pharmacological treatment of diseases (Yu et al., 2020; Li et al., 2021; Choi et al., 2023; Lu et al., 2023).

In cell-based drug delivery systems, the drug to be loaded and the cell types to be preferred as carriers vary according to the targeted area. Mature erythrocyte cells have a much wider range of applications compared to other drug delivery systems. The long lifespan of erythrocytes, which can be easily obtained from blood (100-120 days), allows the blood concentration of the drug loaded into them to remain in the therapeutic range for much longer. Moreover, erythrocytes are much larger than other blood cells, organelle-free, nucleus-free, and biconcave discoid-shaped cells. This situation facilitates substance exchange with increased surface-volume area (Sun et al., 2017). With their flexible structure, erythrocytes can reach small venous capillaries, especially the lung epithelium, and can also be passively targeted to tissues. In addition, the presence of more than 300 different membrane protein structures in erythrocytes allows the membrane surface to be modified with certain reagents or ligands, thus increasing their targeting capabilities (Rong et al., 2022; Fan et al., 2023). Erythrocytes can be loaded with drugs that have various activities such as analgesic, anti-inflammatory, anticancer, and immunosuppressant. Despite these advantages, erythrocytes are sometimes eliminated before reaching the target area by undergoing intravascular hemolysis, where free hemoglobin and erythrocyte contents are released into the bloodstream. They can also undergo phagocytosis in the monocyte-macrophage system of organs such as the liver and spleen through extravascular hemolysis (Thiagarajan et al., 2021). Intravascular and extravascular hemolysis of erythrocytes can lead to many complications including vascular dysfunction, thrombosis, vasoconstriction and decreased renal blood flow. In addition to hemolysis, clinical conditions that may be caused by the number of erythrocytes should also be taken into consideration when using erythrocytes as carriers. Studies in mice and dogs have reported a relationship between increased proinflammatory response, sepsis, pneumonia,

and multiple organ failure after erythrocyte transfusion (Rapido et al., 2017; Sun et al., 2017). It is known that inflammation induced by erythrocyte increase is related to the structure and content of erythrocytes. The heme group in the structure of erythrocytes constitutes a large portion (2.0–2.5 g) of the total amount of iron (3–4 g) found in the human body. Damaged, aged or disintegrated erythrocytes release iron, and with the increased iron concentration, a suitable environment is created for the growth of many infectious agents, especially siderophilic microorganisms. Previous studies have shown that iron injection into rodents significantly increases the virulence of some pathogens. During erythrocyte-related inflammation, circulating iron is shifted to storage to prevent the pathogen in the body from accessing iron, which leads to iron deficiency erythropoiesis, resulting in anemia of inflammation (Pieracci et al., 2005; Ganz, 2018; Shah et al., 2021). This situation, the use of erythrocytes restricted in some cell-based drug delivery systems (Sun et al., 2017).

Platelets, which are blood cells carried in the circulation like erythrocytes, are cells used as drug carriers in various cancers and metastases, especially in wound healing. A large number of platelets are produced every day for physiological events to occur and platelets are renewed quickly (8-10 days). This period has been deemed sufficient for drug transport and platelets have been evaluated as a safe and reproducible carrier (Lu et al., 2019). The shorter half-life of platelets compared to erythrocytes allows optimal drug concentrations to be achieved quickly (Choi et al., 2023). However, it has been reported that platelets injected in high volumes for drug administration may affect the physiology of the circulatory system and cause serious side effects such as thrombosis (Fan et al., 2023).

Platelets circulate in the blood in an inactive form under normal physiological conditions and become active as a result of vascular endothelial damage or various traumas. Active platelets play a role in hemostasis by constricting blood vessels and forming hemostatic plugs. Platelets also perform these effects by secreting procoagulant/antifibrinolytic factors. In the treatment of endothelial damage, including coronary artery stenosis and atherosclerosis, the tendency of platelets to accumulate in these regions has been utilized. Platelets, which have been shown to be involved in chronic inflammation in atherosclerotic diseases, participate in the inflammatory response as well as hemostasis (Dai et al., 2022; Fang et al., 2024). This situation highlights the activity of platelets in wound healing, in which

hemostasis, inflammation, proliferation and regeneration play a role in the mechanism. Platelets, which are responsible for initiating the coagulation cascade during hemostasis, secrete various growth factors, chemokines and cytokines (transforming growth factor (TGF), tumor necrosis factor (TNF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), prostaglandin (PGE2), matrix metalloproteinases (MMP), fibroblast growth factor (FGF), epidermal growth factor (EGF), and interleukin-1 (IL-1). These cytokines and factors regulate the recruitment, proliferation and differentiation of cells that can promote angiogenesis and form granulation tissue (Cecerska-Heryć et al., 2022). However, prolonged inflammation during healing causes the breakdown of extracellular matrix and growth factors that play a role in tissue regeneration by increasing protease activity. It has been reported that these growth factors, which are known to decrease in chronic wounds, increase with external application of platelets. In this context, regulating inflammation with platelet-mediated drugs in wound healing provides a remarkable advantage (Bonferoni et al., 2019; Dai et al., 2022). Platelets are closely related to the pathological processes of tumor formation and metastasis, like erythrocytes. In this process, platelets migrate toward tumor sites to regulate tumor cell growth, differentiation, and angiogenesis. Due to the faster release of the drug in the acidic environment of cancerous tissue and this interaction between platelets and tumor cells, anti-neoplastic drugs are loaded into platelets in specific targeted therapy (Dai et al., 2022). In addition, platelets, which can escape phagocytosis by giving "Don't eat me" signals to macrophages with the CD47 membrane expression on their surface, as in erythrocytes, increase the duration of the drug's residence in the blood when used as a carrier (Choi et al., 2023). Due to their strong activity, extracellular vesicles derived from platelets have been designed for use in the treatment of thrombocytopenia, and platelets have been evaluated as a special targeting mechanism (Fang et al., 2024).

Immune system cells such as leukocytes (neutrophils, eosinophils, basophils, lymphocytes), lymphocytes (T cells, NK cells), and monocytes play an important role in the immune response of pathologies such as autoimmune diseases, infections and especially cancer. However, these cells are used as carriers for drugs with different activities. Monocytes, which have a nucleated structure, create an innate immune response against various pathogens such as bacteria and fungi, unlike erythrocytes and platelets. At the same time, monocytes found in the infection and tumor microenvironment attract other monocytes to the target area via the chemotactic-1 protein (Wu et al., 2022;

Fan et al., 2023). Neutrophils, on the other hand, respond more rapidly in acute inflammation, settling in inflammatory tissue and secreting cytokines for the migration of other cells to the region. However, failure to control neutrophil infiltration can also lead to various inflammatory diseases such as chronic obstructive lung disease (COPD), atherosclerosis, and diabetes mellitus. Due to their ability to migrate to the inflammation region, nanovesicles have been developed from neutrophil membranes to be used in the treatment of diseases such as rheumatoid arthritis, pneumonia, and cancer. It has been shown that these vesicles accumulate in the liver after systemic injection (Chu et al., 2018; Herrero-Cervera et al., 2022). In another study, the migration of the carrier system to the inflammation area was evaluated in the liver fibrosis-inflammation model by loading drugs into neutrophils. According to the results obtained, it was reported that the inflammatory lesion areas in the liver were specifically targeted (Sun et al., 2024). Neutrophils have also been evaluated as cellular carriers in the treatment of atherosclerosis, where neutrophil-mediated inflammation is effective (Zhao et al., 2023). Since the half-life of neutrophils in the bloodstream is 8 hours and the risk of drug toxicity is low, the use of neutrophils as carriers for drug targeting to infected and tumorous areas is promising in treatment (Yang et al., 2022; Zhang et al., 2023). However, like other cellular carrier systems, the difficulties in large-scale production of neutrophils and their safety in clinical applications are among the challenges that need to be overcome (Zhao et al., 2023).

Lymphocytes are specifically targeted to many areas, especially inflammation areas like leukocytes. T lymphocyte cells are produced from pluripotent cells in the bone marrow. T cells, which have chemotactic properties, mediate the secretion of proteins that will trigger the death of the target cell with receptors on their surface. In addition, their properties such as inducing apoptosis and expressing antigen-specific receptors allow the use of T cells as carriers in the detection or treatment of malignancies (Choi et al., 2023). For this purpose, research is in the clinical phase of the study for the treatment of lymphoblastic leukemia as well as Hodgkin lymphoma by loading chimeric antigen receptors onto T cells (Phase-1, NCT05420493).

Another representative of lymphocyte cells, NK cells, constitute 30% of circulating lymphocytes. While T cells can only target specific receptors in the immune response, NK cells can also target tumor cells through alternative receptors and kill them directly. In addition, NK cells can create an immune response independent of antigens and are used to target the immune system

by increasing cell infiltration. Despite these advantages, their use is limited due to the high cost of carrier NK cells and difficulties in storage (Yang et al., 2022; Fan et al., 2023).

Stem cells, which are used as carrier systems as well as blood and immune system cells, are undifferentiated cells that have the ability to self-renew and regenerate functional tissues. Stem cells, which have a wide range of applications, are classified according to their differentiation potential and origin. According to their potential, stem cells are divided into totipotent, pluripotent, multipotent, oligopotent, and unipotent. Pluripotent cells consist of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Multipotent stem cells include hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), neural stem cells (NSCs), and endothelial stem/progenitor cells (EPCs) (Tan et al., 2024). Stem cells can respond to microenvironmental conditions to direct differentiation and cellular activity. Although the mechanism has not been fully elucidated, it has been proven that transplanted stem cells can differentiate into neurons, glia, and vascular cells through trophic factors. Based on these effects, many administration routes such as intraventricular, intrathecal, intranasal, and intravenous have been investigated, and stem cell-based treatments have come to the fore in diseases where multiple nerve cells are damaged, such as cerebral palsy (Jantzie et al., 2018; Cox et al., 2022).

Stem cells, which can renew themselves and differentiate into the cell types required for physiological tissue repair, also play a critical role in wound healing. Due to the difficulty of direct application for skin regeneration, stem cells have been evaluated as drug delivery systems. Stem cells used in this context include ESC, MSC, and iPSC. It has been reported that MSCs act through tissue regeneration and inflammation regulation in various dermatological diseases such as diabetic wounds, burns, atopic dermatitis, lupus erythematosus, and psoriasis. It has been stated that MSCs show their wound healing effect by increasing the levels of factors such as EGF, hepatocyte growth factor (HGF), VEGF, FGF as well as MMPs (Riedl et al., 2021). In a study, MSCs were targeted to the wound area with carboxyethyl chitosan hydrogel for the treatment of diabetic foot wounds and exhibited healing effects by promoting angiogenesis (Geng et al., 2022).

It is known that stem cells also target the lungs, bone marrow, and lymphoid structures, and play a role in neurodegenerative disease-related pathways by regulating the activity of dendritic cells. In addition, anticancer drugs are loaded into stem cells in the form of nanoparticles or vesicles. However, the use of stem cells as drug carriers is limited due to their tendency

to accumulate in non-target tissues, the invasiveness of the harvesting method, the fact that they cause immunogenicity and tumorigenicity, and ethical dilemmas (Wu et al., 2019; Levy et al., 2021; Zhang and Huang, 2023). These limitations, as well as the lack of understanding of the interactions between carrier cells and the human body, make it difficult to assess potential toxicity. More research is needed to clearly understand the safety and toxicity profile of promising cell-based carriers in clinical use (Chan et al., 2022).

Cell Targeting Mechanisms in Cell-Based Drug Delivery Systems

Drug-loaded carrier cells can reach many areas with the bloodstream. Based on the natural targeting mechanism of these cells, which can migrate to pathological areas including cancerous, damaged, ischemic or wounded tissues, disease-specific targeted treatments are being developed. Natural processes such as the rapid migration of leukocytes to tissues infected with lipogenic pathogens and platelets to injury sites have been evaluated as targeting mechanisms (Yu et al., 2020). The ability of MSCs to accumulate in tumors is due to their sensitivity to tissue damage, hypoxia, and inflammation in tumor microenvironments. NK cells, which are widely used for immunosuppressive and antineoplastic treatments, can target tumors by recognizing tumor cells and forming immunological synapses (Lu et al., 2023). Macrophages, like NK cells, can also be targeted to tumor cells. In a previous study conducted for the treatment of glioma, nanoparticles were loaded into macrophages, and it was shown that the nanoparticle concentration in the environment could remain constant with the macrophage-tumor interaction. In targeting erythrocytes, their sensitivity to oxygen is used. Erythrocytes, which are used as carriers for antioxidant nanoparticles in ischemia, are targeted to regulate the oxygen flow in the environment and to cleanse free radicals through nanoparticles (Li et al., 2021; Pang et al., 2018). In this context, the advantages of pathological tissues being different from healthy tissues have been used; cell-based drug delivery systems have been investigated in brain tumors, ischemic stroke and various neurodegenerative diseases (de la Torre et al., 2020; Li et al., 2021).

Significant changes occur in the expression of some specific receptors, especially in pathological tissues. Macrophages, which are found in high numbers in solid tumors, can promote the migration of other macrophages to the tumor region with chemotactic proteins such as chemokine ligand 2 (CCL2) and chemokine receptor 4 (CXCR4) on their

surfaces (Lu et al., 2023). The response of carrier cells to different microenvironments varies depending on the signaling of chemokines and growth factors (Choi et al., 2023). In this context, microenvironmental conditions such as hypoxic environment, increased inflammation and changes in receptor expression are taken into account in the targeting mechanisms of cells.

Hypoxic Microenvironment

Cancer cells consume high amounts of energy due to their rapid proliferation. When their oxygen requirements for metabolism are not supplied, they change their metabolism and form new vascular structures with irregular angiogenesis. Although the expression of erythropoietin and angiogenic factors increases, irregular and dysfunctional vascularization contributes to the formation of a hypoxic environment in the cancer cell environment. The oxygen pressure, which is approximately 40 mm Hg in healthy tissues, can drops to 2.5 mm Hg in tumor regions (Chen et al., 2023; Lu et al., 2023). When the microenvironment of cancerous tissues is examined, monocytes, tumor-associated macrophages, and lymphocytes are generally detected. This shows that white blood cells can penetrate deep into the tumor. In a study conducted in this context, it was reported that monocytes/macrophages can reach hypoxic environments that free nanoparticles cannot reach and that these carriers are used as ‘‘Trojan horses’’ (Choi et al., 2007; Yu et al., 2020).

Another condition where hypoxic environment occurs is ischemic stroke. Ischemic stroke is a fatal condition that results in significant reduction in blood flow and hypoxia after thrombosis or embolism. During ischemic stroke, endogenous antioxidants cannot detoxify the reactive oxygen species (ROS) present in high levels in the environment, and this can lead to brain damage and infarction (Li et al., 2021). Inflammation accompanying hypoxia, promotes the migration of leukocytes to the region, thus enabling the infiltration of cells into the hypoxic environment. An *in vivo* study has shown that monocyte/neutrophil carriers can successfully deliver cyclic arginylglycylaspartic acid (cRGD) liposomes to the brain in a cerebral ischemia model (Hou et al., 2019). In this context, a good definition of the disease microenvironment has become an important starting point for targeting drug carrier cells to tissues.

Inflammation Environment

Inflammation is the body's response to tissue damage caused by physical or ischemic damage, exposure to toxins or pathogens. Chronic inflammation is known to mediate a variety of diseases, including cancer, diabetes, and arthritis, as well as neurodegenerative diseases, cardiovascular diseases, and autoimmune diseases (Singh et al., 2019). Immune system cells such as leukocytes, lymphocytes, and monocytes, which are responsible for generating innate and adaptive immune responses to inflammation, can be rapidly attracted to areas of inflammation (Fan et al., 2023). With the coming together of millions of immune cells that can respond rapidly through activation, adhesion and migration along the endothelial vessels, drug delivery to inflamed areas is possible. Immune system cells that have been shown to be able to pass through the BBB and even the endothelial barrier, which poses difficulties in drug delivery with its structure smaller than 3 nm, work as ‘‘Trojan horses’’ (Choi et al. 2023). It is thought that cells achieve these effects through their chemotactic and phagocytic properties (Rong et al., 2022). For instance, neutrophils provide a rapid response in acute inflammation and can stimulate the migration of other cells to the area by secreting various cytokines (Chu et al., 2018). The natural process of migration of leukocytes such as T cells, macrophages, neutrophils, NK cells, and monocytes to tumor sites allows them to target inflammation and deliver drugs to the tumor (Lu et al., 2023).

In addition to immune cells, stem cells can also generate inflammatory responses. MSCs are promising candidates for the development of cell-based drug delivery systems for the treatment of autoimmune inflammatory diseases such as multiple sclerosis (MS) (Pozo et al., 2021). In mice with an experimental autoimmune encephalomyelitis model, an anti-inflammatory compound (Ro-31-8425) loaded into MSCs was administered intravenously. It has been reported that the effect of the drug using the MSC carrier is much greater than the free effect of the drug alone and that MSCs are targeted to the inflamed area (Levy et al., 2021).

Receptor-Ligand Interaction

Receptor-ligand interactions play an important role in the effect of drugs. Active targeting in particular is based on ligand-receptor interactions of receptors and modified carriers that mediate cellular uptake. Uptake of drugs into the cell is initiated by the recognition of specific ligands by cell

surface receptors. Thus, target-specific drug transport can be achieved by ligands that will interact with receptors on the targeted cell type (Steffens et al., 2023). Drugs are directed to the target area based on significant changes in the expression of some specific receptors, especially in pathological tissues (Lu et al., 2023). It is known that vascular cell adhesion molecule-1 (VCAM-1) is found at higher levels on the surface of breast cancer cells, which are among the cells with increased specific receptor expression, compared to healthy tissues. The interaction between α4-integrins on the surface of macrophages and VCAM-1, has led to the idea of using macrophages as carriers. Indoximod, an indoleamine 2,3-dioxygenase (IDO-1) inhibitor used as an antineoplastic agent in breast cancer, has been targeted to tumor areas by coating with macrophage membranes and has been shown to regress metastasis (Liu et al., 2020). In addition, it has been shown that classically activated macrophages (M1) target the tumor site more specifically than alternatively activated macrophages (M2). This is due to the fact that M1 macrophages can identify tumor cells by upregulating specific chemokines (Hu et al., 2020). Furthermore, based on the specific binding between CD44, whose expression is increased in breast cancer cells, and platelet P-selectin, doxorubicin covered with platelet membranes was targeted to the tumor region and anticancer activity was increased. Based on this study, bionic gold nanocages coated with platelet and neutrophil hybrid membranes were developed (Ye et al., 2020). In this context, it has been shown that it is possible to direct the cells to be used as carriers to different targets with various membrane modifications (Sun et al., 2017).

Drug Loading Methods in Cell-Based Drug Delivery Systems

Complexes formed by drug/drug-loaded nanoparticles and cells for an effective and safe treatment;

- Should not be toxic to healthy cells,
- Should have a long blood circulation time,
- Should form strong bonds,
- Should be able to release the drug in the target area,
- Should be eliminated when the effect ends.

In this context, methods such as modification of the cell surface and loading of drugs into the cell are used to form complexes with living cells. Each of the methods has advantages and disadvantages, and should be selected by evaluating the characteristics of the cell selected as the carrier and the drug to be loaded (Yu et al., 2022; Choi et al., 2023). Drug loading methods in cell-based drug delivery systems are shown in Table 1 (Table1). Also, in studies using carrier cells, cell and drug types, drug loading methods and the therapeutic applications are compiled in Table 2 (Table 2).

Table 1. Drug Loading Methods in Cell-Based Drug Delivery Systems (Wu et al., 2022; Choi et al., 2023)

Cell surface drug modification	**Intracellular drug loading**
1. **Chemical modification** Ligand-Receptor Binding Biotin-avidin linkage Glycoengineering Cell backpack **2. Physical modification**	**1. Encapculation** **2. Phagocytosis** **3. Electroporation** **4. Hypotonic Swelling** **5. Genetic transfection** **6. Loading in the open canalicular system**

Table 2. Applications of Cell-Based Drug Delivery Systems and Drug Loading Methods

Cell Types	Loading Drugs	Therapeutic applications	Drug Loading Methods	References
Erythrocyte	Ce6 conjugated-Doxorubicin	Breast cancer	Physical modification	Gao et al., 2017
MSC	Doxorubicin	Lung Cancer	Biotin-avidin linkage	Yao et al., 2017
NSC	Docetaxel	Intratumoral therapy	Biotin-avidin linkage	Mooney et al., 2014
MSC	Paclitaxel-loaded nanoparticles	Ovarian cancer	Glycoengineering	Layek et al., 2020
T cells	Protein nano-gels	Glioblastoma, melanoma	Cell backpack	Tang et al., 2018
Monocytes	mRNA	Cell-based therapies	Electroporation	Dannull and Nair, 2016
Platelets	Iron oxide magnetic nanoparticles	Anti-platelet therapy	Loading in the open canalicular system	Cabrera et al., 2020
MSC	Oncolytic reovirus	Acute myeloid leukemia	Genetic Transfection	Wang et al., 2021
Platelets	Doxorubicin, CD22 monoclonal antibody	B-cell lymphoma	Ligand-Receptor Binding	Xu et al., 2017
ADSCs	Paclitaxel	Ovarian Cancer	Cell surface drug modification	Borghese et al., 2020
Monocytes	Astaxanthin, trametinib loaded-nanoparticles	Idiopathic pulmonary fibrosis	Bioconjugation	Chang et al., 2020

Abbreviations: ADSCs: Adipose-derived stem cells, Ce6: Chlorin e6, mRNA: Messenger ribonucleic acid

Living cells have been investigated as carriers for drug targeting in various clinical studies. Clinical studies on cell-based drug delivery systems are compiled in Table 3 (Table 3).

Table 3. Clinical Applications of Cell-Based Drug Delivery Systems

Cell Type	**Loading Drugs**	**Therapeutic applications**	**Phase**	**ClinicalTrials.Gov Identifier/Ref**
Erythrocytes	L-Asparaginase	Pancreatic Adenocarcinoma	Phase 1	NCT01523808
Erythrocytes	L-Asparaginase	Acute Lymphoblastic Leukemia	Phase 3	NCT01518517
Erythrocytes	Dexamethasone sodium phosphate	Cystic fibrosis, Inflammatory bowel disease	Phase 1	NCT02380924
Erythrocytes	Pemrolizumab	Solid tumors, AML	Phase 2	NCT04372706
Monocytes	CMV pp65-LAMP mRNA	Glioblastoma	Phase 1	NCT04741984
Macrophages	Anti-HER2 CAR-M	HER2-positive adenocarcinoma	Phase 1	NCT04660929
MSCs	TRAIL	Lung Cancer	Phase 2	NCT03298763
MSCs	Interferon beta	Ovarian Cancer	Phase 1	NCT02530047
MSCs	Prochymal™	Crohn's Disease	Phase 3	NCT01233960
ADSCs	Cx601	Crohn's Disease	Phase 3	NCT03279081
PBMCs	siRNA	Solid Tumors	Phase 1	NCT06172894
NKs	FT538 – Daratumumab combination	AML	Phase 1	NCT04714372
T Cells	CAR-T	Colorectal cancer	Phase 2	NCT02959151
NSCs	SN-38	Glioblastoma	Phase 1	NCT02192359

Abbreviations: AML: Acute myeloid leukemia, CAR-T: Chimeric antigen -T receptors, CMV: Cytomegalovirus, HER2: Human epidermal growth factor 2, LAMP:

Lysosome-associated membrane protein, PBMCs: Peripheral blood mononuclear cells, siRNA: Small interfering ribonucleic acid, TRAIL: TNF-related apoptosis-inducing ligand

Advantages of Cell-Based Drug Delivery Systems

In addition to the low solubility and bioavailability of the drugs used in the treatment, there is a risk of toxicity in healthy cells due to their effect outside the target tissue. At the same time, various immunogenicity reactions can be observed because the body perceives the drug as an antigen. Nanoformulations have been developed to overcome these problems. However, their undesirable toxicity has made their effectiveness and safety in clinical use controversial. In addition, the difficulties in specific targeting, as well as the difficulties in production and high costs, limit the use of nanoformulations (Yang et al., 2022; Wu et al., 2022). Recent studies have shown that using autologous cells or their membranes as a carrier has several advantages over nanoformulations. When the drug is loaded in the cell by various methods or covered with the cell membrane, its biological structure and functions are preserved. Since they are part of the physiological structure, drug carrier cells, which show high biocompatibility, also undergo apoptosis through a natural process after releasing the drug (Timin et al., 2018). When biodegradable cells are used as carriers, the risk of metabolite-induced toxicity in healthy cells is reduced. Especially hemolyzed erythrocytes are recognized by RES and rapidly destroyed without producing toxic byproducts (Rong et al., 2022). Since erythrocytes with intact cell integrity can escape phagocytosis with specific receptors, their potential to reach the target tissue increases and the risk of toxicity for healthy cells decreases. It has been reported that erythrocytes show this effect without any protein accumulation, immunoglobulin G (IgG) or immunoglobulin M (IgM) interaction. This situation, which increases the circulation time of the drug in the blood, allows the cells to escape opsonization and increase targeting (Della Pelle and Kostevšek, 2021). In addition, the wide range of circulating red blood cells, the ability of stem cells, macrophages and neutrophils to find inflammatory targets, the ability of T cells and NK cells to recognize and kill tumors, and the tendency of platelets to accumulate in damaged tissues make targeting specific and reduce the risk of toxicity (Yang et al., 2022; Choi et al., 2023).

Physiological barriers are an important obstacle for nanoformulations. However, macrophages, NSCs, and MSCs can cross the BBB, so they are targeted for neurodegenerative diseases such as Parkinson's Disease and Alzheimer's Disease (Rong et al., 2022). The physiological tendency of cells to migrate to various regions provides an advantage in drug targeting. The retention of erythrocytes with impaired cell integrity by RES has enabled the use of erythrocytes to target the RES of the liver, spleen and bone marrow (Koleva et al., 2020). It is known that this migration is due to the sensitivity of carrier cells to microenvironment differences between healthy and pathological tissues. It has been shown that macrophages and platelets respond to specific receptors that increase on the surface of tumor cells, especially in cancer (Liu et al., 2020; Ye et al., 2020). Once more, in contrast to healthy tissues, increased inflammation in the injury area accelerates the chemotaxis of platelets and the wound healing process accordingly (Bonferoni et al., 2019; Dai et al., 2022). In this context, specific treatment of the disease with the cells' natural targeting mechanisms is an important strategy (Chen et al., 2023; Zhao et al., 2023).

Cell surface features provide several advantages for drug loading, in contrast to nanoformulations. Among these advantages are the relatively easy cell isolation and high drug loading capacity. A wide variety of materials can be loaded into cells, such as liposomes and nanoparticles, bioactive substances (nucleoside/nucleotide analogs, glucocorticoid analogs, enzymes, peptides, toxins) and some simple vectors (Sun et al., 2017). Particularly erythrocytes are an appropriate cell type for drug loading due to their large surface-volume area. 1000-2000 nano-erythrosomes of 200 nm can be obtained from a single erythrocyte. It has also been reported that the polarity and water solubility of the drug do not affect the loading efficiency in isotonic conditions (Della Pelle and Kostevšek, 2021). All these advantages make cells stand out for use as a drug delivery system.

Disadvantages of Cell-Based Drug Delivery Systems

There are advantages as well as disadvantages in using cells as drug delivery systems. Dosing is one of the most important disadvantages of delivery systems. The amount of dose to be applied has a critical role in ensuring that drugs remain in the therapeutic range for an ideal period and reach therapeutic concentrations. In delivery systems where living cells are

used, the bioactivity of the cells used should also be evaluated in addition to the dosing of drugs (Villa et al., 2017). There is a risk of thrombosis, especially after the application of platelets as carriers (Fan et al., 2023). Once more, excessive infiltration and activation of neutrophils damages vascular endothelial cells and extravascular tissues through microcirculatory blockage. This results in inflammatory reactions and systemic side effects (Rong et al., 2022). The unpredictable biological functionality of the cells used as carriers may bind to non-target structures and lead to unwanted accumulation in other tissues (Chen et al., 2023). For instance, erythrocytes may undergo hemolysis before the drug reaches the tissue and may be released outside the target area, causing cytotoxicity (Yu et al., 2020). Modifications to be made in cell membranes to increase their stability may negatively affect the biocompatibility of cells (Sun et al., 2017; Chen et al., 2023). Furthermore, after excessive drug loading on their membranes, erythrocytes can be more easily removed from the bloodstream. This makes it difficult to use erythrocytes as carriers for non-RES targets (Yu et al., 2020; Fan et al., 2023). Although it has been reported that carrier cells to be used in targets other than RES escape phagocytosis with receptors on their surfaces, some can still be removed by the immune system. It has been reported that platelet membrane drug delivery systems cannot accumulate in the liver and kidneys, and therefore it is difficult to target platelets in these areas. Similarly, this situation is also present in other surface modified cells. Therefore, it is important to evaluate the size and morphology of the cells used as carriers and optimize them for the carrier system (Fang et al., 2024). In addition, the possibility of drugs to be loaded into phagocytic cells such as macrophages being broken down in the cytoplasm should be taken into account when selecting drugs and cells (Fan et al., 2023).

Chimeric antigen-T receptor (CAR-T) cell therapies, which have attracted attention in anticancer treatment in recent years, involve the re-modification of T cells isolated from patients with a specific ‘‘CAR’’ to effectively target tumor cells. However, when T cells are re-transplanted into patients as drug carriers with various modifications, it has been reported that other T cells experience a decrease in cell viability and function (Fan et al., 2023). Stem cells have regenerative, anti-inflammatory, and immunomodulatory properties, and while their natural tumor tropism offers several advantages in tumor targeting, it can also intricately trigger inflammation, immunogenicity, and carcinogenesis. Stem cells also have

challenges to overcome, such as poor post-transplant retention and low drug loading efficiency (Choi et al., 2023; Fan et al., 2023).

Successful results have been achieved in studies conducted with cell-based drug delivery systems that are promising in treatment, despite the various difficulties. However, in order to elucidate the unpredictable relationships between carrier cells and the complex physiology of the body and to find a place in the clinic, the production, characterization and applications of cell-based drug delivery systems need to be standardized (Chan et al., 2022; Mozafari et al., 2023).

Conclusion

Cell-based drug delivery systems are systems that use cells such as erythrocytes, platelets, macrophages, and neutrophils to transport various bioactive compounds to target sites. In addition to their advantages such as immunogenicity and low risk of toxicity in healthy cells, biocompatibility, and ability to accumulate in the targeted area, there are various difficulties in their dosing and production. Further research is needed to evaluate the potential risks of using cells for drug delivery and to elucidate the mechanisms.

REFERENCES

Bonferoni, M. C., Rossi, S., Sandri, G., Caramella, C., Del Fante, C., Perotti, C., … & Ferrari, F. (2019). Bioactive medications for the delivery of platelet derivatives to skin wounds. *Current Drug Delivery, 16*(5), 472–483.

Borghese, C., Casagrande, N., Corona, G., & Aldinucci, D. (2020). Adipose-derived stem cells primed with paclitaxel inhibit ovarian cancer spheroid growth and overcome paclitaxel resistance. *Pharmaceutics*, *12*(5), 401.

Cabrera, D., Walker, K., Moise, S., Telling, N. D., & Harper, A. G. S. (2020). Controlling human platelet activation with calcium-binding nanoparticles. *Nano Research*, *13*(10), 2697–2705.

Cecerska-Heryć, E., Goszka, M., Serwin, N., Roszak, M., Grygorcewicz, B., Heryć, R., & Dołęgowska, B. (2022). Applications of the regenerative capacity of platelets in modern medicine. *Cytokine & Growth Factor Reviews*, *64*, 84–94.

Chan, W. J., Li, H., & Dehestani, S. (2022). Cell-based drug-delivery systems: a possible solution to improve nanomedicine for cancer treatment?. *Nanomedicine*, *17*(6), 349–352.

Chang, X., Xing, L., Wang, Y., Yang, C. X., He, Y. J., Zhou, T. J., … & Jiang, H. L. (2020). Monocyte-derived multipotent cell delivered programmed therapeutics to reverse idiopathic pulmonary fibrosis. *Science Advances*, *6*(22), eaba3167.

Chen, Z., Han, F., Du, Y., Shi, H., & Zhou, W. (2023). Hypoxic microenvironment in cancer: molecular mechanisms and therapeutic interventions. *Signal Transduction and Targeted Therapy*, *8*(1), 70.

Choi, A., Javius-Jones, K., Hong, S., & Park, H. (2023). Cell-based drug delivery systems with innate homing capability as a novel nanocarrier platform. *International Journal of Nanomedicine*, *18*, 509–525.

Chu, D., Dong, X., Shi, X., Zhang, C., & Wang, Z. (2018). Neutrophil-based drug delivery systems. *Advanced materials*, *30*(22), e1706245.

ClinicalTrials.gov (2016). *A study of chimeric antigen receptor T cells combined with interventional therapy in advanced liver malignancy*. (Accessed on 10.08.2024, https://clinicaltrials.gov/study/NCT02959151)

ClinicalTrials.gov (2016). *Recovery and survival of erydex in non-patient volunteers.* (Accessed on 10.08.2024, https://clinicaltrials.gov/study/NCT02380924?term=Dexamethasone&intr=RBC&rank=1

ClinicalTrials.gov (2019). *Mesenchymal stem cells (MSC) for ovarian cancer*. (Accessed on 10.08.2024, https://clinicaltrials.gov/study/NCT02530047

ClinicalTrials.gov (2021). *Administration of GRASPA (Suspension of erythrocytes encapsulating L-asparaginase) in patients with pancreatic cancer*. (Accessed on 10.08.2024, https://clinicaltrials.gov/study/NCT01523808)

ClinicalTrials.gov (2021). *Evaluation of Prochymal® for treatment-refractory moderate-to-severe Crohn's disease.* (Accessed on 10.08.2024, https://clinicaltrials.gov/study/NCT01233960?intr=mesenchymal%20stem%20cells&term=prochymal&cond=Crohn%27s%20Disease&rank=2)

ClinicalTrials.gov (2021). *Targeted stem cells expressing trail as a therapy for lung cancer (Tactical).* (Accessed on 10.08.2024, https://clinicaltrials.gov/study/NCT03298763?term=stem%20cell&intr=TRAIL&rank=1)

ClinicalTrials.gov (2022). *GRASPA (erythrocytes encapsulating L-asparaginase) in patients with relapse of acute lymphoblastic leukemia (Graspivotall).* (Accessed on 10.08.2024, https://clinicaltrials.gov/study/NCT01518517)

ClinicalTrials.gov (2022). *RTX-240 monotherapy and in combination with pembrolizumab.* (Accessed on 12.08.2024, https://clinicaltrials.gov/study/NCT04372706)

ClinicalTrials.gov (2023). *Monocyte antigen carrier cells for newly diagnosed GBM (Demand).* (Accessed on 12.08.2024, https://clinicaltrials.gov/study/NCT04741984)

ClinicalTrials.gov (2024). Carboxylesterase-*expressing allogeneic neural stem cells and irinotecan hydrochloride in treating patients with recurrent high-grade gliomas.* (Accessed on 12.08.2024, https://clinicaltrials.gov/study/NCT02192359?term=SN-38&intr=stem%20cell&rank=1)

ClinicalTrials.gov (2024). *CAR-macrophages for the treatment of HER2 overexpressing solid tumors.* (Accessed on 12.08.2024, https://clinicaltrials.gov/study/NCT04660929?cond=HER2-%20adenocarcinoma&term=macrophages&rank=1)

ClinicalTrials.gov (2024). *Clinical study of chimeric antigen receptor T lymphocytes (CAR-T) in the treatment of relapsed/refractory non-hodgkin lymphoma.* https://clinicaltrials.gov/study/NCT05420493?cond=hodgkin&intr=t%20cells&rank=1)

ClinicalTrials.gov (2024). *FT538 in combination with daratumumab in AML acute myeloid leukemia.* (Accessed on 12.08.2024, https://clinicaltrials.gov/study/NCT04714372?a=3)

ClinicalTrials.gov (2024). *PBMC-based leukocyte immunotherapy (Palindrom).* (Accessed on 12.08.2024, https://clinicaltrials.gov/study/NCT06172894?intr=leukocyte-based&rank=2)

ClinicalTrials.gov (2024). *Study to assess efficacy and safety of CX601, adult allogeneic expanded adipose-derived stem cells (EASC) for the treatment of complex perianal fistula(s) in participants with Crohn's disease (CD) (admire-cd-ii).* (Accessed on 14.08.2024, https://clinicaltrials.gov/study/NCT03279081)

Cox, C. S., Jr, Juranek, J., Kosmach, S., Pedroza, C., Thakur, N., Dempsey, A., … & Savitz, S. I. (2022). Autologous cellular therapy for cerebral palsy: A randomized, crossover trial. *Brain Communications*, *4*(3), fcac131.

Dai, Z., Zhao, T., Song, N., Pan, K., Yang, Y., Zhu, X., … & Xia, C. (2022). Platelets and platelet extracellular vesicles in drug delivery therapy: A review of the current status and future prospects. *Frontiers in Pharmacology*, *13*, 1026386.

Dannull, J., & Nair, S. K. (2016). Transfecting human monocytes with RNA. *Methods in Molecular Biology*, *1428*, 177–186.

de la Torre, P., Pérez-Lorenzo, M. J., Alcázar-Garrido, Á., & Flores, A. I. (2020). Cell-based nanoparticles delivery systems for targeted cancer therapy: Lessons from anti-angiogenesis treatments. *Molecules*, *25*(3), 715.

Della Pelle, G., & Kostevšek, N. (2021). Nucleic acid delivery with red-blood-cell-based carriers. *International Journal of Molecular Sciences*, *22*(10), 5264.

Fan, X., Wang, K., Lu, Q., Lu, Y., & Sun, J. (2023). Cell-based drug delivery systems participate in the cancer immunity cycle for improved cancer immunotherapy. *Small*, *19*(4), e2205166.

Fang, M., Liu, R., Fang, Y., Zhang, D., & Kong, B. (2024). Emerging platelet-based drug delivery systems. *Biomedicine & Pharmacotherapy*, *177*, 117131.

Ganz T. (2018). Iron and infection. *International Journal of Hematology*, *107*(1), 7–15.

Gao, M., Hu, A., Sun, X., Wang, C., Dong, Z., Feng, L., & Liu, Z. (2017). Photosensitizer decorated red blood cells as an ultrasensitive light-responsive drug delivery system. *ACS Applied Materials & Interfaces*, *9*(7), 5855–5863.

Geng, X., Qi, Y., Liu, X., Shi, Y., Li, H., & Zhao, L. (2022). A multifunctional antibacterial and self-healing hydrogel laden with bone marrow mesenchymal stem cell-derived exosomes for accelerating diabetic wound healing. *Biomaterials Advances*, *133*, 112613.

Herrero-Cervera, A., Soehnlein, O., & Kenne, E. (2022). Neutrophils in chronic inflammatory diseases. *Cellular & Molecular Immunology*, *19*(2), 177–191.

Hou, J., Yang, X., Li, S., Cheng, Z., Wang, Y., Zhao, … & Qin, J. (2019). Accessing neuroinflammation sites: Monocyte/neutrophil-mediated drug delivery for cerebral ischemia. *Science Advances*, *5*(7), eaau8301.

Hu, C., Lei, T., Wang, Y., Cao, J., Yang, X., Qin, L., … & Gao, H. (2020). Phagocyte-membrane-coated and laser-responsive nanoparticles control primary and metastatic cancer by inducing anti-tumor immunity. *Biomaterials*, *255*, 120159.

Jantzie, L. L., Scafidi, J., & Robinson, S. (2018). Stem cells and cell-based therapies for cerebral palsy: A call for rigor. *Pediatric Research*, *83*(1-2), 345–355.

Koleva, L., Bovt, E., Ataullakhanov, F., & Sinauridze, E. (2020). Erythrocytes as carriers: From drug delivery to biosensors. *Pharmaceutics*, *12*(3), 276.

Layek, B., Shetty, M., Nethi, S. K., Sehgal, D., Starr, T. K., & Prabha, S. (2020). Mesenchymal stem cells as guideposts for nanoparticle-mediated targeted drug delivery in ovarian cancer. *Cancers*, *12*(4), 965.

Levy, O., Rothhammer, V., & Mascanfroni, I. (2021). A cell-based drug delivery platform for treating central nervous system inflammation. *Journal of Molecular Medicine*, *99*(5), 663–671.

Li, Y. J., Wu, J. Y., Liu, J., Qiu, X., Xu, W., Tang, T., & Xiang, D. X. (2021). From blood to brain: Blood cell-based biomimetic drug delivery systems. *Drug Delivery*, *28*(1), 1214–1225.

Liu, R., An, Y., Jia, W., Wang, Y., Wu, Y., Zhen, Y., … & Gao, H. (2020). Macrophage-mimic shape changeable nanomedicine retained in tumor for multimodal therapy of breast cancer. *Journal of Controlled Release*, *321*, 589–601.

Lu, Q., Liu, T., Han, Z., Zhao, J., Fan, X., Wang, H., … & Sun, J. (2023). Revolutionizing cancer treatment: The power of cell-based drug delivery systems. *Journal of Controlled Release*, *361*, 604–620.

Lu, Y., Hu, Q., Jiang, C., & Gu, Z. (2019). Platelet for drug delivery. *Current Opinion in Biotechnology*, *58*, 81–91.

Mooney, R., Weng, Y., Garcia, E., Bhojane, S., Smith-Powell, L., Kim, S. U., … & Berlin, J. M. (2014). Conjugation of pH-responsive nanoparticles to neural stem cells improves intratumoral therapy. *Journal of Controlled Release*, *191*, 82–89.

Mozafari, N., Mozafari, N., Dehshahri, A., & Azadi, A. (2023). Knowledge gaps in generating cell-based drug delivery systems and a possible meeting with artificial intelligence. *Molecular Pharmaceutics*, *20*(8), 3757–3778.

Pang, L., Zhu, Y., Qin, J., Zhao, W., & Wang, J. (2018). Primary M1 macrophages as multifunctional carrier combined with PLGA nanoparticle delivering anticancer drug for efficient glioma therapy. *Drug Delivery*, *25*(1), 1922–1931.

Pieracci, F. M., & Barie, P. S. (2005). Iron and the risk of infection. *Surgical Infections*, *6*(1), 41–46.

Pozo D. (2021). Cell-based drug delivery harnesses inflammatory and autoimmune responses in neurodegeneration. *Journal of Molecular Medicine*, *99*(5), 673–674.

Rapido F. (2017). The potential adverse effects of haemolysis. *Blood Transfusion*, *15*(3), 218–221.

Riedl, J., Popp, C., Eide, C., Ebens, C., & Tolar, J. (2021). Mesenchymal stromal cells in wound healing applications: Role of the secretome, targeted delivery and impact on recessive dystrophic epidermolysis bullosa treatment. *Cytotherapy*, *23*(11), 961–973.

Rong, R., Raza, F., Liu, Y., Yuan, W. E., Su, J., & Qiu, M. (2022). Blood cell-based drug delivery systems: A biomimetic platform for antibacterial therapy. *European Journal of Pharmaceutics and Biopharmaceutics*, *177*, 273–288.

Shah, A. A., Donovan, K., Seeley, C., Dickson, E. A., Palmer, A. J. R., Doree, C., … & Stanworth, S. J. (2021). Risk of infection associated with administration of intravenous iron: A systematic review and meta-analysis. *Journal of the American Medical Association*, *4*(11),e2133935.

Singh, N., Baby, D., Rajguru, J. P., Patil, P. B., Thakkannavar, S. S., & Pujari, V. B. (2019). Inflammation and cancer. *Annals of African medicine*, *18*(3), 121–126.

Steffens, R. C., & Wagner, E. (2023). Directing the way-receptor and chemical targeting strategies for nucleic acid delivery. *Pharmaceutical Research*, *40*(1), 47–76.

Sun, D., Du, X., Cao, X., Wu, B., Li, S., Zhao, Y., … & Huang, H. (2024). Neutrophil-based bionic delivery system breaks through the capillary barrier of liver sinusoidal endothelial cells and inhibits the activation of hepatic stellate cells. *Molecular Pharmaceutics*, *21*(4),2043–2057.

Sun, Y., Su, J., Liu, G., Chen, J., Zhang, X., Zhang, R., … & Qiu, M. (2017). Advances of blood cell-based drug delivery systems. *European Journal of Pharmaceutical Sciences*, *96*, 115–128.

Tan, F., Li, X., Wang, Z., Li, J., Shahzad, K., & Zheng, J. (2024). Clinical applications of stem cell-derived exosomes. *Signal Transduction and Targeted Therapy*, *9*(1), 17.

Tang, L., Zheng, Y., Melo, M. B., Mabardi, L., Castaño, A. P., Xie, Y. Q., … & Irvine, D. J. (2018). Enhancing T cell therapy through TCR-signaling-responsive nanoparticle drug delivery. *Nature Biotechnology*, *36*(8), 707–716.

Timin, A. S., Litvak, M. M., Gorin, D. A., Atochina-Vasserman, E. N., Atochin, D. N., & Sukhorukov, G. B. (2018). Cell-based drug delivery and use of nano-and microcarriers for cell functionalization. *Advanced Healthcare Materials*, *7*(3), 10.1002/adhm.201700818.

Thiagarajan, P., Parker, C. J., & Prchal, J. T. (2021). How do red blood cells die?. *Frontiers in Physiology*, *12*, 655393.

Villa, C. H., Cines, D. B., Siegel, D. L., & Muzykantov, V. (2017). Erythrocytes as carriers for drug delivery in blood transfusion and beyond. *Transfusion Medicine Reviews*, *31*(1), 26–35.

Wang, X., Zhao, X., & He, Z. (2021). Mesenchymal stem cell carriers enhance anti-tumor efficacy of oncolytic virotherapy. *Oncology Letters*, *21*(4), 238.

Wu, H. H., Zhou, Y., Tabata, Y., & Gao, J. Q. (2019). Mesenchymal stem cell-based drug delivery strategy: From cells to biomimetic. *Journal of Controlled Release*, *294*, 102–113.

Wu, Y., Liu, Y., Wang, T., Jiang, Q., Xu, F., & Liu, Z. (2022). Living cell for drug delivery. *Engineered Regeneration*, *3*(2), 131-148.

Xu, P., Zuo, H., Zhou, R., Wang, F., Liu, X., Ouyang, J., & Chen, B. (2017). Doxorubicin-loaded platelets conjugated with anti-CD22 mAbs: a novel targeted delivery system for lymphoma treatment with cardiopulmonary avoidance. *Oncotarget*, *8*(35), 58322–58337.

Yang, L., Yang, Y., Chen, Y., Xu, Y., & Peng, J. (2022). Cell-based drug delivery systems and their *in vivo* fate. *Advanced Drug Delivery Reviews*, *187*, 114394.

Yao, S., Li, X., Liu, J., Sun, Y., Wang, Z., & Jiang, Y. (2017). Maximized nanodrug-loaded mesenchymal stem cells by a dual drug-loaded mode for the systemic treatment of metastatic lung cancer. *Drug Delivery*, *24*(1), 1372–1383.

Ye, H., Wang, K., Lu, Q., Zhao, J., Wang, M., Kan, Q., … & Sun, J. (2020). Nanosponges of circulating tumor-derived exosomes for breast cancer metastasis inhibition. *Biomaterials*, *242*, 119932. *Advance online publication.*

Yu, H., Yang, Z., Li, F., Xu, L., & Sun, Y. (2020). Cell-mediated targeting drugs delivery systems. *Drug Delivery*, *27*(1), 1425–1437.

Zhang, F., Xu, Z., & Jolly, K. J. (2023). Myeloid cell-mediated drug delivery: From nanomedicine to cell therapy. *Advanced Drug Delivery Reviews*, *197*, 114827.

Zhang, W., & Huang, X. (2023). Stem cell-based drug delivery strategy for skin regeneration and wound healing: Potential clinical applications. *Inflammation and Regeneration*, *43*(1), 33.

Zhao, Y., Zhang, H., Zhang, Q., & Tao, H. (2023). Research progress of neutrophil-mediated drug delivery strategies for inflammation-related disease. *Pharmaceutics*, *15*(7), 1881.

EXOSOMES: POTENTIAL APPLICATIONS ON DRUG DELIVERY SYSTEMS

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Introduction

Extracellular vesicles (EVs) have been increasingly important in recent years for both understanding different disease processes and developing therapeutic targets for diseases. Small membrane-bound vesicles known as extracellular vesicles (EVs) are exuded from the cells of a variety of organisms, including prokaryotes and higher multicellular eukaryotes like plants. EVs are adept at carrying out the intra- and intercellular communications necessary for the proper operation of tissues and multiple organ systems (Rokad et al., 2020). Exosomes were initially identified in 1985 by Pan et al. (Pan et al., 1985). They are the smallest EVs, with a size range of roughly 50–150 nm (Samanta et al., 2018). Understanding exosomal characteristics and functions, as well as how size, shape, and surface proteins affect drug loading and delivery, requires characterization of exosomes. The shape, size, number, and surface protein compositions of exosomes can all be found using standard exosome characterization procedures (Patil et al., 2020). When exosomes are inside multivesicular bodies, they are referred to as intraluminal vesicles and have an endocytic origin. The merger of multivesicular entities with the plasma membrane releases them into the extracellular area. It was previously believed that exosomes were only waste of undesirable proteins, lipids, and other compounds. Immune cells, including tumour cells, red blood cells, platelets, lymphocytes, and dendritic cells, are the primary producers of exosomes in animals (Akuma et al., 2019). They can be found in biological fluids such blood plasma, milk, and urine. There is growing evidence that exosomes play essential roles in molecular transport and cellular communication, with broad implications for human health related to immunological activation, neurotoxicity, and oncogenesis.

Isolation and Separation Techniques of Exosomes

Since exosomes are always linked to biological components, it is essential to isolate them in order to comprehend their function and potential uses in biologics. Numerous techniques have been created to make it easier to separate exosomes from bodily fluids (Patil et al., 2020). There are numerous methodologies available to produce high recovery rates, high purity, and high throughput separation of exosomes. Some of these can be listed as follows; Ultracentrifugation, Immunological Separation, Ultrafiltration, Dielectrophoretic (DEP) Separation etc.

Ultracentrifugation-based separation

Centrifugation is the most often used isolation method which was basis for ultracentrifugation due to difference in sedimentation coefficient between extracellular content and exosomes. Prior to the initiation of isolation, human plasma or serum typically undergoes a cleaning phase to remove big bioparticles, and the sample is spiked with protease inhibitors to stop exosomal protein breakdown (Rechavi et al., 2009). The centrifugal force employed in ultracentrifugation usually varies between approximately 100,000 and 120,000 × g (Yang et al., 2019). Differential and gradient density ultracentrifugation are two additional subcategories of ultracentrifugation. Gradient ultracentrifugation is a viable solution to address the low separation purity of this approach. This technique makes use of two or more distinct gels or solutions with varying densities. Because the EVs are separated based on their densities, a high separation rate is attained (Patil et al., 2020).

Immunological Separation

By leveraging the immunoaffinities interactions between those proteins (antigens) and their antibodies, as well as the specific interactions between the receptors and ligands, the abundance of proteins and receptors found in the membrane of exosomes presents a fantastic opportunity to develop highly specific techniques for the isolation of exosomes (Yang et al., 2019). The immunological separation concept relies on the exosomes being captured by the antigen-antibody response. To capture exosomes, this technique takes advantage of the different proteins that are present on their membrane. Due to the need for special reagents and cell-free samples, this method is costly and cannot be used for large-scale sampling (Patil et al., 2020).

Ultrafiltration

To capture particles of a particular size, the ultrafiltration process makes use of porous membranes, also known as centrifugal filters, with varying membrane types and pore diameters (Ansari et al., 2024). The membrane separation method known as ultrafiltration operates on the basis of the size and molecular weight of exosomes and other materials to be separated. Membranes with pores the same size as an exosome—100 nm—can be used to segregate exosomes from macromolecules, allowing the exosome to flow through while the other components are trapped on the membrane (Patil et al.,

2020). However, this is a labour-intensive process that requires isolation using either Amicon or Vivaspin filters (Kornilov et al., 2018).

Dielectrophoretic (DEP) Separation

The basis of dielectrophoretic separation is the idea that polarised particles in a nonuniform electric field experience dielectric force. The size and intrinsic dielectric characteristics of the cells, as well as the strength and frequency of the applied electric field, determine how much force is given to the particles through DEP. Larger particles migrate towards low electric field areas, while exosomes, which are nanoparticles, are drawn to high electric field (Patil et al., 2020). Although the usage of this technology is limited because to its necessity for electrothermal heating, it can be investigated for its potential benefits in terms of speed and throughput (Aghilinejad et al., 2018).

Functions of Exosomes on Cell Interaction

Exosomes are 40–100 nm in diameter nanoscale extracellular vesicles that are discharged from a number of cell types along with their plasma membranes. Exosomes were first widely found in bodily fluids like blood, urine, and body fluid (Yu et al., 2015a). Because exosomes differ in composition depending on where they originate, the cell from which they originate plays a crucial role in determining the roles that these particles play in the various biological processes that they are involved in (Gutierrez-Millan et al., 2021). Exosomes, can include a variety of cell components, such as DNA, RNA, lipids, metabolites, cytosolic and cell-surface proteins, depending on the parent cell (Kalluri & LeBleu, 2020). Utilizing exosomes' capacity to mediate specific physiological and pathological processes, medicinal drugs are delivered to a target in a way that minimizes systemic damage (Patil et al., 2020). Additionally, numerous discoveries suggested that exosomes are essential for cancer research in terms of carcinogenesis, progression, and metastasis (Yu et al., 2015b). Exosomes may be crucial in controlling regular biological processes like immunological monitoring, blood coagulation, tissue repair, and stem cell maintenance (Samanta et al., 2018). Their ability to regulate intricate intracellular pathways has expanded their potential use in the therapeutic management of a wide range of illnesses, including cancer and neurological diseases.

Exosomes and modified liposome membranes' biochemical characteristics can be utilised to target delivery to particular cell populations (Ohno et al., 2013). When consider the functions of exosomes, they appear to have versatile uses. Research in this field is being motivated by the promise of EVs as therapeutic and diagnostic tools for the treatment of many illnesses, such as cancer, cardiovascular dysfunction, and neurodegeneration. The diverse processes and routes linked to exosome uptake along with the potential cell type selectivity of exosomes contribute to the intricacy of its activity in intercellular communication (Mulcahy et al., 2014; McKelvey et al., 2015). It has been reported that this communication state has a critical functional effect between different neural cell types. It has been demonstrated that neurons can endocytose and internalise exosomes generated from oligodendrocytes. Exosomes play role as another way that neurons and glia can communicate with each other. Additionally, neurons control synaptic activity and neurogenesis through exosomes, as well as intracellular communication and homeostasis (Xiao et al., 2014). The production of neuroprotective effects against oxidative stress in neurons by exosomes derived from stressed astrocytes subjected to oxygen and glucose deprivation was found to be dependent on Prion protein (Guitart et al., 2016).

Exosomes are widely dispersed in human secretions and function as messengers between cells by exchanging proteins, RNA, and DNA (Xiao et al., 2021). However, the factors and stimuli that regulate exosome release are not fully understood. Fascinatingly, exosome secretion can also be impacted by cellular homeostasis. The majority of research focuses on the impacts that result from exosome materials being delivered to the receptor cell, but it's important to remember that exosome secretion is a process that removes materials from generating cells, changing the cells' properties and homeostasis (Vidal, 2019). For instance, hematopoietic cell line K562 cells secreted more exosomes when there was an increase in intracellular Ca^{2+} (Parolini et al., 2009). Exosome secretion has also been demonstrated to be influenced by environmental pH. Furthermore, exosome release may be enhanced by cellular stressors such as radiation, cisplatin therapy, hypoxia exposure, and ER stress (King et al., 2012; Xiao et al., 2014). Exposures can modify the cargo of exosomes, which gives exposed cells a way to rewire healthy cells in both proximal and distal regions. Therefore, the possibility that pathogenic exosome release plays a key role in the processes and modes of action linking toxic chemicals with unfavorable exposure outcomes is another reason why exosome biology can be useful to toxicology (Bowers et al., 2020). Exosomes

have been successfully used in both in vitro and in vivo experiments for drug administration, and the majority of investigations on this topic have demonstrated that exosomes exhibit effective biological activity (Zhu et al., 2017). Innovative exosome indicators of liver and kidney damage have been discovered by in vivo techniques including animal exposure models and human clinical trials (Bowers et al., 2020). Exosome profiles that are specific to hepatitis-ischemic injury in the liver, inflammatory liver injury, and various forms of acute kidney damage have been discovered (Cho et al., 2017).

Role of Exosomes in Drug Delivery Systems

Drug delivery systems are innovative approaches developed to make drugs more effective and less toxic compared to traditional treatments. This has been the case in systems developed with nano-sized materials in recent years. Exosomes, one of these nanosized vesicles, have unique characteristics that make them interesting for use in drug delivery applications. Exosomes have a range of adhesion proteins on their surface, which gives them an edge over other systems when used as drug carriers. These benefits include the ability to transfer substances across great distances, stability, safety, and specificity (Gutierrez-Millan et al., 2021). Through the exchange of bioactive molecules with recipient cells, such as proteins, mRNAs, and miRNAs, exosomes participate in intercellular interactions that impact signaling pathways and biological processes (Sharma & Mukhopadhyay, 2024). Exosomes' propensity to cross the blood-brain barrier (BBB) improves nervous system neurological and motor function and makes it possible to repeatedly inject medications containing exosomes intravenously without experiencing any unfavourable side effects (Sen et al., 2023).

Therapeutic chemicals must be efficiently loaded into exosomes in order for them to be exploited as drug delivery systems. Currently, a number of small molecules—both hydrophobic and hydrophilic—have already been combined to exosomes, showing improvements in molecular stability as well as an increase in the amount of medicine collected inside target cells and the duration of blood circulation (Luan et al., 2017). There are two main methods for adding therapeutic compounds to exosomes: passive or active encapsulation or loading (Balachandran & Yuana, 2019; Patil et al., 2020). Either incubating the medication with exosomes or with donor cells will result in passive loading. In contrast to incubating with donor cells, where the cells are first treated with medications before secreting exosomes containing the

drug, incubating with exosomes allows the pharmaceuticals to permeate into the exosomes according to a concentration gradient (Patil et al., 2020). The active drug loading approach improves drug loading efficiency and makes it easier to load big molecules by methods like sonication, extrusion, electroporation, or drug conjugation (Akuma et al., 2019). To deliver their pharmacological cargo, they might also manage to breach the plasma membrane. Additionally, ligands or antibody fragments that bind to particular cell types can be shown on liposomal membranes to improve medication delivery tailored to individual cell types (Lai et al., 2013). In a Shank3B Knockout autism mouse model, intrathecal administration of exosomes produced from mesenchymal stem cells decreased repetitive behaviors and increased behavioral traits similar to autism, such as social vocalization (Perets et al., 2020). Extracellular vesicles have been utilized recently to deliver the CRISPR/Cas9 genome editing apparatus to cells. This may provide a novel strategy for the delivery of Cas9/sgRNA in the treatment of several genetically illnesses, including neurological diseases (Elsharkasy et al., 2020). Exosomes are also interesting drug delivery vehicles because of their capacity to carry biomolecules and medications to target cells. Furthermore, exosomal proteins and nucleic acids (miRNA, mRNA, and DNA) have demonstrated promise as prognostic, predictive, and diagnostic biomarkers for a variety of malignancies (Xia et al., 2021). Exosomes also made from macrophages have also been investigated as carriers of antitumor medications in the quest to enhance antitumor therapies, capitalizing on the critical role that macrophages play in the genesis of cancer (Cheng et al., 2019).

Exosome-based therapeutic drug delivery systems and therapeutic products are currently in pre-clinical and clinical research (Kučuk et al., 2021; Sharma & Mukhopadhyay, 2024). The FDA has approved this drug for the treatment of severe dermatological conditions, such as burns and wounds. Capricor Therapeutics has developed two exosome-based vaccinations against SARS-CoV-2- the exosome mRNA vaccine and the exosome VLP Display Vaccine. Both vaccines are presently in the pre-clinical stage (Sharma & Mukhopadhyay, 2024). Also, a Phase 1 clinical trial for Plexaris, a natural exosome-based medication developed by Exopharm Pty Ltd to aid in wound healing (Sharma & Mukhopadhyay, 2024). Extensive study in the realm of exosomes has produced a multitude of novelties and intellectual properties. Patent certifications have been granted to innovations in the production, isolation, transportation, and therapeutic application of exosomes. Currently, researchers are assessing the many therapeutic uses of mesenchymal stem

cells (MSCs) as a result of the ongoing hunt for novel treatments in the field of cell therapy. The studies have demonstrated that the majority of these cells' activity, including their ability to modulate immunity, can be attributable to the exosomes that are generated from them (Gutierrez-Millan et al., 2021). Since the released exosomes operate as paracrine mediators by sharing genetic information among cells, mesenchymal cells and their exosomes hold great promise for the development of novel cancer treatments (Zhou et al., 2018). Some of research indicates that MSC-exosomes may deliver proteins, messenger RNA, and microRNA to recipient cells, where they may then influence the development, metastasis, and responsiveness of distinct tumor cells to drugs (Zhang et al., 2017). Surprisingly, exosomes produced from MSCs can penetrate most tumor regions and offer an environment favorable for the development of cancer, including immunological regulation, angiogenesis, metastasis, treatment resistance, and cell proliferation (Zhou et al., 2018). Due to the medication's more regulated and targeted release to tumor cells, using this method of administration rather than the traditional one has the advantage of reducing adverse effects. Furthermore, the exosomes themselves include chemicals from the tumor cells that are physiologically active. The methods that have been created from exosomes and the experiment outcomes are positive and undoubtedly very significant for prospective science and medicine.

Conclusion

The researches on exosome is a brand-new, quickly expanding discipline that is gaining popularity. Exosomes are effective therapeutic agents that have drawn a lot of attention recently due to their qualities, which include being more stable, less toxic, and biodegradable. Exosomes have several benefits for therapeutic and medication delivery, but there are still some limitations. Many obstacles must be overcome before widely used in clinical studies. These include the high expense of post-drug approval, the dearth of effective techniques for defining the safety parameters of exosomal designs, and the potential for some exosomes to cause immune reactions.

REFERENCES

Aghilinejad, A., Aghaamoo, M., Chen, X., & Xu, J. (2018). Effects of electrothermal vortices on insulator-based dielectrophoresis for circulating tumor cell separation. *Electrophoresis*, *39*(5–6), 869–877.

Akuma, P., Okagu, O. D., & Udenigwe, C. C. (2019). Naturally occurring exosome vesicles as potential delivery vehicle for bioactive compounds. *Frontiers in Sustainable Food Systems*, *3*.

Ansari, F. J., Tafti, H. A., Amanzadeh, A., Rabbani, S., Shokrgozar, M. A., Heidari, R., Behroozi, J., Eyni, H., Uversky, V. N., & Ghanbari, H. (2024). Comparison of the efficiency of ultrafiltration, precipitation, and ultracentrifugation methods for exosome isolation. *Biochemistry and Biophysics Reports*, *38*, 101668.

Balachandran, B., & Yuana, Y. (2019). Extracellular vesicles-based drug delivery system for cancer treatment. *Cogent Medicine*, *6*(1), 1635806.

Bowers, E. C., Hassanin, A. A. I., & Ramos, K. S. (2020). In vitro models of exosome biology and toxicology: New frontiers in biomedical research. *Toxicology in Vitro*, *64*, 104462.

Cheng, H., Wang, Z., Fu, L., & Xu, T. (2019). Macrophage polarization in the development and progression of ovarian cancers: An Overview. *Frontiers in Oncology*, *9*.

Cho, Y., Mezey, E., Hardwick, J. P., Salem, N., Clemens, D. L., & Song, B. (2017). Increased ethanol-inducible cytochrome P450-2E1 and cytochrome P450 isoforms in exosomes of alcohol-exposed rodents and patients with alcoholism through oxidative and endoplasmic reticulum stress. *Hepatology Communications*, *1*(7), 675–690.

Elsharkasy, O. M., Nordin, J. Z., Hagey, D. W., de Jong, O. G., Schiffelers, R. M., Andaloussi, S. EL, & Vader, P. (2020). Extracellular vesicles as drug delivery systems: Why and how? *Advanced Drug Delivery Reviews*, *159*, 332–343.

Guitart, K., Loers, G., Buck, F., Bork, U., Schachner, M., & Kleene, R. (2016). Improvement of neuronal cell survival by astrocyte-derived exosomes under hypoxic and ischemic conditions depends on prion protein. *Glia*, *64*(6), 896–910.

Gutierrez-Millan, C., Calvo Díaz, C., Lanao, J. M., & Colino, C. I. (2021). Advances in exosomes-based drug delivery systems. *Macromolecular Bioscience*, *21*(1).

Kalluri, R., & LeBleu, V. S. (2020). The biology , function , and biomedical applications of exosomes. *Science*, *367*(6478).

King, H. W., Michael, M. Z., & Gleadle, J. M. (2012). Hypoxic enhancement of exosome release by breast cancer cells. *BMC Cancer*, *12*(1), 421.

Kornilov, R., Puhka, M., Mannerström, B., Hiidenmaa, H., Peltoniemi, H., Siljander, P., Seppänen-Kaijansinkko, R., & Kaur, S. (2018). Efficient ultrafiltration-based protocol to deplete extracellular vesicles from fetal bovine serum. *Journal of Extracellular Vesicles*, *7*(1).

Kučuk, N., Primožič, M., Knez, Ž., & Leitgeb, M. (2021). Exosomes engineering and their roles as therapy delivery tools, therapeutic targets, and biomarkers. *International Journal of Molecular Sciences*, *22*(17), 9543.

Lai, R. C., Yeo, R. W. Y., Tan, K. H., & Lim, S. K. (2013). Exosomes for drug delivery — a novel application for the mesenchymal stem cell. *Biotechnology Advances*, *31*(5), 543–551.

Luan, X., Sansanaphongpricha, K., Myers, I., Chen, H., Yuan, H., & Sun, D. (2017). Engineering exosomes as refined biological nanoplatforms for drug delivery. *Acta Pharmacologica Sinica*, *38*(6), 754–763.

McKelvey, K. J., Powell, K. L., Ashton, A. W., Morris, J. M., & McCracken, S. A. (2015). Exosomes: Mechanisms of uptake. *Journal of Circulating Biomarkers*, *4*, 7.

Mulcahy, L. A., Pink, R. C., & Carter, D. R. F. (2014). Routes and mechanisms of extracellular vesicle uptake. *Journal of Extracellular Vesicles*, *3*(1).

Ohno, S., Takanashi, M., Sudo, K., Ueda, S., Ishikawa, A., Matsuyama, N., Fujita, K., Mizutani, T., Ohgi, T., Ochiya, T., Gotoh, N., & Kuroda, M. (2013). Systemically ınjected exosomes targeted to EGFR deliver antitumor microRNA to breast cancer cells. *Molecular Therapy*, *21*(1), 185–191.

Pan, B. T., Teng, K., Wu, C., Adam, M., & Johnstone, R. M. (1985). Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes. *The Journal of Cell Biology*, *101*(3), 942–948.

Parolini, I., Federici, C., Raggi, C., Lugini, L., Palleschi, S., De Milito, A., Coscia, C., Iessi, E., Logozzi, M., Molinari, A., Colone, M., Tatti, M., Sargiacomo, M., & Fais, S. (2009). Microenvironmental pH ıs a key factor for exosome traffic in tumor cells. *Journal of Biological Chemistry*, *284*(49), 34211–34222.

Patil, S. M., Sawant, S. S., & Kunda, N. K. (2020). Exosomes as drug delivery systems: A brief overview and progress update. *European Journal of Pharmaceutics and Biopharmaceutics*, *154*, 259–269.

Perets, N., Oron, O., Herman, S., Elliott, E., & Offen, D. (2020). Exosomes derived from mesenchymal stem cells improved core symptoms of genetically modified mouse model of autism Shank3B. *Molecular Autism*, *11*(1), 65.

Rechavi, O., Erlich, Y., Amram, H., Flomenblit, L., Karginov, F. V., Goldstein, I., Hannon, G. J., & Kloog, Y. (2009). Cell contact-dependent acquisition of cellular and viral nonautonomously encoded small RNAs. *Genes & Development*, *23*(16), 1971–1979.

Rokad, D., Jin, H., Anantharam, V., Kanthasamy, A., & Anumantha, G. (2020). *HHS Public Access*. *6*(3), 73–79.

Samanta, S., Rajasingh, S., Drosos, N., Zhou, Z., Dawn, B., & Rajasingh, J. (2018). Exosomes: new molecular targets of diseases. *Acta Pharmacologica Sinica*, *39*(4), 501–513.

Sen, S., Xavier, J., Kumar, N., Ahmad, M. Z., & Ranjan, O. P. (2023). Exosomes as natural nanocarrier-based drug delivery system: recent insights and future perspectives. *3 Biotech*, *13*(3), 101.

Sharma, V., & Mukhopadhyay, C. Das. (2024). Exosome as drug delivery system: Current advancements. *Extracellular Vesicle*, *3*, 100032.

Vidal, M. (2019). Exosomes: Revisiting their role as "garbage bags." *Traffic*, *20*(11), 815–828.

Xia, Z., Qing, B., Wang, W., Gu, L., Chen, H., & Yuan, Y. (2021). Formation, contents, functions of exosomes and their potential in lung cancer diagnostics and therapeutics. *Thoracic Cancer*, *12*(23), 3088–3100.

Xiao, L., Hareendran, S., & Loh, Y. P. (2021). Function of exosomes in neurological disorders and brain tumors. *Extracellular Vesicles and Circulating Nucleic Acids*.

Xiao, X., Yu, S., Li, S., Wu, J., Ma, R., Cao, H., Zhu, Y., & Feng, J. (2014). Exosomes: Decreased sensitivity of lung cancer A549 cells to cisplatin. *PLoS ONE*, *9*(2), e89534.

Yang, X.-X., Sun, C., Wang, L., & Guo, X.-L. (2019). New insight into isolation, identification techniques and medical applications of exosomes. *Journal of Controlled Release*, *308*, 119–129.

Yu, D. dan, Wu, Y., Shen, H. yu, Lv, M. meng, Chen, W. xian, Zhang, X. hui, Zhong, S. liang, Tang, J. hai, & Zhao, J. hua. (2015a). Exosomes in development, metastasis and drug resistance of breast cancer. *Cancer Science*, *106*(8), 959–964.

Yu, D. dan, Wu, Y., Shen, H. yu, Lv, M. meng, Chen, W. xian, Zhang, X. hui, Zhong, S. liang, Tang, J. hai, & Zhao, J. hua. (2015b). Exosomes in development, metastasis and drug resistance of breast cancer. In *Cancer Science* (Vol. 106, Issue 8, pp. 959–964).

Zhang, X., Tu, H., Yang, Y., Fang, L., Wu, Q., & Li, J. (2017). Mesenchymal stem cell-derived extracellular vesicles: roles in tumor growth, progression, and drug resistance. *Stem Cells International*, *2017*, 1–12.

Zhou, J., Tan, X., Tan, Y., Li, Q., Ma, J., & Wang, G. (2018). Mesenchymal stem cell derived exosomes in cancer progression, metastasis and drug delivery: A Comprehensive review. *Journal of Cancer*, *9*(17), 3129–3137.

Zhu, X., Badawi, M., Pomeroy, S., Sutaria, D. S., Xie, Z., Baek, A., Jiang, J., Elgamal, O. A., Mo, X., La Perle, K., Chalmers, J., Schmittgen, T. D., & Phelps, M. A. (2017). Comprehensive toxicity and immunogenicity studies reveal minimal effects in mice following sustained dosing of extracellular vesicles derived from HEK293T cells. *Journal of Extracellular Vesicles*, *6*(1).

TOPICAL BIOMATERIALS TO PROMOTE WOUND HEALING

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Abstract

Fast and effective treatment of wounds formed on the skin due to trauma is precious in enabling tissues and organs to perform their functions correctly and ensuring body integrity. Wound treatment varies depending on the degree and severity of the injury, the patient's age, accompanying diseases, and physiological condition, and wound treatments are performed with various application methods. However, topical treatment has various advantages over other application methods, such as maintaining moisture balance in the wound area, providing growth factors that accelerate healing, and supporting vascularization. In recent years, the use of biomaterials with proliferative, antioxidant, anti-inflammatory, and antimicrobial activities that support wound healing in the production of topical agents have attracted attention. Topical biomaterials containing antimicrobial agents offer a good alternative by preventing colonization in combating microorganism infections that delay wound healing and develop antibiotic resistance. Also, topical biomaterials containing nucleic acids that play a role in expressing growth factors have important effects on the healing of chronic wounds. Biomaterials suitable for meeting the physiological needs of the skin are preferred not only for their bioactivity but also for preserving the stability of the active substance, increasing skin permeability, and reducing toxic effects. For this purpose, bioactive substances that support wound healing are loaded into nanoparticles produced by green synthesis, and the effectiveness of topical agents in wound healing is increased. Although topical biomaterials have disadvantages such as difficulty in dosing, unpredictable immunogenicity, and toxicity risks, they are seen to be promising in wound healing. In this chapter, suitable for topical use to support wound healing green synthesis nanoparticle-containing biomaterials, nucleic acid-containing biomaterials, antibacterial agent-containing biomaterials, biomaterials derived from natural sources, and extract-containing hydrogel biomaterials are evaluated.

Introduction

The skin is a protective barrier that separates the body from the external environment. This barrier can be disrupted by various traumas such as surgical interventions, stab wounds, heat, chemical, radiation-induced burns. Disrupted skin integrity is classified as acute and chronic wounds depending on the underlying causes and consequences (Kolimi et al., 2022). Acute wounds are usually small wounds that can heal with the body's own repair mechanism. Chronic wounds are difficult to treat due to inadequate wound care, concomitant diseases (diabetes, edema, nutritional deficiencies, anemia, cardiovascular diseases, inadequate blood supply, etc.), and infection of the wound area. If the necessary intervention is delayed or there is no response to the applied treatments, it results in amputation and/or systemic effects in extremity wounds (Beyene et al., 2022). Wound healing is a highly complex process involving molecular, biochemical, and cellular mechanisms as well as the immune response (Gwarzo et al., 2022).

The healing process is basically examined in four consecutive and intertwined stages: hemostasis, inflammation, proliferation, and remodeling (Sezen et al., 2023). The inflammatory phase begins immediately after the hemostasis process, which begins with platelets accumulating in the damaged area and stopping bleeding, depending on the type of injury. Inflammation that develops after injury is a protective response in which the body tries to eliminate the factors that cause cell damage and necrotic cells and tissues through the synchronized activation of blood vessels, defense cells and mediators. The main goal at this stage is to remove pathogens from the wound area. Increased vascular permeability allows immune cells such as neutrophils and monocytes to settle in the damaged area. During the process where chemotaxis comes to the forefront, environmental conditions are prepared for the proliferation phase with the help of cytokines ((Tumor necrosis factor alpha (TNF-α), CXC chemokine ligand (CXCL), interleukins (IL)), growth factors ((Transforming growth factor alpha (TGF-α), platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), fibroblast growth factor (FGF), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF)) and other regulators ((Tissue inhibitor of metalloproteinases (TIMPs), matrix metalloproteinases (MMPs)). During the proliferative phase, fibroblasts, keratinocytes, stem cells, and other cells that will form the basis of the tissue skeleton in the wound area begin to proliferate rapidly. This is followed by the formation of the extracellular matrix (ECM), angiogenesis,

and the formation of granulation tissue. The remodeling phase is the phase in which the number of fibroblasts in the wound area decreases, collagen production reaches balance, epithelialization is completed, the color of the wound area becomes pale due to the decrease in capillary density, wound tension resistance increases and ultimately healed scar tissue is formed (Figure 1) (Broughton et al., 2006; Kumar et al., 2017; Sezen et al., 2021). Problems that may occur during the complex wound healing process may result in hypertrophic scars, keloids, or wounds that do not heal (Enoch and Leaper 2008). The most common and difficult wounds to treat are chronic wounds of various types, such as pressure sores, diabetic ulcers, and vascular ulcers. These wounds usually take a long time to heal and are associated with significant morbidity and mortality (Han et al., 2017). After cancer and diabetes, wound care expenditure constitutes the highest national health service (NHS) healthcare expense (Guest et al., 2020). Research has stated that 2% of the population in developed countries receives treatment for chronic wounds at least once in their lives. It has also been reported that in Europe this rate is 0.3-0.4% and that the treatment of these wounds constitutes a significant portion of healthcare expenditures (Tatarusanu et al., 2023; Ansari and Darvishi, 2024).

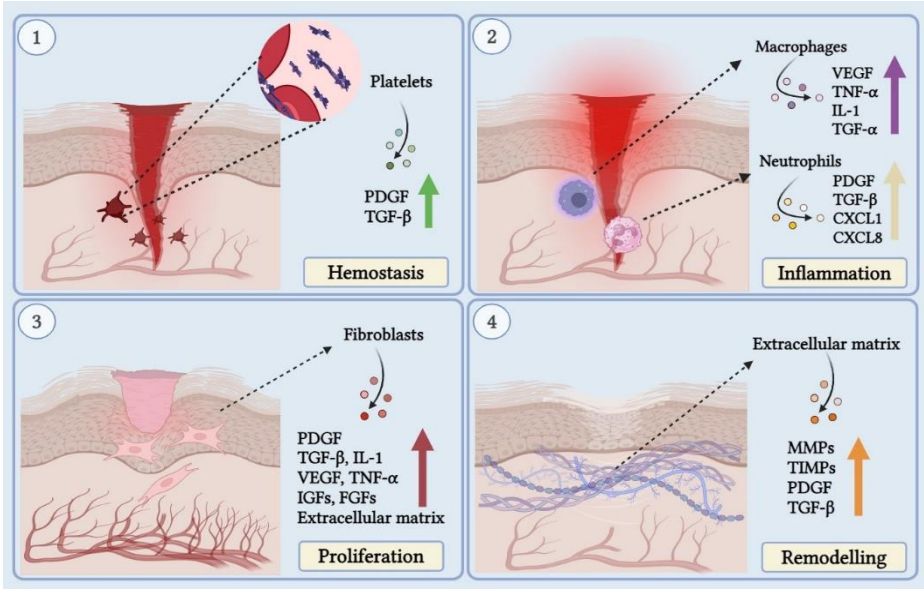

Figure 1. Wound Healing Steps

*Graphical abstract created with BioRender.com

Topical drugs that support wound healing are widely used to accelerate wound healing, prevent wound infection and chronicity, and reduce hospital stay and healthcare expenses. Natural and synthetic biomaterials are frequently preferred in topical products due to their high activity, providing a good alternative against developing antibiotic resistance, and the ease of using different active ingredients together. These topical products, which are formulated depending on the type of injury, severity, and location of the damage, are increasingly important in wound healing and tissue engineering due to their advantages such as being effective, biocompatible, and low toxicity (Ansari and Darvishi, 2024). In this section, the bioactivities, areas of use, and advantages and disadvantages of green synthesis nanoparticle (NP)-containing biomaterials, nucleic acid-containing biomaterials, antibacterial agent-containing biomaterials, biomaterials derived from natural sources, and extract-containing hydrogel biomaterials are evaluated.

Green Synthesis NP-Containing Biomaterials

Topical formulations are important in treatment as they ensure the penetration of the active ingredient into the skin barrier and sufficient absorption by the skin. Various formulations are being investigated to increase the solubility of the drug, to provide controlled release, to deliver the active ingredients to the target area at the desired concentration, and to provide high stability to the drug. Due to these advantages, NPs are widely used in topical applications. Metal NPs are preferred especially in skin injuries due to their antibacterial, antioxidant, anti-inflammatory and anti-aging effects. Well-known metal NPs include various NPs such as silver (Ag NPs), gold (Au NPs), zinc (Zn NPs), zinc oxide (ZnO NPs), copper (Cu NPs) and copper oxide (CuO NPs) (Paiva-Santos et al., 2021). Despite their wound healing bioactivity and superior physicochemical properties, metal NPs have been identified as risky for human/environmental health due to the toxicity of the solvents used and the resulting by-products when produced through traditional chemical synthesis (Jamkhande et al., 2019). This situation has rendered the development of new environmentally friendly and economical alternative methods to the chemical synthesis method, which has low production efficiency and high cost (Sezen et al., 2023). In this context, the green synthesis method developed has recently started to replace chemical synthesis because it avoids the use of harmful chemicals during the reaction, is human/environment friendly and has a reasonable cost. Green synthesis is a natural synthesis method in which various phytochemicals such as flavonoids,

phenolic acids, alkaloids, saponins, carbohydrates, amino acids, proteins, and terpenoids obtained from plant extracts, bacteria, fungi, enzymes, algae, and other natural sources are used as stabilizers or reducing agents (Rehana et al., 2017; Dada et al., 2019; Paiva-Santos et al., 2021; Aldakheel et al., 2023). In this process, natural metabolic products are reduced with metallic ions to produce NPs. The components in the biological material act as reductants, providing stabilization for NPs during synthesis. Therefore, the green synthesis method does not require the use of external chemical stabilizers. Green synthesized metal/metal oxide NPs are included in many topical formulations applied in such as wound healing, tissue therapy, and regenerative medicine (Ahmed et al., 2017). It has been reported that green synthesized ZnO NPs from *Capparis spinosa* extract exhibited strong inhibition zones against Gram (+) bacteria including *Enterococcus faecium* and methicillin-resistant *Staphylococcus aureus* (MRSA) strains. In addition, these NPs accelerated healing in human dermal fibroblast cells created in an *in vitro* wound model in a dose-dependent manner and increased the expressions of fibroblast growth factor receptor 2 (Bek/FGFR2), IGF, and TGF-β associated with wound healing. In the study, the increase in antioxidant status together with the decrease in oxidant status was associated with the size and stability of green synthesized ZnO NPs (Sezen et al., 2023).

Low-toxicity metal NPs produced by the green synthesis method using biological materials have critical importance in the development of potential topical agents that will accelerate wound healing. In a study examining the effect of metal NPs on wound healing, Ag NPs and Au NPs were produced from *Coleus forskohlii* root extract by green synthesis. Ag NPs and Au NPs accelerated the healing process in excision wounds created in rats and also significantly reduced scars. It has been reported that green synthesized NPs show this effect by increasing proliferation and migration as well as anti-inflammatory and antioxidant activities (Naraginti et al., 2016). In a clinical study examining the wound healing effect of green synthesis Ag NPs, Ag NPs prepared using *Delonix elata* leaves were topically applied to the post-operative wound areas of anorectal surgery patients. After 10 days of application, a visible improvement was observed in the wound areas of the patients, and on the 17th day, it was determined that the wounds were almost completely closed. In this study, it was reported that inflammation and cytokine modulation played a role in the rapid epithelialization of plant-based metal NPs (Chai et al., 2018). In this context, the superior physicochemical properties of NP structures combined with the bioactivity of the biological

material used in green synthesis are promising in wound healing. Despite all their advantages, green synthesized NPs can combine with various side metabolites during synthesis and turn into undesirable structures. In addition, due to the complex structure of biological systems and the elimination components of the body, NPs can accumulate in non-target tissues. In this context, more research is needed to evaluate the safety of green synthesized NPs and to eliminate concerns about the effects they may cause in the long term (Nandhini et al., 2023).

Nucleic Acid-Containing Biomaterials

It is known that nucleic acids support wound healing by regulating cell proliferation, migration, angiogenesis, and collagen production (Hamblin et al., 2016). Nucleic acids achieve these effects by enabling the expression or silencing of genes involved in the wound healing process. During wound healing, the expression of many proteases, especially MMP-9, increases in the skin microenvironment. Proteases are also responsible for the destruction of ECM proteins such as collagen, fibronectin, vitronectin, and therapeutic proteins such as growth factors, cytokines, and chemokines that have positive effects on wound healing (Das et al., 2016). In chronic wounds where the healing process is disrupted, it has been reported that some proteases show activity for much longer periods than in normally healing wounds. In this context, treatments targeting the reduction of the expression of some proteases involved in the process to accelerate wound healing have been evaluated as an important strategy (Westby et al., 2020). Among these new strategies, nucleic acids such as aptamers, antisense oligonucleotides, messenger ribonucleic acid (mRNA), small interfering RNA (siRNA), microRNA (miRNA), plasmid deoxyribonucleic acid (pDNA) and clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR-Cas9) have emerged as biomaterials for the purpose of regeneration (Sharma et al., 2022; Zhuang and Cui, 2022). However, the inability to maintain the stability of nucleic acids in the wound area and their rapid degradation by enzymes in the area is a significant problem. Topical application of nucleic acids by encapsulating them with NP prevents the destruction of nucleic acids by providing continuous release in the target tissue. This approach, which combines nanotechnology and gene therapy for the successful healing of a chronic wound, is an important step in achieving effective wound treatment (Ezhilarasu et al., 2020).

It has been determined that nucleic acids whose release kinetics are modified by smart polymer NPs exhibit healing effects in bone, cartilage, cardiac, neural, and skin regeneration (Wang et al., 2017; Aldayel et al., 2018; Yu et al., 2020; Tan et al., 2021). Biomaterials accomplish these effects by altering the protein expression that plays a role in the tissue regeneration process, the production of bioactive molecules, and the regulation of genes that regulate regeneration (Sharma et al., 2022). siRNA selectively targets genes known to be increased in chronic wounds, such as MMP-9, ganglioside-monosialic acid 3 synthase (GM3S), macrophage chemoattractant protein-1 (MCP-1), and TNF-α, thereby blocking gene expression (Kasiewicz and Whitehead, 2016). It has been determined that siRNA-loaded lipid NPs used in the treatment of full-thickness excisional skin wounds of diabetic mice accelerated the wound healing process by reducing TNF-α mRNA expression in the wound area by 40-55% (Kasiewicz and Whitehead, 2018). It has been determined that siRNAs can silence gene targets such as p53, MMP, and TGF-β receptor 1 (TGFβR1) associated with wound healing (Nguyen et al., 2010; Wang et al., 2014; Lan et al., 2021). Similar to siRNAs, various miRNAs have been shown to contribute to wound healing by regulating angiogenesis, ECM remodeling, scar formation, fibroblast, and keratinocyte proliferation (Fang et al., 2016; Icli et al., 2016).

In chronic wounds such as diabetic foot wounds, many factors including oxidative stress, ischemia, high protease, and enzyme activities, immune cell infiltration, mechanical stress, and biofilm/infection in the wound microenvironment significantly limit the effectiveness of the drug in the target area. Biomaterials using nucleic acids are promising in the repair and regeneration of chronic wounds by regulating angiogenesis, epithelialization, oxidative stress, and inflammation-related parameters. In addition, therapeutically used nucleic acids have advantages such as being able to easily design their sequences, being able to regulate protein expression for a long time, and being able to target transcription factors that other drugs cannot target (Berger et al., 2021; Sharma et al., 2022). Despite their advantages, nucleic acids to be used topically cannot be easily taken into the cell due to the negative charge of cell membranes. This situation caused by electrostatic interactions also leads to some difficulties in the dosage of nucleic acid-encapsulated NPs (Ezhilarasu et al., 2020; Zhuang and Cui, 2022). Considering the dynamic process of wound healing, research continues on new strategies that will deliver the appropriate nucleic acid to the right cell

type at the most appropriate time for use in different wound types (Berger et al., 2021).

Antibacterial Agent-Containing Biomaterials

One of the most common complications of skin injuries is infections associated with chronic wounds. The disruption of the integrity of the skin barrier with the injury makes this area vulnerable to infections caused by pathogenic microorganisms. Microorganisms multiply in the damaged area and trigger an inflammatory response through the toxins they produce. This leads to delayed fibroplasia, tissue damage, delayed healing, and chronicity of the wound (Kizhakkekalam et al., 2022; Liu et al., 2022). Wound areas are often infected by bacteria found in the skin flora. Although antibacterial treatments have been developed to accelerate the healing of infected wounds, it has been reported that 70% of the bacteria responsible for wound infections are resistant to at least one of the most commonly used antibiotics. Another important contamination cause that delays wound healing is hospital-acquired bacterial infections, a significant portion of which are highly resistant to antibiotics. The hydrated biofilm layer, consisting of extracellular polymeric substances produced by bacteria on the wound surface, is another obstacle that increases antibiotic resistance and makes treatment difficult (Chen et al., 2022; Liu et al., 2022). The biofilm layer, which is commonly encountered in chronic wounds, disrupts the skin barrier function, causing the wound to close defectively or not at all. Accompanied by debridement and aseptic approach effective, potent antibacterial topical treatments are required to overcome this situation which complicates treatment and delays wound healing (Caldwell, 2020). In addition, the increase in the number of multidrug-resistant microbial strains and the inability to develop new antibiotic classes have accelerated research on the use of antimicrobial biomaterials in treatment (Kizhakkekalam et al., 2022).

Antimicrobial biomaterials can provide high concentrations in the wound area after topical application, and they can also be effective against resistant strains due to their different mechanisms of action than antibiotics. On the other hand, antimicrobial biomaterials can have a cytotoxic effect on cells such as fibroblast cells and endothelial cells that play a critical role in the wound healing process after topical application and can cause local pain and skin rash (Punjataewakupt et al., 2019). New topical antimicrobials are being developed that do not cause toxic effects on healthy cells and ensure that the

wound healing process progresses appropriately. Plants, bacteria, algae, and various biological materials or metabolites of these materials are important sources in biomaterial production in addition to their antibacterial activities. Among the ointment extracts prepared using different solvents of *Bergia ammannioides* plant, it was determined that the hexane fraction showed strong antibacterial activity against *Staphylococcus aureus* ATCC4175. It was reported that the formulation provided significant wound healing on the 10th day by increasing collagen synthesis in the wound model created in rats (Ezzat et al., 2016). It was determined that ZnO NPs synthesized using *Mentha longifolia* L. essential oil had antimicrobial effects against MRSA ATCC 67106, *Escherichia coli* ATCC BAA-2523, *Acinetobacter baumannii* ATCC BA1609, *Pseudomonas aeruginosa* ATCC 9070, *Streptococcus salivarius* ATCC13419, and *Streptococcus mutans* ATCC35668 strains under *in vitro* conditions. It was also shown that the formulation increased proliferation and migration in human dermal fibroblast cells depending on the dose (Sezen et al., 2020). In another study comparing the topical gel formulation prepared using the macroalgae-associated *Bacillus amyloliquefaciens* MTCC 12716 polysaccharide with clindamycin, it was recorded that the formulation showed strong antimicrobial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, and *Escherichia coli* strains. In the scratch wound test modeled on mouse fibroblast cells (L929), it was reported that the 48th-hour wound closure was 94% in the formulation group and 84% in the clindamycin group (Kizhakkekalam et al., 2022). In the preparation of a formulation, the microorganism itself or its metabolite is used. It has been reported that ZnO NPs coated with the antibacterial effective microbial metabolite rhamnolipid obtained from *Acinetobacter calcoaceticus* have healing effects in a *Staphylococcus aureus* contaminated wound model created in rats. This effect of ZnO NPs has been associated with the promotion of dermal proliferation, neovascularization and epithelialization (Malakar et al., 2023).

In topical formulations, encapsulation of bioactive compounds with NPs is one of the most applicable and advantageous ways to provide drug delivery to the lesioned area. NPs, which can pass through the skin barrier and reach the inside of the cell with their small size and high surface-to-volume ratio, are methods used for topical drug delivery. With topical NP application, the skin barrier passage of the antimicrobial nanomaterial is facilitated, and the interaction with host cells and microbial pathogens increases, and when the drug is released from the NP in a controlled manner, the risk of toxicity in

healthy cells decreases (Krausz et al., 2015). ZnO NPs, which are generally accepted as safe by the International Agency for Research on Cancer (IARC), are included in topical products for various uses, including sunscreens. The ongoing toxicity concerns regarding NPs have led researchers to prefer the green synthesis method in the production of these NPs (Girigoswami 2018; Khabir et al., 2021). Topical application of Au NPs obtained from *Tragopogon dubius* leaf extract by green synthesis method has been shown to accelerate wound healing without causing toxicity in *Staphylococcus aureus* ATCC 6538 contaminated burn wound model in rats. Au NPs have also been reported to have strong antibacterial effects against *Klebsiella pneumoniae* ATCC 700603, *Bacillus cereus* ATCC 14579, *Escherichia coli* ATCC 35218 strains (Layeghi-Ghalehsoukhteh et al., 2018). In another study, curcumin which has anti-inflammatory and antioxidant effects as well as antimicrobial effects, was encapsulated with NP, and the antibacterial activity of the formulation against MRSA was demonstrated. The wound healing effects of curcumin NPs were evaluated in a full-thickness burn wound model created in mice and it was reported that they showed healing effects over the proliferative phase by increasing re-epithelialization, collagen deposition, and fibronectin production (Krausz et al., 2015).

Despite the various advantages of NPs that can easily penetrate the skin and accelerate wound healing, there are also potential toxicity risks. NPs smaller than 10 nm or 600 Da can passively pass through skin barriers and reach systemic circulation, even with topical application. Systemic exposure to NPs causes cytotoxicity and genotoxicity with an increase in reactive oxygen species (ROS). In a study where ZnO NPs were shown to cause dose-dependent DNA damage in human hepatocytes and kidney cells, this effect of ZnO NPs was associated with increased oxidative stress (Guan et al., 2012). In another study evaluating the genotoxic potential of NPs, it was stated that ZnO NPs obtained from *Symphoricarpos albus* L. extract did not show any toxic effects up to a concentration of 640 µg/ml (Demir et al., 2023). Considering the fact that antimicrobial resistance cannot be stopped, it seems that different biomaterials will continue to be used in the development of new low-toxicity, proliferative antimicrobial topical formulations (Caldwell, 2020).

Biomaterials Derived from Natural Sources

Wound healing is a process that involves complex interactions between the ECM and various cell populations (fibroblasts, keratinocytes, endothelial cells, macrophages, and platelets). In this process, coordinated activation of various growth factors, cytokines, and chemokines plays an active role. Biomaterials that provide physical support in the wound area with their ECM-like structures and facilitate regeneration by promoting proliferation and migration exhibit wound healing effects. Due to these effects, natural biomaterials such as collagen, silk, eggshell membrane, and chitosan are used as dressings/wound dressings in addition to synthetic materials (Wilkinson and Hardman, 2020; Downer et al., 2023). Natural biomaterials hold promise in wound healing due to their mechanical strength, biocompatibility, biodegradability, and porous structure that allows nutrient/oxygen passage (Niculescu et al., 2022).

Collagen, one of the most important components of the ECM, is a polypeptide produced by fibroblasts and provides tensile strength in tissue formation. There are approximately 28 different subtypes, while type 1 collagen is most commonly found in skin, tendons, and bones (Chaudhari et al., 2016). Type 1 collagen, which can be isolated from animals such as cattle, rats, birds, and pigs, is used to create sponge structures that take the shape of the wound area and provide an environment suitable for regeneration. Collagen sponges, which are generally reported to be effective in chronic wounds, are preferred in tissue engineering due to their low risk of causing immunogenicity and their easy modification (Davison-Kotler et al., 2019). It has been reported that gentamicin-loaded type-1 collagen sponges support wound healing by promoting epithelialization in a wound model created in rats. Compared to the control group, it has been shown to reduce the expression of proinflammatory cytokines IL-6 and TNF-α, but increase IL-10 levels (Aravinthan et al., 2018). In a study, the wound healing activity of polyvinylpyrrolidone/polylactic acid-polyethylene oxide (PVP/PLA-PEO)-collagen complex nanofibers loaded with cefazolin was evaluated in mice with a wound model. The findings showed that nanofibers containing 10% and 20% collagen significantly increased the healing rate, while samples containing 40% collagen caused a decrease in the healing rate (Hajikhani et al., 2021). Despite its dose-dependent wound healing potential, collagen carries a risk of infection, which can lead to a vicious cycle in treatment (Li et al., 2021).

Silk is a protein biopolymer produced by various insects such as silkworms, flies, spiders, mites, and scorpions. It is considered a biocompatible material because it does not create a long-term or permanent inflammatory response and facilitates tissue migration. When initially placed in living tissue, it triggers an inflammatory response that decreases over time, indicating that silk is also immune-compatible (Chouhan et al., 2020). Water-insoluble silk consists of two main proteins fibroin, which makes up about 70% of the structure, and sericin, which binds the fibroins together. Silk fibroin has been reported to promote the proliferation, differentiation, and adhesion of various cell types, including epithelial, endothelial, fibroblast, keratinocyte, osteoblast, and glial cells (Mazurek et al., 2018). Fibroin, known to increase tissue regeneration, has been included in the Phase-1 study as a new carrier system for the treatment of open wounds in the palatal mucosa (Phase 1, NCT05171400). It has been shown that the hydrogel prepared with silk fibroin provides rapid re-epithelialization in rats with a third-degree burn model (Chouhan et al., 2018). Its hemostatic effect, non-allergenicity, and ability to retain exudate in the inflammation area make wound healing biomaterials using silk fibroin advantageous (Chouan et al., 2018). Sericin, which exhibits antioxidant and antimicrobial effects, promotes cell migration and collagen production, which play a critical role in wound healing (Mazurek et al., 2018). In studies, sericin NP formulations, which also have anti-inflammatory effects, have been combined with various antibacterial agents and have been shown to provide strong proliferation in an infected diabetic foot wound model (Diab et al., 2022). Additionally, sericin and chitosan are used to mask these undesirable effects by coating bioactive materials such as Ag NPs, which cause staining and irritation in the wound area. In a study, it was reported that effective wound healing was achieved without irritation with the synergistic antimicrobial effect of sericin and Ag NPs in a full-thickness excisional wound model created in rats (Verma et al., 2017). Although there is a lot of evidence for its wound healing effects, the low stability and solubility of sericin and the complex and costly extraction processes of fibroin are among the features that need to be improved for their clinical use (Mazurek et al., 2024).

Eggshell membrane stands out as a multi-biomolecular complex in wound healing with its collagen (Type I, V, X), glycoprotein, keratin, and hyaluronic acid content. The pores (0.1–7 μm) in its structure allow gas exchange, while the intertwined fibrous network structure protects against bacterial invasion. Due to its structure similar to ECM, the eggshell membrane

is used as a potential biomaterial in wound healing (Chen et al., 2022). In a study, AgNPs were loaded into eggshell membranes and used as a wound dressing in mice where a full-thickness excisional wound model was created. It has been shown that biomaterials support granulation tissue formation as well as epithelialization through inflammatory parameters (Liu et al., 2017). Moreover, it is very important to perform quality controls of animal-derived biomaterials to prevent pathogen transmission from the animal they are obtained from. Additionally, concerns about animal-derived biomaterials include variability that may occur during commercial production, long-term immunogenicity, standardization, and possible risks to clinical safety (Das et al., 2016).

Chitosan is a glucosamine-derived polysaccharide that can be obtained from the shells of creatures such as lobsters, shrimps, and crabs, as well as some fungi, algae, and microorganisms. It is widely used in wound healing applications, hemostasis, and surgical dressings due to its bioadhesion (Hamedi et al., 2022; Priya et al., 2024). It prevents pathogens from entering the wound area with its bioadhesive effect and exhibits an antibacterial effect by breaking down the negatively charged bacterial membrane with its cationic structure (Liu et al., 2018). Chitosan increases platelet and erythrocyte aggregation during the hemostasis phase of wound healing while exhibiting antibacterial effects during inflammation. It is also frequently used as a regenerative component since it promotes granulation formation (Raina et al., 2022). A wound healing film layer containing Ag-ZnO NP was developed using chitosan and guar gum, and it was shown that the chitosan film exhibited a strong inhibitory effect against Gram (–) and Gram (+) bacteria. After, it was reported that this film layer significantly increased migration in an *in vitro* wound model in L929 cells (Mohanty et al., 2023). In another study using pentoxifylline-loaded chitosan as a wound healing film component, it was shown that the formulation prepared at different concentrations released more than 80% of the drug by diffusion kinetics after 72 hours, providing sufficient concentration in the region. The formulation was also applied topically to rats in a wound model and significant healing was reported in the wound areas on the ninth day (Moreira et al., 2023). Some formulations of chitosan, which are considered safe in the Generally Recognized as Safe (GRAS) category, have been approved by the Food and Drug Administration (FDA) (Yan et al., 2021; Abourehab et al., 2022).

The fact that wound physiology varies from patient to patient reveals the importance of the biomaterial to be preferred in wound management. While each has its advantages and disadvantages, natural biomaterials generally have difficulties such as low biostability and high production costs. Biostability can be increased by cross-linking with other polymers, but this limits the infiltration of cells into the area, which plays a critical role in wound healing. In this context, research continues to develop new biomaterials that have high biostability, and low toxicity, and will accelerate wound healing efficiently and effectively (Chaudhari et al., 2016; Shivakumar et al., 2021; Niculescu et al., 2022).

Extract-Containing Hydrogel Biomaterials

Hydrogels are hydrophilic polymers with a three-dimensional porous network structure that can bind to 20-95% of the water in the environment. Hydrogels, which create a strong protective barrier in the wound area with their polymeric structure, can also adhere strongly to the wound area with the elastic and transparent film layer they form. This increases the release of the drug and skin penetration. In addition, the moist environment created by hydrogels in the wound area reduces swelling and pain due to inflammation and accelerates the wound healing process. They are used topically in various injuries such as burn wounds, ulcers, and surgical incisions due to their gas permeability, biocompatibility, and exudate absorption. In addition, hydrogels with a structure similar to ECM are loaded with stem cells or growth factors as well as anti-inflammatory, antioxidant, and antimicrobial compounds that play a critical role in wound healing (Stan et al., 2021; Huang et al., 2022; Yuniarsih et al., 2023). Besides these, although many medicinal plants support wound healing, there are various difficulties in applying plant extracts alone to the skin. Hydrogels have gained importance in the formulations of these herbal materials containing wound healing components due to their advantages in topical application (Stan et al., 2021; Yuniarsih et al., 2023). Hydrogel formulations of *Sansevieria trifasciata* leaf extract with strong bioactive properties were prepared at different concentrations (15%, 20%, 25%) and their healing effects were evaluated in a wound model created in rats. It was shown that the 25% hydrogel concentration of the plant extract increased the wound closure rate more than other formulations. The wound healing effect of the hydrogel formulation, which was reported to remain stable for 90 days, was associated with the plant content that can increase angiogenesis as well as collagen accumulation (Yuniarsih et al., 2023). In

another study, it was shown that *Calendula officinalis* L. extract, known for its anti-inflammatory and antioxidant effects, supported tissue regeneration in rats modeled with polyacrylamide (PAM)-based hydrogel decubitus ulcer wounds without causing any acute toxicity. It was also reported that the formulation accelerated wound healing through growth factors as well as proinflammatory cytokine expression (Ferreira et al., 2023).

In addition to their advantages in topical application, hydrogels can also respond to changes in the microenvironment at the wound site. Chronic wounds, especially diabetic foot wounds, are characterized by low pH, high ROS, and irregular MMP levels. This makes the wound susceptible to infection and results in impaired angiogenesis and wound healing. Hydrogels can show their effects by being stimulated by external factors such as temperature, light, and magnetism, as well as changes in ROS levels and pH at the lesion site. Thus, the bioactive components loaded into the hydrogel are effectively delivered to the wound site and a controlled release is provided (Stan et al., 2021; Chen et al., 2023). The crosslinkers utilized in the manufacturing process of hydrogels, which are referred to as ‘‘smart hydrogel’’ because of their functional characteristics, are responsible for these properties. In addition to natural polymers such as gelatin, hyaluronic acid, alginate, and dextran, synthetic polymers such as polyacrylic acid (PAA), poloxamer, polyvinyl alcohol (PVA), polyethylene glycol (PEG), and polyacrylamide are among the crosslinkers used in hydrogel preparation. Hydrogels formed with physical, chemical, or biological crosslinks respond to environmental changes by changing their permeability, network structure, swelling index, and sol-gel transitions (Liang et al., 2021; Aliakbar Ahovan et al., 2022; Wu et al., 2022).

There are also smart hydrogels developed using plant extracts to detect microenvironment changes in wound management in addition to treatment. It has been emphasized that pH-sensitive smart hydrogels have an important role in wound management since various parameters such as protease activity, oxygen availability, and angiogenesis are closely related to pH in the wound healing process. In this context, a chitosan hydrogel with red cabbage extract was prepared as a wound dressing based on the fact that red cabbage, used as a natural indicator, gives a red color in an acidic environment and a green color in a basic environment. It is known that the skin pH, which is 4.5-6.5, increases to 7.4 or even 9 pH in the event of bacterial infection. With this knowledge in mind, it was aimed to observe the changes in the

wound area with the hydrogel dressing prepared in the study (Arafa et al., 2021). Another hydrogel prepared using a combination of carrageenan, carob bean gum, and cranberry extract showed color change due to bacterial wound infections. The hydrogel, which was shown to increase cell viability in the mouse embryonic fibroblast cell line (NIH 3T3), was also reported to support wound healing with the antibacterial effect of its herbal content (Zepon et al., 2019).

The effectiveness of hydrogels and the speed of wound healing vary depending on the type of wound, depth, severity, blood flow rate in the area, presence of infection, and the patient's health status. When applied correctly at the right time, topical biomaterials have the potential to increase patient compliance and improve treatment outcomes. In this context, the development of new and modern topical biomaterials that will support wound healing in clinical use in wound management is quite valuable (Stan et al., 2021; Yuniarsih et al., 2023).

Conclusion

Around the world, injuries that compromise the integrity of the skin barrier pose a serious health problem worldwide. Topical biomaterials used in the treatment of wound healing accelerate the wound healing process with their biocompatibility and low toxicity risks. Recently, biomaterials containing antimicrobial agents, nucleic acids, plant extracts and green synthesis NPs have attracted attention with their wound healing supporting effects. Topical application of natural biomaterials obtained from natural sources such as collagen, eggshell membrane, and silk have also been reported to have significant wound healing effects. Although there are numerous advantages in increasing treatment efficacy, more research is needed to determine the effects of topical biomaterials produced from different sources that support wound healing in clinical use.

REFERENCES

Abourehab, M. A. S., Pramanik, S., Abdelgawad, M. A., Abualsoud, B. M., Kadi, A., Ansari, M. J., & Deepak, A. (2022). Recent advances of chitosan formulations in biomedical applications. *International Journal of Molecular Sciences*, *23*(18), 10975.

Ahmed, S., Annu, Chaudhry, S. A., & Ikram, S. (2017). A review on biogenic synthesis of ZnO nanoparticles using plant extracts and microbes: A prospect towards green chemistry. *Journal of Photochemistry and Photobiology. B, Biology*, *166*, 272–284.

Aldakheel, F. M., Sayed, M. M. E., Mohsen, D., Fagir, M. H., & El Dein, D. K. (2023). Green synthesis of silver nanoparticles loaded hydrogel for wound healing; systematic review. *Gels*, *9*(7), 530.

Aldayel, A. M., O'Mary, H. L., Valdes, S. A., Li, X., Thakkar, S. G., Mustafa, B. E., & Cui, Z. (2018). Lipid nanoparticles with minimum burst release of TNF-α siRNA show strong activity against rheumatoid arthritis unresponsive to methotrexate. *Journal of Controlled Release*, *283*, 280–289.

Aliakbar Ahovan, Z., Esmaeili, Z., Eftekhari, B. S., Khosravimelal, S., Alehosseini, M., Orive, G., … & Gholipourmalekabadi, M. (2022). Antibacterial smart hydrogels: New hope for infectious wound management. *Materials Todays Bio*, *17*, 100499.

Ansari, M., & Darvishi, A. (2024). A review of the current state of natural biomaterials in wound healing applications. *Frontiers in Bioengineering and Biotechnology*, *12*, 1309541.

Arafa, A. A., Nada, A. A., Ibrahim, A. Y., Sajkiewicz, P., Zahran, M. K., & Hakeim, O. A. (2021). Preparation and characterization of smart therapeutic pH-sensitive wound dressing from red cabbage extract and chitosan hydrogel. *International Journal of Biological Macromolecules*, *182*, 1820–1831.

Aravinthan, A., Park, J. K., Hossain, M. A., Sharmila, J., Kim, H. J., Kang, C. W., … & Kim, J. H. (2018). Collagen-based sponge hastens wound healing via decrease of inflammatory cytokines. *3 Biotech*, *8*(12), 487.

Berger, A. G., Chou, J. J., & Hammond, P. T. (2021). Approaches to modulate the chronic wound environment using localized nucleic acid delivery. *Advances in Wound Care*, *10*(9), 503–528.

Beyene, R. T., Derryberry, S. L., & Barbul, A. (2020). The effect of comorbidities on wound healing. *The Surgical Clinics of North America*, *100*(4), 695–705.

Broughton, G., Janis, J. E., & Attinger, C. E. (2006). The basic science of wound healing. *Plastic and Reconstructive Surgery*. *117*(7):12–34.

Caldwell, M. D. (2020). Bacteria and antibiotics in wound healing. *Surgical Clinics*, *100*(4), 757-776.

Chai, S. H., Wang, Y., Qiao, Y., Wang, P., Li, Q., Xia, C., & Ju, M. (2018). Bio fabrication of silver nanoparticles as an effective wound healing agent in the wound care after anorectal surgery. *Journal of Photochemistry and Photobiology. B, Biology*, *178*, 457–462.

Chaudhari, A. A., Vig, K., Baganizi, D. R., Sahu, R., Dixit, S., Dennis, V., … & Pillai, S. R. (2016). Future prospects for scaffolding methods and biomaterials in skin

tissue engineering: A review. *International Journal of Molecular Sciences*, *17(*12), 1974.
Chen, J., Cao, Z., Cannon, J., Fan, Y., Baker, J. R., Jr, & Wang, S. H. (2022). Effective treatment of skin wounds co-infected with multidrug-resistant bacteria with a novel nanoemulsion. *Microbiology Spectrum*, *10*(2), e0250621.
Chen, X., Chen, Y., Fu, B., Li, K., Huang, D., Zheng, C., … & Yang, D. P. (2022). Eggshell membrane-mimicking multifunctional nanofiber for in-situ skin wound healing. *International Journal of Biological Macromolecules*, *210*, 139–151.
Chen, Y., Wang, X., Tao, S., Wang, Q., Ma, P. Q., Li, Z. B., … & Li, D. W. (2023). Research advances in smart responsive-hydrogel dressings with potential clinical diabetic wound healing properties. *Military Medical Research*, *10*(1), 37.
Chouhan, D., & Mandal, B. B. (2020). Silk biomaterials in wound healing and skin regeneration therapeutics: From bench to bedside. *Acta Biomaterialia*, *103*, 24–51.
Chouhan, D., Lohe, T. U., Samudrala, P. K., & Mandal, B. B. (2018). In situ forming injectable silk fibroin hydrogel promotes skin regeneration in full thickness burn wounds. *Advanced Healthcare Materials*, *7*(24), e1801092.
ClinicalTrials.gov (2021). *A new drug delivery system - silk fibroin film loaded or not with insulin on palatal mucosa wound healing.* (Accessed on 29.08.2024, https://clinicaltrials.gov/study/NCT05171400?cond=wound&intr=fibroin&rank=2)
Dada, A. O., Adekola, F. A., Dada, F. E., Adelani-Akande, A. T., Bello, M. O., Okonkwo, C. R., … & Adetunji, C. O. (2019). Silver nanoparticle synthesis by *Acalypha wilkesiana* extract: Phytochemical screening, characterization, influence of operational parameters, and preliminary antibacterial testing. *Heliyon*, *5*(10), e02517.
Das, S., & Baker, A. B. (2016). Biomaterials and nanotherapeutics for enhancing skin wound healing. *Frontiers in Bioengineering and Biotechnology*, *4*, 82.
Davison-Kotler, E., Marshall, W. S., & García-Gareta, E. (2019). Sources of collagen for biomaterials in skin wound healing. *Bioengineering*, *6*(3), 56.
Demir, A. Y., Karadayi, M., Isaoglu, M., Karadayi, G., & Gulluce, M. (2023). *In vitro* genotoxicity assessment of biosynthesized zinc oxide nanoparticles. *Toxicology and Industrial Health*, *39*(7), 345–355.
Diab, S. E., Tayea, N. A., Elwakil, B. H., Gad, A. A. E. M., Ghareeb, D. A., & Olama, Z. A. (2022). Novel Amoxicillin-Loaded Sericin Biopolymeric Nanoparticles: Synthesis, Optimization, Antibacterial and Wound Healing Activities. *International Journal of Molecular Sciences*, *23*(19), 11654.
Downer, M., Berry, C. E., Parker, J. B., Kameni, L., & Griffin, M. (2023). Current biomaterials for wound healing. *Bioengineering*, *10*(12), 1378.
Enoch, S., & Leaper, D. J. (2008). Basic science of wound healing. *Surgery*, *26*(2), 31-37.
Ezhilarasu, H., Vishalli, D., Dheen, S. T., Bay, B. H., & Srinivasan, D. K. (2020). Nanoparticle-based therapeutic approach for diabetic wound healing. *Nanomaterials*, *10*(6), 1234.
Ezzat, S. M., Choucry, M. A., & Kandil, Z. A. (2016). Antibacterial, antioxidant, and topical anti-inflammatory activities of *Bergia ammannioides*: A wound-healing plant. *Pharmaceutical Biology*, *54*(2), 215–224.

Fang, S., Xu, C., Zhang, Y., Xue, C., Yang, C., Bi, H., … & Xing, X. (2016). Umbilical cord-derived mesenchymal stem cell-derived exosomal microRNAs suppress myofibroblast differentiation by inhibiting the transforming growth factor-β/SMAD2 pathway during wound healing. *Stem Cells Translational Medicine*, *5*(10), 1425–1439.

Ferreira, L. M. M. C., Bandeira, E. S., Gomes, M. F., Lynch, D. G., Bastos, G. N. T., Silva-Júnior, J. O. C., & Ribeiro-Costa, R. M. (2023). Polyacrylamide hydrogel containing calendula extract as a wound healing bandage: *In vivo* test. *International Journal of Molecular Sciences*, *24*(4), 3806.

Girigoswami K. (2018). Toxicity of metal oxide nanoparticles. Advances *in Experimental Medicine and Biology*, 1048, 99–122.

Guan, R., Kang, T., Lu, F., Zhang, Z., Shen, H., & Liu, M. (2012). Cytotoxicity, oxidative stress, and genotoxicity in human hepatocyte and embryonic kidney cells exposed to ZnO nanoparticles. *Nanoscale Research Letters*, *7*(1), 602.

Guest, J. F., Fuller, G. W., & Vowden, P. (2020). Cohort study evaluating the burden of wounds to the UK's National Health Service in 2017/2018: Update from 2012/2013. *British Medical Journal*, *10*(12), e045253.

Gwarzo, I. D., Mohd Bohari, S. P., Abdul Wahab, R., & Zia, A. (2022). Recent advances and future prospects in topical creams from medicinal plants to expedite wound healing: A review. *Biotechnology & Biotechnological Equipment*, *36*(1), 82-94.

Hajikhani, M., Emam-Djomeh, Z., & Askari, G. (2021). Fabrication and characterization of mucoadhesive bioplastic patch via coaxial polylactic acid (PLA) based electrospun nanofibers with antimicrobial and wound healing application. *International Journal of Biological Macromolecules*, *172*, 143–153.

Hamblin MR, Avci P, Prow TW. (2016). Nanoscience in Dermatology. (Ed. Hamblin, MR, Avci, P, Prow, TW) London: Academic Press-Elsevier.

Hamedi, H., Moradi, S., Hudson, S. M., Tonelli, A. E., & King, M. W. (2022). Chitosan based bioadhesives for biomedical applications: A review. *Carbohydrate Polymers*, *282*, 119100.

Han, G., & Ceilley, R. (2017). Chronic Wound healing: A review of current management and treatments. *Advances in Therapy*, *34*(3), 599–610.

Huang, C., Dong, L., Zhao, B., Lu, Y., Huang, S., Yuan, Z., … & Qian, W. (2022). Anti-inflammatory hydrogel dressings and skin wound healing. *Clinical and Translational Medicine*, *12*(11), e1094.

Icli, B., Nabzdyk, C. S., Lujan-Hernandez, J., Cahill, M., Auster, M. E., Wara, A. K., … & Feinberg, M. W. (2016). Regulation of impaired angiogenesis in diabetic dermal wound healing by microRNA-26a. *Journal of Molecular and Cellular Cardiology*, *91*, 151–159.

Jamkhande P.G., Ghule N.W., Bamer A.H., Kalaskar M.G. (2019). Metal nanoparticles synthesis: An overview on methods of preparation, advantages and disadvantages, and applications. *Journal of Drug Delivery Science and Technology*, *53*, 101174.

Kasiewicz, L. N., & Whitehead, K. A. (2016). Silencing TNFα with lipidoid nanoparticles downregulates both TNFα and MCP-1 in an *in vitro* co-culture model of diabetic foot ulcers. *Acta Biomaterialia*, *32*, 120–128.

Kasiewicz, L. N., & Whitehead, K. A. (2018). Lipid nanoparticles silence tumor necrosis factor α to improve wound healing in diabetic mice. *Bioengineering & Translational Medicine*, *4*(1), 75–82.

Khabir, Z., Holmes, A. M., Lai, Y. J., Liang, L., Deva, A., Polikarpov, M. A., … & Zvyagin, A. V. (2021). Human epidermal zinc concentrations after topical application of zno nanoparticles in sunscreens. *International Journal of Molecular Sciences*, *22*(22), 12372.

Kizhakkekalam, V. K., Chakraborty, K., & Krishnan, S. (2022). Antibacterial and wound healing potential of topical formulation of marine symbiotic *Bacillus*. *Archives of Microbiology*, *204*(10), 648.

Kolimi, P., Narala, S., Nyavanandi, D., Youssef, A. A. A., & Dudhipala, N. (2022). Innovative treatment strategies to accelerate wound healing: Trajectory and recent advancements. *Cells*, *11*(15), 2439.

Krausz, A. E., Adler, B. L., Cabral, V., Navati, M., Doerner, J., Charafeddine, R. A., … & Friedman, A. J. (2015). Curcumin-encapsulated nanoparticles as innovative antimicrobial and wound healing agent. *Nanomedicine : Nanotechnology, Biology, and Medicine*, *11*(1), 195–206.

Kumar V, Abbas AK, Aster JC. (2017). Robbins Basic Pathology. (Ed. Kumar, V, Abbas, AK, Aster, JC) Canada: Elsevier Health Sciences.

Lan, B., Zhang, L., Yang, L., Wu, J., Li, N., Pan, C., … & Ren, M. (2021). Sustained delivery of MMP-9 siRNA via thermosensitive hydrogel accelerates diabetic wound healing. *Journal of Nanobiotechnology*, *19*(1), 130.

Layeghi-Ghalehsoukhteh, S., Jalaei, J., Fazeli, M., Memarian, P., & Shekarforoush, S. S. (2018). Evaluation of 'green' synthesis and biological activity of gold nanoparticles using *Tragopogon dubius* leaf extract as an antibacterial agent. *IET Nanobiotechnology*, *12*(8), 1118–1124.

Li, D., Ren, J. W., Xu, T., Li, L., Liu, P., & Li, Y. (2021). Effect of bovine bone collagen oligopeptides on wound healing in mice. *Aging*, *13*(6), 9028–9042.

Li, H., Xue, Y., Jia, B., Bai, Y., Zuo, Y., Wang, S., … & Tang, H. (2018). The preparation of hyaluronic acid grafted pullulan polymers and their use in the formation of novel biocompatible wound healing film. *Carbohydrate Polymers*, *188*, 92–100.

Liang, Y., He, J., & Guo, B. (2021). Functional hydrogels as wound dressing to enhance wound healing. *ACS Nano*, *15*(8), 12687–12722.

Liu, M., Luo, G., Wang, Y., Xu, R., Wang, Y., He, W., … & Wu, J. (2017). Nano-silver-decorated microfibrous eggshell membrane: Processing, cytotoxicity assessment and optimization, antibacterial activity and wound healing. *Scientific Reports*, *7*(1), 436.

Liu, Y. F., Ni, P. W., Huang, Y., & Xie, T. (2022). Therapeutic strategies for chronic wound infection. *Chinese Journal of Traumatology*, *25*(1), 11–16.

Malakar, C., Kashyap, B., Kalita, M. C., & Deka, S. (2023). Wound healing efficacy of rhamnolipid-coated zinc oxide nanoparticle along with its *in vivo* antibacterial efficacy against *Staphylococcus aureus*. *Experimental Dermatology*, *32*(2), 154–164.

Mazurek, Ł., Rybka, M., Jurak, J., Frankowski, J., & Konop, M. (2024). Silk sericin and its effect on skin wound healing: A state of the art. *Macromolecular Bioscience*, e2400145.

Mazurek, Ł., Szudzik, M., Rybka, M., & Konop, M. (2022). Silk fibroin biomaterials and their beneficial role in skin wound healing. *Biomolecules*, *12*(12), 1852.

Mohanty, S., Bharadwaj, T., Verma, D., & Paul, S. (2023). Development of Ag doped ZnO nanostructure and tranexamic acid infused chitosan-guargum film: A multifunctional antimicrobial dressing with haemostatic and wound closure potential. *Chemical Engineering Journal*, *472*, 144976.

Moreira, V. M., Leite, J. M. D. S., Medeiros, K. D. A., Assis, K. M. A. D., Borges, J. C., Santana, L. M. B., ... & Damasceno, B. P. G. D. L. (2023). Pentoxifylline/chitosan films on wound healing: *In vitro/in vivo* evaluation. *Pharmaceutics*, *15*(4), 1122.

Nandhini, S. N., Sisubalan, N., Vijayan, A., Karthikeyan, C., Gnanaraj, M., Gideon, D. A. M., … & Sadiku, R. (2023). Recent advances in green synthesized nanoparticles for bactericidal and wound healing applications. *Heliyon*, *9*(2), e13128.

Naraginti, S., Kumari, P. L., Das, R. K., Sivakumar, A., Patil, S. H., & Andhalkar, V. V. (2016). Amelioration of excision wounds by topical application of green synthesized, formulated silver and gold nanoparticles in albino Wistar rats. *Materials for Biological Applications*, *62*, 293–300.

Nguyen, P. D., Tutela, J. P., Thanik, V. D., Knobel, D., Allen, R. J., Jr, Chang, C. C., … & Saadeh, P. B. (2010). Improved diabetic wound healing through topical silencing of p53 is associated with augmented vasculogenic mediators. *Wound Repair and Regeneration*, *18*(6), 553–559.

Niculescu, A. G., & Grumezescu, A. M. (2022). An up-to-date review of biomaterials application in wound management. *Polymers*, *14*(3), 421.

Paiva-Santos, A. C., Herdade, A. M., Guerra, C., Peixoto, D., Pereira-Silva, M., Zeinali, M., … & Veiga, F. (2021). Plant-mediated green synthesis of metal-based nanoparticles for dermopharmaceutical and cosmetic applications. *International Journal of Pharmaceutics*, *597*, 120311

Priya, S., Choudhari, M., Tomar, Y., Desai, V. M., Innani, S., Dubey, S. K., & Singhvi, G. (2024). Exploring polysaccharide-based bio-adhesive topical film as a potential platform for wound dressing application: A review. *Carbohydrate Polymers*, *327*, 121655.

Punjataewakupt, A., Napavichayanun, S., & Aramwit, P. (2019). The downside of antimicrobial agents for wound healing. *European Journal of Clinical Microbiology & Infectious Diseases*, *38*(1), 39–54.

Raina, N., Pahwa, R., Thakur, V. K., & Gupta, M. (2022). Polysaccharide-based hydrogels: New insights and futuristic prospects in wound healing. *International Journal of Biological Macromolecules*, *223*(Pt A), 1586–1603.

Rehana, D., Mahendiran, D., Kumar, R. S., & Rahiman, A. K. (2017). Evaluation of antioxidant and anticancer activity of copper oxide nanoparticles synthesized using medicinally important plant extracts. *Biomedicine & Pharmacotherapy*, *89*, 1067–1077.

Sezen, S. (2021). *Investigation of the therapeutic effects of essential oil from Origanum majorana (white oregano / Alanya oregano) on burn-injured wounds in Sprague-dawley rats using in-vivo and in-vitro methods.* Unpublished Doctoral Thesis, Erzurum: Atatürk University.

Sezen, S., Ertuğrul, M. S., Balpınar, Ö., Bayram, C., Özkaraca, M., Okkay, I. F., … & Güllüce, M. (2023). Assessment of antimicrobial activity and *in vitro* wound healing potential of ZnO nanoparticles synthesized with *Capparis spinosa*

extract. *Environmental Science and Pollution Research International*, *30*(55), 117609–117623.

Sezen, S., Nalcı, K.A., Demir, A.Y., Bayram, C., Çiçek, B., Okkay, U., … & Hacımüftüoğlu A. (2020). Investigation of wound healing and antimicrobial effects of zno nanoparticles synthesized using *Mentha longifolia* L. essential oil. *International Biotechnology Congress*, 05- 07 March, İstanbul, 56.

Sharma, P., Kumar, A., Agarwal, T., Dey, A. D., Moghaddam, F. D., Rahimmanesh, I., … & Sharifi, E. (2022). Nucleic acid-based therapeutics for dermal wound healing. *International Journal of Biological Macromolecules*, *220*, 920–933.

Shivakumar, P., Gupta, M. S., Jayakumar, R., & Gowda, D. V. (2021). Prospection of chitosan and its derivatives in wound healing: Proof of patent analysis (2010-2020). *International Journal of Biological Macromolecules*, *184*, 701–712.

Stan, D., Tanase, C., Avram, M., Apetrei, R., Mincu, N. B., Mateescu, A. L., & Stan, D. (2021). Wound healing applications of creams and "smart" hydrogels. *Experimental Dermatology*, *30*(9), 1218–1232.

Tan, L., Bogush, N., Naqvi, E., Calvert, J. W., Graham, R. M., Taylor, W. R., … & Husain, A. (2021). Thyroid hormone plus dual-specificity phosphatase-5 siRNA increases the number of cardiac muscle cells and improves left ventricular contractile function in chronic doxorubicin-injured hearts. *Theranostics*, *11*(10), 4790–4808.

Tatarusanu, S. M., Lupascu, F. G., Profire, B. S., Szilagyi, A., Gardikiotis, I., Iacob, A. T., … & Profire, L. (2023). Modern approaches in wounds management. *Polymers*, *15*(17), 3648.

Verma, J., Kanoujia, J., Parashar, P., Tripathi, C. B., & Saraf, S. A. (2017). Wound healing applications of sericin/chitosan-capped silver nanoparticles incorporated hydrogel. *Drug Delivery and Translational Research*, *7*(1), 77–88.

Wang, Y. W., Liou, N. H., Cherng, J. H., Chang, S. J., Ma, K. H., Fu, E., … & Dai, N. T. (2014). siRNA-targeting transforming growth factor-β type I receptor reduces wound scarring and extracellular matrix deposition of scar tissue. *The Journal of Investigative Dermatology*, *134*(7), 2016–2025.

Wang, Y., Malcolm, D. W., & Benoit, D. S. W. (2017). Controlled and sustained delivery of siRNA/NPs from hydrogels expedites bone fracture healing. *Biomaterials*, *139*, 127–138.

Westby, M. J., Norman, G., Watson, R. E. B., Cullum, N. A., & Dumville, J. C. (2020). Protease activity as a prognostic factor for wound healing in complex wounds. *Wound Repair and Regeneration*, *28*(5), 631–644.

Wilkinson, H. N., & Hardman, M. J. (2020). Wound healing: Cellular mechanisms and pathological outcomes. *Open Biology*, *10*(9), 200223.

Wu, Y., Wang, Y., Long, L., Hu, C., Kong, Q., & Wang, Y. (2022). A spatiotemporal release platform based on pH/ROS stimuli-responsive hydrogel in wound repairing. *Journal of Controlled Release*, *341*, 147–165.

Yan, D., Li, Y., Liu, Y., Li, N., Zhang, X., & Yan, C. (2021). Antimicrobial properties of chitosan and chitosan derivatives in the treatment of enteric infections. *Molecules*, *26*(23), 7136.

Yu, D., Ma, M., Liu, Z., Pi, Z., Du, X., Ren, J., & Qu, X. (2020). MOF-encapsulated nanozyme enhanced siRNA combo: Control neural stem cell differentiation and ameliorate cognitive impairments in Alzheimer's disease model. *Biomaterials*, *255*, 120160.

Yuniarsih, N., Hidayah, H., Gunarti, N. S., Kusumawati, A. H., Farhamzah, F., Sadino, A., & Alkandahri, M. Y. (2023). Evaluation of wound-healing activity of hydrogel extract of *Sansevieria trifasciata* leaves (Asparagaceae). *Advances in Pharmacological and Pharmaceutical Sciences*, 7680518.

Zepon, K. M., Martins, M. M., Marques, M. S., Heckler, J. M., Dal Pont Morisso, F., Moreira, M. G., … & Kanis, L. A. (2019). Smart wound dressing based on κ-carrageenan/locust bean gum/cranberry extract for monitoring bacterial infections. *Carbohydrate Polymers*, *206*, 362–370.

Zhuang, Y., & Cui, W. (2021). Biomaterial-based delivery of nucleic acids for tissue regeneration. *Advanced Drug Delivery Reviews*, *176*, 113885.

BRYOPHYTES IN BIOTECHNOLOGY

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Introduction

Bryophytes, the ancestors of terrestrial plants, constitute the second largest plant group after Angiosperms in terms of the number of species. They are represented by about 20.000 species in the world (WFO, 2024). The term of 'bryophyte' is generally used to refer collectively to mosses (Bryophyta), liverworts (Marchantiophyta) and hornworts (Anthocerotophyta) (Glime and Chavoutier, 2017). From tropical regions to subarctic and subantarctic regions, bryophytes can be found in a wide variety of climates where there is sufficient moisture to sustain their life and are important components of many ecosystems (Walsh et al., 2024). Lacking true roots, stems and leaves, these plants depend on water for sexual reproduction. They live on a variety of substrates such as soil, rocks, tree trunks, dead wood and obtain the mineral nutrients needed for their growth directly from the substances dissolved in the moist substrates in which they live (Spitale, 2017). The ability of bryophytes to endure prolonged dry spells, even in extremely hot weather, and to quickly resume photosynthesis when dampened by rain or dew is a remarkable adaptation. The majority of bryophytes are ectohydric, meaning they can take up water, inorganic fertilizers, and minerals straight from the atmosphere instead of through the substratum and soil (Bahuguna et al., 2013). Additionally, thanks to their poikilohydric properties, they balance their internal water balance according to the external environment. Since they do not have a developed vascular system, they take water from the environment by diffusion from all their surfaces (Glime, 2017a). Therefore, since their interaction with the environment is very high, they are very sensitive to the smallest ecological factor changes occurring in their habitats and are directly affected. This sensitivity increases their probability of being effective bioindicators and provides valuable information about environmental conditions.

Historically, studies have generally focussed on their role in ecosystems and their use in traditional medicine, but advances in molecular biology, genomics and biotechnology have created a different perspective on bryophytes and their potential as powerful tools for modern science and industry. In addition, the biochemical mechanisms they have evolved to survive for millions of years, their diverse physiological properties and unique biological properties such as metabolites have made them attractive candidates for environmental remediation, drug production and more biotechnological applications (Decker and Reski, 2020). They also have

immense promise for biomapping atmospheric precipitation (Saxena and Harinder, 2004).

Bryophytes in Environmental Biotechnology

Phytoremediation

Phytoremediation, one of the methods applied to clean up contaminated soil, air and water, is the use of plants and associated microbial communities to reduce the concentrations and toxic effects of various environmental pollutants (Pilon-Smits, 2005; McCutcheon and Jørgensen, 2008). Bryophytes are used in the remediation of various environmental pollutants through phytoremediation, thanks to their ability to survive in harsh environments with high pollution levels and various climatic conditions where other plants cannot thrive, and their ability to do diffusion with their entire surface. Moreover, bryophytes with strong drought tolerance, are particularly effective at absorbing and accumulating heavy metals, organic pollutants and radioactive elements in soil and water. When it comes to monitoring air pollution caused by pollutants in urban areas, moss species are more useful than the leaves of vascular plants. Therefore, bryophytes are thought to be the most effective biomonitoring agent for pollution in the environment (Barukial and Hazarika, 2023).

Bryophytes are highly effective in removing various environmental pollutants. For example, they can accumulate heavy metals such as cadmium (Cd), copper (Cu), lead (Pb), mercury (Hg) and zinc (Zn) from contaminated soils and water (Fasani et al., 2022). This ability makes them useful in cleaning up contaminated areas, especially in areas affected by mining or industrial activities. When metals are in ionic form, they bind to extracellular anionic exchange sites found mostly on the cell wall and plasma membrane surfaces of mosses, and moss cells survive metal toxicity by immobilising most of these toxic ions outside the cell (Basile et al., 2012).

Mosses such as *Funaria hygrometrica*, examples of which are given in Table 1, have been extensively studied for their ability to accumulate heavy metals such as copper (Cu), lead (Pb), and zinc (Zn) from polluted environments. These species can retain heavy metals in their cell walls and vacuoles, thus reducing the bioavailability of these toxins in the environment (Basile et al., 2008). Furthermore, the dense mat-like growth of mosses on soil

surfaces can stabilise contaminated soils, preventing the spread of pollutants through erosion or leaching.

Table 1. Some bryophytes used in phytoremediation

Species	Metals	References
Marchantia polymorpha L.	Al, As, Cd, Cu, Fe, Pb, Zn	Ares et al., 2018; Vásquez et al., 2019
Funaria hygrometrica Hedw.	Pb, Zn, Cu	Basile et al., 2008
Scorpiurum circinatum (Brid.) M.Fleisch. & Loeske	Cd, Cu, Pb, Zn	Basile et al., 2012
Leptodictyum riparium (Hedw.) Warnst	Cu, Zn, Cd, Pb	Maresca et al., 2022
Imbribryum muehlenbeckii (Bruch & Schimp.) N. Pedersen	Fe, Cr	Tesser et al., 2021
Ricciocarpos natans (L.) Corda		
Sphagnum perichaetiale Hampe		
Philonotis thwaitessiic Mitt.	Cd	Taeprayoon et al., 2023

Some bryophytes can absorb and break down organic pollutants, including hydrocarbons and pesticides, making them valuable in remediation of contaminated soils and water bodies (Nirmala and Sugathan, 2022). Moreover, mosses have been used locally and nationally in the past as biomonitors of particularly persistent organic pollutants (POPs), polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) (Harmens et al., 2013).

Bioindicators of Environmental Health

Bryophytes are also recognised as excellent bioindicators due to their sensitivity to environmental changes, especially air and water quality. Their lack of a vascular system makes them directly dependent on the atmosphere and water for nutrients, making them highly sensitive to changes in environmental chemistry (Sinha et al., 2021). Furthermore, different approaches have been put forward to demonstrate the use of bryophytes as important pollution indicators and these can be categorised as follows:

Sensitivity to Pollution

Bryophytes are highly sensitive to air and water pollution, especially heavy metals, acidic deposition and nitrogen compounds. Their lack of vascular systems exposes them more directly to environmental pollutants that can accumulate in their tissues (Sinha et al., 2021). In Japan (Taoda, 1972) and Europe (Greven, 1992), bryophytes have long been utilised to determine the impact of environmental pollution. In addition, mosses play an important role in nutrient cycling; immobilisation of nitrogen (N) in some habitats, such as bogs and heathlands, effectively traps the accumulation of reactive nitrogen (N), preventing its leaching into pore water and making it unavailable to the roots of higher plants (Curtis et al., 2005).

Indicator of Air Quality

Due to their high capacity to retain metals and other pollutants, bryophytes are widely used in active air quality monitoring studies in temperate and tropical regions (Benítez et al., 2021; Sinha et al., 2021). The presence or absence of certain bryophyte species can reveal levels of pollutants such as sulphur dioxide or ammonia in the air. One of the important air pollutants for terrestrial and epiphytic bryophytes is considered to be SO_2 (sulphur dioxide). This is because it causes chlorophyll plasmolysis and severely affects plant growth (Govindapyari et al., 2010). In addition, bryophytes are effective in capturing airborne pollutants, including nitrogen oxides, sulphur dioxide and particulate matter, and contribute to improving air quality in urban environments (Govindapyari et al., 2010; Pescott et al., 2015).

Water Quality Indicators

In aquatic environments, bryophytes can be used to assess water quality and ecological status. Aquatic bryophytes are used as indicators of the presence or absence of a pollutant or as monitors to measure pollutant concentrations (Gecheva and Yurukova, 2014). Using bryophytes for biomonitoring water quality (also called ‘bryomonitoring’) also enables simultaneous monitoring of a large number of compounds (i.e. organic, inorganic and even radioactive compounds) by analysing a single sample, and assessment of water quality at small (e.g. around pollutant sources) and large scales (e.g. regional) (Debén et al., 2017). Changes in species composition or health of aquatic bryophytes can indicate problems such as nutrient pollution or heavy metal contamination in the water. *Fontinalis antipyretica* and

Platyhypnidium riparioides, two common aquatic moss found in the northern hemisphere, are the most common bryomonitors (Gecheva and Yurukova, 2014; Debén et al., 2017).

Habitat and Climate Indicators

Bryophytes are highly sensitive to microclimatic conditions, including changes in humidity and temperature, making them reliable indicators for monitoring climate change impacts at local and regional scales (Táborská et al., 2020). Their sensitivity stems from their physiology and their dependence on external water sources for survival and growth (Spitale, 2017). There are two ways that bryophytes can be utilised as indicators: (a) their existence or absence in the environment, and (b) morphological and physiological changes that can be tracked (Gignac, 2001; Ruklani et al., 2021). In regions where humidity levels fluctuate (due to deforestation, urbanisation or climate change), bryophytes can serve as indicators of microclimatic shifts. A decrease in humidity can lead to the decline or extinction of species adapted to humid environments, while drought-tolerant species may become more dominant (Gignac, 2001). Although bryophytes can be used as climate indicators, it has been reported that the main factor contributing to the temporal variation of differences in epiphytic bryophyte communities is not climate change but air quality change (Hutsemékers et al., 2023).

Biodiversity Indicators

Bryophytes serve as important biodiversity indicators because of their sensitivity to environmental changes and their ability to reflect the health of ecosystems. A diverse and healthy bryophyte community is often a sign of a well-preserved ecosystem (Bahuguna et al., 2013). Conversely, a reduction in bryophyte diversity may indicate environmental stress, habitat degradation or ecosystem change (He et al., 2016). Additionally, many bryophytes have specific habitat requirements such as moisture, light levels and substrate type (e.g. rocks, soil or bark). Changes in the abundance and diversity of bryophytes may reflect changes in habitat quality (Pitkämäki, 2015). On the other hand, in forests, bryophytes are particularly important indicators of forest integrity (Frego, 2007). In healthy, mature forests, a high diversity of bryophyte species can be found due to stable, moist and shady conditions. Logging, forest fragmentation or land use changes that alter canopy cover can lead to a reduction in bryophyte diversity (Hernández-Rodríguez et al., 2021).

Bryophytes in Pharmaceutical Biotechnology

Secondary Metabolites: A Source of Bioactive Compounds

Bryophytes synthesise a large number of secondary metabolites with a variety of nutraceutical and pharmacological properties because of their unique morphology and physiology (Cianciullo et al., 2022). Among the metabolites synthesised by bryophytes, flavonoids protect from light (Davies et al., 2020), flavonoids and sesquiterpenoids help to resist biotic stress (Cheng et al., 2009; Davies et al., 2020), most glycosides protect against low temperature and desiccation (Takezawa, 2018), and methoxyphenols and cinnamic acid derivatives provide anatomical structures that promote growth and development (Peter et al., 2019). These compounds, which also contain terpenoids, alkaloids, and phenolics, exhibit a range of biological activities, such as antimicrobial, antiviral, anti-inflammatory, antifungal, antioxidant, and anticancer effects (Glime, 2017c; Sabharwal et al., 2023).

Bryophytes contain different phytochemicals such as fatty acids, terpenoids, small phenolic molecules (Sen et al., 2023). Bibenzyls, small phenolic molecules, are colourless solid ethane derivatives obtained by the flavonoid biosynthetic route, and different bibenzyl and bisbibenzyl subtypes exist in different bryophyte species (Nandy and Dey, 2020). Liverworts contain high amounts of the cytotoxic bibenzyl compound (Jain et al., 2023).

The production of bioactive compounds by bryophytes is often influenced by environmental factors such as light, temperature, and moisture (Dziwak et al., 2022), which suggests that controlling growth conditions could enhance the yield of these valuable metabolites. This presents an exciting opportunity for the development of sustainable production systems for pharmaceuticals derived from bryophytes.

Traditional and Modern Medicinal Uses

In addition to the term ethnobotany - the multidisciplinary study of the relationship between plants and humans (Martin, 2010) - which is used for all plants, the term ‘Ethnobryology’ has been used to refer to the medicinal uses of bryophytes (Flowers, 1957). About 150 ethnobryological species are listed (Benek et al., 2022), a few of which are given in Table 2. While Native

Americans, Chinese and Indians have long utilised bryophytes for their therapeutic benefits, their use among Europeans became widespread later.

Traditionally, bryophytes have been used to treat liver diseases, ringworm, heart issues, inflammation, fever, urinary and digestive issues, female issues, infections, lung disease, skin issues, and as filters and pollutants-cleaning agents (Glime, 2017d). In modern medicine, on the other hand, bryophytes are being explored for their potential to treat a variety of conditions, including infections, inflammation, and cancer (Das et al., 2022). Secondary metabolites such as terpenoids, flavonoids and phenolic acids produced by bryophytes have bioactive properties with antibacterial, antifungal and anti-inflammatory effects (Glime, 2017c; Sabharwal et al., 2023). Furthermore, some studies have highlighted the potential of liverworts to produce anti-tumour compounds that can inhibit the growth of cancer cells (Cianciullo et al., 2022).

Advances in analytical techniques, including high performance liquid chromatography (HPLC) and mass spectrometry (MS), have significantly improved the ability to characterise these bryophyte-derived compounds in detail. These techniques allow the sensitive isolation and identification of new compounds, while also facilitating the study of their mechanisms of action. (Nandy and Dey, 2020).

Many antibiotics have been isolated from bryophytes, but few have been developed for medical use, despite their proven efficacy. In Germany, *Ceratodon purpureus* and *Bryum argenteum* are used to treat fungal infections of horses. Various medicinal uses such as antileukemic properties and anticancer agents seem promising (Glime, 2017d). With the increasing global threat to antibiotic resistance, the pharmaceutical industry is increasingly interested in exploring bryophytes as a source of novel antimicrobial agents (Cianciullo et al., 2021). Given the chemical diversity and proven antimicrobial activities of bryophyte secondary metabolites, they represent a promising avenue for the development of new antibiotics, especially against multidrug-resistant pathogens.

Table 2. Traditional medical uses of some bryophytes

Taxa	Medical uses	References
Bryum argenteum Hedw.	Fever	Asakawa, 2015; Chandra et al., 2017
	Fungal infections	Frahm, 2004
Ceratodon purpureus (Hedw.) Brid.	Fungal infections	Frahm, 2004
Conocephalum conicum (L.) Dumort.	Fever	Glime, 2017d
	Gallstones	Asakawa, 2015
	Snake bites	Asakawa, 2015
Cratoneuron filicinum (Hedw.) Spruce	Heart disease	Chandra et al., 2017
Dawsonia superba Grev.	Diuretics	Chandra et al., 2017
	Grow hair	Azuelo et al., 2011
Ditrichum pallidum (Hedw.) Hampe	Convulsions (especially in infants)	Chandra et al. 2017
Entodon flavescens (Hook.) A. Jaeger	Earache	Lubaina et al., 2014
Entodon myurus (Hook.) Hampe	Antibacterial	Singh et al., 2011
Fissidens nobilis Griff.	Jaundice	Asakawa, 2015
	Diuretics	Asakawa, 2015; Chandra et al., 2017
Fontinalis antipyretica Hedw.	Antipyretic	Drobnik and Stebel, 2014
Frullania ericoides (Nees) Mont.	Hair, scalp	Remesh and Manju, 2009
Funaria hygrometrica Hedw.	Pulmonary tuberculosis	Asakawa 2015; Chandra et al., 2017
Haplocladium microphyllum (Sw. ex Hedw.) (=*Haplocladium capillatum* (Mitt.) Broth.)	Cystitis, uropathy	Asakawa, 2015
	Adenopharyngitis	Asakawa, 2015
	Bronchitis, tonsillitis, tympanitis, cystitis	Ding, 1982
Hydrogonium orientale (F. Weber) Jan Kučera (= *Barbula indica* (Hook.) Spreng.)	Intermittent fever	Lubaina et al., 2014
	Menstrual pain	Chandra et al., 2017
Hyophila involuta (Hook.) A. Jaeger (=*Hyophila attenuata* Broth.)	Cough suppressant, neck pain	Lubaina et al., 2014
	Cuts, burns	Dagar and Dagar, 1999
Leptodictyum riparium (Hedw.) Warnst.	Fever	Asakawa, 2015; Chandra et al., 2017
	Uropathy	Asakawa, 2015

Marchantia paleacea Bertol.	Swelling, fever	Sabovljević et al., 2011
Marchantia polymorpha L.	Pulmonary tuberculosis	Asakawa, 2012
	Liver disorders, hepatitis jaundice, inflammation	Glime, 2017d
Oreas martiana (Hoppe & Hornsch.) Brid.	Menorrhagia	Chandra et al., 2017
	External Wounds	Asakawa, 2007, 2015; Chandra et al., 2017
Philonotis fontana (Hedw.) Brid.	Fever	Asakawa 2015; Chandra et al., 2017
	Adenopharyngitis	Asakawa, 2015
Plagiochasma appendiculatum Lehm. & Lindenb.	Burns, skin diseases	Kumar et al., 2007
Polytrichum commune Hedw.	Inflammation, fever	Chandra et al., 2017
	Hemostasis, wounds, hair care, diuretic, urinary tract infections, and as tea to treat colds	Herrick, 1995; Glime, 2017d; Ozturk et al., 2018; Benek et al., 2022
Polytrichum juniperinum Hedw.	Burns, diuretic, prostate	Karpiński and Adamczak, 2017
***Polytrichum* sp.**	Stop bleeding and night sweats, presumably associated with menopause	Fu et al. 2009; Chandra et al., 2017
Reboulia hemisphaerica (L.) Raddi	Skin blotches, external wounds, and bruises	Asakawa, 2007, 2015; Chandra et al., 2017
Rhodobryum giganteum (Schwägr.) Paris	Nervous prostration and cardio-vascular diseases	Wu, 1982
	Uropathy	Asakawa, 2015
	Sedative	Chandra et al., 2017
Rhodobryum roseum (Hedw.) Limpr.	Neurasthenia	Asakawa, 2015
	Sedative	Chandra et al., 2017
	Nervous prostration, cardio-vascular diseases	Wu, 1982
***Sphagnum* sp.**	Boils and mosquito bites	Glime, 2017d
Sphagnum teres (Schimp.) Ångstr. ex Hartm.	Eye diseases and hemorrhoids	Ding, 1982
Taxiphyllum taxirameum (Mitt.) M. Fleisch.	Anti-inflammatory	Asakawa, 2015
Weissia controversa Hedw.	Intermittent fever	Lubaina et al., 2014
Wiesnerella denudata (Mitt.) Steph.	Gall stones	Kumar et al., 2007

Bryophytes in Molecular Biology and Genetic Engineering

Bryophytes as Model Organisms

Model organisms are widely used to investigate various biological processes. Bryophytes can be useful models for understanding the chronology of land plant evolution and for studying functional genomics because they have low cellular complexity and a dominant haploid gametophytic phase. These distinctive features and progress in sequencing technology have led to the development of some bryophytes, including *Physcomitrium patens*, *Marchantia polymorpha*, *Anthoceros agrestis*, as modern model plants (Yadav et al., 2023). Also, for molecular genetic studies, several mosses such as *Funaria hygrometrica* and *Syntrichia ruralis* have been used as effective experimental tools (Wood et al., 2000). In addition, the potential of *Ceratodon purpureus* as a model for research on plant photosynthesis, quantitative genetics, sex-specific markers, sexual dimorphism, and genome expansion has also been considered by several studies (Shaw and Beer, 1999; Thornton et al., 2005; McDaniel, 2005; Norrell et al., 2014; Eppley et al., 2018; Nieto-Lugilde et al., 2018; Kollar et al., 2021).

Initially, however, the successful demonstration of efficient homologous recombination processes in *P. patens* (Schaefer and Zryd, 1997) provided an advantage over other model bryophytes in research on functional analysis of plant genes. *P. patens* was the first bryophyte whose genome was sequenced (Rensing et al., 2008). With the availability of its complete genome sequence and various molecular tools, this plant was developed as the first modern model organism for bryophyte research (Rensing et al., 2020). *M. polymorpha*, for which a complete genome sequence has been published, is a well-developed model plant for the liverwort lineage (Bowman et al., 2017). On the other hand, the development of tools and resources to investigate hornworts is still ongoing despite the availability of whole genome sequences for some *Anthoceros* species (*A. punctatus* (Li et al., 2020) and *A. angustus* (Zhang et al., 2020).

Metabolic Engineering in Bryophytes

Bryophytes, which have developed different metabolic pathways vital for survival in a variety of environments, are increasingly being utilised in

metabolic engineering research (Kulshrestha et al., 2022). The various secondary metabolites bryophytes produce, many of which have medicinal and ecological importance, help them cope with environmental stresses such as drought, UV radiation and temperature fluctuations (Davies et al., 2020). These compounds have potential applications in medicine, cosmetics, and agriculture (Rao, 2021). Metabolic engineering can increase or modify the production of these compounds for industrial use. Furthermore, metabolic engineering can be applied to increase photosynthetic efficiency or alter carbon fixation pathways. This could be useful for understanding fundamental photosynthetic mechanisms and for applications in synthetic biology (Bar-Even, 2018).

Moreover, bryophytes are known for their resilience to environmental stresses such as desiccation, high salinity, and extreme temperatures. Metabolic engineering will contribute to the understanding of the function of genes involved in environmental adaptation and the application for the production of useful metabolites derived from stress-resistant mosses (Byun et al., 2021).

Bryophytes can be engineered to produce biofuels or other valuable biomolecules (Sirohi et al., 2019). Their ability to grow in nutrient-poor environments and their simple growth requirements make them attractive candidates for sustainable bioproduction systems.

Bryophytes have therapeutic potential due to the presence of phenols, flavonoids, terpenoids, polysaccharides, lipids, amino acids, quinines and phenylpropanoids (Bandyopadhyay and Dey, 2022). They are also sources of unique bioactive compounds with anti-inflammatory, antimicrobial and antioxidant properties (Sabharwal et al., 2023). In addition, bryophytes are used to produce proteins that clot human blood, while some are known to reduce thrombin activity (Glime, 2017b). Engineering studies to produce bryophytes with higher yields may make them suitable for pharmaceutical production.

The metabolic flexibility of bryophytes can also be utilised for environmental sustainability, including carbon sequestration and pollution reduction. Their ability to thrive in extreme environments allows the use of engineered bryophytes in harsh agricultural environments or for ecological restoration (Kulshrestha et al., 2022).

Bryophytes in Agriculture and Horticulture

Soil Health, Erosion Control and Biofertilizers

The role of bryophytes in soil formation and stabilisation has long been known. In sustainable agricultural practices, bryophytes are increasingly being studied as potential biofertilisers. Some bryophyte species, such as liverworts and hornworts, can form symbiotic relationships with nitrogen-fixing cyanobacteria that contribute to soil fertility by making nitrogen available to other plants (Adams, 2002). The ability to naturally enrich soils with essential nutrients makes bryophytes an environmentally friendly alternative to chemical fertilisers.

Bryophytes are highly effective in improving soil health and controlling erosion due to their ability to colonise barren and degraded soils. Mosses in particular are pioneer species that thrive in nutrient-poor environments and can initiate soil formation. Their dense cover helps stabilise the soil surface, reducing water runoff and erosion on slopes as well as protecting the soil from compaction and drying out (Glime, 2017f). *Sphagnum* mosses, for example, are widely used in horticulture as soil improvers. It has the ability to retain large amounts of water, making it an excellent medium for germinating seeds and growing plants that need a moist environment. Furthermore, the use of bryophytes in agriculture can contribute to sustainable agricultural practices by reducing the need for chemical fertilisers and improving soil health.

Horticultural and Landscaping Applications

Bryophytes, especially mosses, have both aesthetic and functional applications in horticulture and landscaping, making them increasingly popular in sustainable urban design (Radu et al., 2015). They are widely used for green roofs, living walls and terrariums because they require little maintenance, and their water retention properties are highly valued. One of the best-known uses of bryophytes is in Japanese gardens, where they are used to minimise the landscape and give a sense of distance (Glime, 2017e).

In modern horticulture, mosses are frequently used in terrariums due to their compact growth habit, ability to thrive in moist and enclosed environments, and aesthetic appeal (Nordström, 2019). *Sphagnum* moss in particular is widely used in the cultivation of epiphytic plants such as orchids

due to its superior water-holding capacity, which helps to maintain humidity levels (Younis et al., 2022).

Beyond their ornamental value, bryophytes also have important functional roles in reducing environmental impacts, especially in urban landscapes. Green roofs incorporating mosses and other bryophytes provide an efficient, low-maintenance solution for regulating building temperatures, reducing energy consumption and mitigating the effects of stormwater runoff. Mosses are particularly suitable for this purpose due to their ability to absorb and retain water, thus reducing the load on urban drainage systems during heavy rainfall (Oberndorfer et al., 2007).

In addition to their hydrological benefits, green roofs containing bryophytes help reduce urban heat islands by cooling the surrounding environment through evapotranspiration. Studies have shown that green roofs can significantly lower surface temperatures, which reduces the heat absorbed by buildings, leading to lower energy demand for cooling (Getter and Rowe, 2006).

The inclusion of bryophytes in urban landscapes represents a growing trend in sustainable architecture and urban planning, as these plants not only beautify spaces, but also provide ecological services that improve the quality of life in cities (Spangler, 2021).

Bryophytes in Industrial Biotechnology

Production of Biopolymers and Enzymes

The distinctive chemical makeup of bryophyte cell walls comprises polysaccharides, including cellulose, hemicellulose, and pectins, which display different structural features relative to those in vascular plants (Sarkar et al., 2009). These polysaccharides are of particular interest due to their potential applications in the development of bioplastics, adhesives, and other biodegradable materials, offering sustainable alternatives to traditional synthetic polymers (Benalaya et al., 2024).

In addition to their polysaccharide content, bryophytes are also a promising source for new enzymes of industrial importance. Enzymes such as cellulases, pectinases and glycoside hydrolases isolated from bryophytes have shown activity in the breakdown of complex carbohydrates, making them

valuable for industries such as biofuel production, waste management and textile processing (Marko et al., 2001). Moreover, these enzymes show exceptional thermostability and resistance to extreme environmental conditions, further increasing their applicability in demanding industrial processes. The ability of bryophyte-derived enzymes to catalyse the synthesis of fine chemicals adds another dimension to their potential, particularly in the pharmaceutical and speciality chemical sectors (Ndochinwa et al., 2024).

Bryophytes in Bioenergy Production

The potential of bryophytes for their use in bioenergy production and sustainable energy solutions is being investigated due to their various properties, such as their high durability, viability and production of valuable compounds (Bonhomme and Guillory, 2022). The ability and capacity of bryophytes to synthesise long-chain polyunsaturated fatty acids (vl-PUFAs) such as eicosapentaenoic acid (EPA) and arachidonic acid (AA), which are usually found in marine organisms, makes them a sustainable source of bioenergy (Degola et al., 2022). In addition, their biomass, which can be used in bioenergy production, can be converted into biofuels by various processes such as anaerobic digestion, pyrolysis or gasification (Proctor, 1990). Moreover, they synthesise a wide range of phenolic compounds with potential applications in bioenergy, which can improve the stability and efficiency of biofuels and make them more viable as a green energy alternative. The presence of these synthesised compounds not only helps to protect bryophytes against environmental stresses and pathogens, but also supports their use in bioenergy production (Commisso et al., 2021).

Despite the many advantages of bryophytes, there are challenges in optimising their cultivation and processing for large-scale bioenergy production. Ongoing research is focused on improving bryophyte biomass yields, increasing fatty acid production, and developing efficient methods to harvest and convert bryophyte biomass into usable forms of energy (Harris et al., 2020).

Conclusion

Bryophytes, one of the elements of biodiversity, are very important for many ecosystem services. They could hold 12 times their own weight in water and therefore act as a moisture buffer in many ecosystems. Moreover, these plants provide habitat for many microorganisms and invertebrates and

assimilate atmospheric nitrogen, especially in association with symbiotic cyanobacteria. They have the potential to capture large amounts of particulate matter, both organic and inorganic, from the atmosphere and can be used in air pollution monitoring. Due to their poikilohydric habits, bryophytes are excellent model organisms for detecting and monitoring subtle changes in local ecosystems, especially in habitats such as forests, wetlands and alpine zones, where microclimatic conditions play an important role on species composition and ecosystem health.

Global energy demand, one of the most important problems of our time, has led countries to renewable energy sources as a sustainable solution. This increasing demand can be met by using alternative sources such as biofuels instead of depending on fossil-based non-renewable energy sources such as oil, coal and natural gas, which are exhaustible and scarce resources.

Bryophytes have significant potential in bioenergy production due to their ability to produce valuable fatty acids, accumulate biomass in various environments and synthesize protective phenolic compounds. Research and studies should continue to fully realize the capabilities of these primitive plants and integrate them into sustainable energy systems. In this way, each step will open a new horizon to alleviate the energy crisis.

The use of bryophytes in horticulture and landscaping reflects their dual role as both ornamental and functional elements in urban environments. Their ability to thrive in a variety of settings, from Japanese gardens where they are particularly popular in Japanese gardens due to their high water-holding capacity and air permeability, to modern green roofs and living walls, emphasises their versatility and value in promoting sustainable and aesthetically pleasing urban designs.

As a result, the knowledge on the uses of bryophytes has deepened considerably in recent years. Future studies will contribute to revealing the real value and importance of these primitive plants, are referred as amphibians of plant kingdom, in many fields of biotechnology.

REFERENCES

Adams, D. G. (2002). Cyanobacteria in symbiosis with hornworts and liverworts. *Cyanobacteria in Symbiosis* (pp. 117-135). Dordrecht: Springer Netherlands.

Ares, Á., Itouga, M., Kato, Y., & Sakakibara, H. (2018). Differential metal tolerance and accumulation patterns of Cd, Cu, Pb and Zn in the liverwort *Marchantia polymorpha* L. *Bulletin of Environmental Contamination and Toxicology*, 100(3), 444-450.

Asakawa, Y. (2007). Biologically active compounds from bryophytes. *Pure and Applied Chemistry*, 79(4), 557-580.

Asakawa, Y. (2012). Liverworts-potential source of medicinal compounds. *Medicinal & Aromatic Plants*, 1, 1-2.

Asakawa, Y. (2015). Search for new liverwort constituents of biological interest. *Natural Products: Recent Advances*, 25-92.

Azuelo, A. G., Sariana, L. G., & Pabualan, M. P. (2011). Some medicinal bryophytes: their ethnobotanical uses and morphology. *Asian Journal of Biodiversity*, 2(1).

Bahuguna, Y. M., Gairola, S., Semwal, D. P., Uniyal, P. L., & Bhatt, A. B. (2013). Bryophytes and ecosystem. *Biodiversity of Lower Plants*, 2013, 279-296.

Bandyopadhyay, A., & Dey, A. (2022). The ethno-medicinal and pharmaceutical attributes of Bryophytes: A review. *Phytomedicine Plus*, 2(2), 100255.

Bar-Even, A. (2018). Daring metabolic designs for enhanced plant carbon fixation. *Plant Science*, 273, 71-83.

Barukial, J., & Hazarika, P. (2023). Bryophytes as an accumulator of toxic elements from the environment: recent advances. *Bioactive Compounds in Bryophytes and Pteridophytes*, 165-182.

Basile, A., Sorbo, S., Bassi, P., Napolitano, E., Cogoni, A. E., & Castaldo Cobianchi, R. (2008). Effects of heavy metals on protonemal development and ultrastructure in populations of the moss *Funaria hygrometrica* Hedw. (Bryophyta) from a mine and an unpolluted site. *Fresenius Environmental Bulletin*, 17, 1956-1963.

Basile, A., Sorbo, S., Pisani, T., Paoli, L., Munzi, S., & Loppi, S. (2012). Bioacumulation and ultrastructural effects of Cd, Cu, Pb and Zn in the moss *Scorpiurum circinatum* (Brid.) Fleisch. & Loeske. *Environmental Pollution*, 166, 208-211.

Benalaya, I., Alves, G., Lopes, J., & Silva, L. R. (2024). A review of natural polysaccharides: Sources, characteristics, properties, food, and pharmaceutical applications. *International Journal of Molecular Sciences*, 25(2), 1322.

Benek, A., Canlı, K., & Altuner, E. M. (2022). Traditional medicinal uses of mosses. *Anatolian Bryology*, 8(1), 57-65.

Benítez, Á., Armijos, L., & Calva, J. (2021). Monitoring air quality with transplanted bryophytes in a neotropical Andean city. *Life*, 11(8), 821.

Bonhomme, S., & Guillory, A. (2022). Synthesis and signalling of strigolactone and KAI2-ligand signals in bryophytes. *Journal of Experimental Botany*, 73(13), 4487-4495.

Bowman, J. L., Kohchi, T., Yamato, K. T., Jenkins, J., Shu, S., Ishizaki, K., ... & Schmutz, J. (2017). Insights into land plant evolution garnered from the *Marchantia polymorpha* genome. *Cell*, 171(2), 287-304.

Byun, M. Y., Seo, S., Lee, J., Yoo, Y. H., & Lee, H. (2021). Transfection of arctic *Bryum* sp. KMR5045 as a model for genetic engineering of cold-tolerant mosses. *Frontiers in Plant Science*, 11, 609847.

Chandra, S., Chandra, D., Barh, A., Pandey, R. K., & Sharma, I. P. (2017). Bryophytes: Hoard of remedies, an ethno-medicinal review. *Journal of Traditional and Complementary Medicine*, 7(1), 94-98.

Cheng, A., Sun, L., Wu, X., & Lou, H. (2009). The inhibitory effect of a macrocyclic bisbibenzyl riccardin D on the biofilms of *Candida albicans*. *Biological and Pharmaceutical Bulletin*, 32(8), 1417-1421.

Cianciullo, P., Cimmino, F., Maresca, V., Sorbo, S., Bontempo, P., & Basile, A. (2022). Anti-Tumour Activities from Secondary Metabolites and Their Derivatives in Bryophytes: A Brief Review. *Applied Biosciences*, 1(1), 73-94.

Cianciullo, P., Maresca, V., Sorbo, S., & Basile, A. (2021). Antioxidant and antibacterial properties of extracts and bioactive compounds in bryophytes. *Applied Sciences*, 12(1), 160.

Commisso, M., Guarino, F., Marchi, L., Muto, A., Piro, A., & Degola, F. (2021). Bryo-activities: a review on how bryophytes are contributing to the arsenal of natural bioactive compounds against fungi. *Plants*, 10(2), 203.

Curtis, C. J., Emmett, B. A., Grant, H., Kernan, M., Reynolds, B., & Shilland, E. (2005). Nitrogen saturation in UK moorlands: the critical role of bryophytes and lichens in determining retention of atmospheric N deposition. *Journal of Applied Ecology*, 42(3), 507-517.

Dagar, J. C., & Dagar, H. S. (1999). *Ethnobotany of aborigines of Andaman-Nicobar Islands*.

Das, K., Kityania, S., Nath, R., Das, S., Nath, D., & Talukdar, A. D. (2022). Bioactive compounds from Bryophytes. *Bioactive Compounds in Bryophytes and Pteridophytes* (pp. 1-15). Cham: Springer International Publishing.

Davies, K. M., Jibran, R., Zhou, Y., Albert, N. W., Brummell, D. A., Jordan, B. R., Bowman, J. L., & Schwinn, K. E. (2020). The evolution of flavonoid biosynthesis: a bryophyte perspective. *Frontiers in Plant Science*, 11, 7.

Debén, S., Aboal, J. R., Carballeira, A., Cesa, M., & Fernández, J. A. (2017). Monitoring river water quality with transplanted bryophytes: A methodological review. *Ecological Indicators*, 81, 461-470.

Decker, E. L., & Reski, R. (2020). Mosses in biotechnology. *Current Opinion in Biotechnology*, 61, 21-27.

Degola, F., Sanità di Toppi, L., & Petraglia, A. (2022). Bryophytes: how to conquer an alien planet and live happily (ever after). *Journal of Experimental Botany*, 73(13), 4267-4272.

Ding, H. (1982). Medicinal spore-bearing plants of China. *Shanghai Science and Technology Press*, Shanghai, 1-409.

Drobnik, J., & Stebel, A. (2014). Medicinal mosses in pre-Linnaean bryophyte floras of central Europe. An example from the natural history of Poland. *Journal of Ethnopharmacology*, 153(3), 682-685.

Dziwak, M., Wróblewska, K., Szumny, A., & Galek, R. (2022). Modern use of bryophytes as a source of secondary metabolites. *Agronomy*, 12(6), 1456.

Eppley, S. M., Rosenstiel, T. N., Chmielewski, M. W., Woll, S. C., Shaw, Z. M., & Shortlidge, E. E. (2018). Rapid population sex-ratio changes in the moss *Ceratodon purpureus*. *American Journal of Botany*, 105(7), 1232-1238.

Fasani, E., Li, M., Varotto, C., Furini, A., & DalCorso, G. (2022). Metal detoxification in land plants: From bryophytes to vascular plants. STATE of the art and opportunities. *Plants*, 11(3), 237.

Flowers, S. (1957). Ethnobryology of the Gosuite Indians of Utah. *The Bryologist*, 60(1), 11-14.

Frahm, J. P. (2004). Recent developments of commercial products from bryophytes. *The Bryologist*, 107(3), 277-283.

Frego, K. A. (2007). Bryophytes as potential indicators of forest integrity. *Forest Ecology and Management*, 242(1), 65-75.

Fu, P., Lin, S., Shan, L., Lu, M., Shen, Y. H., Tang, J., Liu, R. H., Zhang, X., Zhu, R. L., & Zhang, W. D. (2009). Constituents of the moss *Polytrichum commune*. *Journal of Natural Products*, 72(7), 1335-1337.

Gecheva, G., & Yurukova, L. (2014). Water pollutant monitoring with aquatic bryophytes: a review. *Environmental Chemistry Letters*, 12, 49-61.

Getter, K. L., & Rowe, D. B. (2006). The role of extensive green roofs in sustainable development. *HortScience*, 41(5), 1276-1285.

Gignac, L. D. (2001). Bryophytes as indicators of climate change. *The Bryologist*, 104(3), 410-420.

Glime, J. M. (2017a). *Bryophyte Ecology*. Volume 1: Physiological Ecology. Chapter 1: Introduction. Ebook sponsored by Michigan Technological University and the International Association of Bryologists.

Glime, J. M. (2017b). *Bryophyte Ecology*. Volume 5: Uses. Chapter 1-1: Household and Personal Uses. Ebook sponsored by Michigan Technological University and the International Association of Bryologists.

Glime, J. M. (2017c). *Bryophyte Ecology*. Volume 5: Uses. Chapter 2-2: Biologically Active Substances. Ebook sponsored by Michigan Technological University and the International Association of Bryologists.

Glime, J. M. (2017d). *Bryophyte Ecology*. Volume 5: Uses. Chapter 2-1: Medical Uses: Medical Conditions. Ebook sponsored by Michigan Technological University and the International Association of Bryologists.

Glime, J. M. (2017e). *Bryophyte Ecology*. Volume 5: Uses. Chapter 7-2: Gardening: Japanese Moss Gardens. Ebook sponsored by Michigan Technological University and the International Association of Bryologists.

Glime, J. M. (2017f). Bryophyte Ecology. Volume 5: Uses. Chapter 7-1: Gardening: Horticultural Uses. Ebook sponsored by Michigan Technological University and the International Association of Bryologists.

Glime, J. M. & Chavoutier, L. (2017). Glossary. *Bryophyte Ecology*. Ebook sponsored by Michigan Technological University and the International Association of Bryologists.

Govindapyari, H., Leleeka, M., Nivedita, M., & Uniyal, P. L. (2010). Bryophytes: indicators and monitoring agents of pollution. *NeBIO*, 1(1), 35-41.

Greven, H.C. (1992). Changes in the Dutch bryophyte flora and air pollution. *Diss. Bot. J. Cramer*, Berlin, Germany.

Harmens, H., Foan, L., Simon, V., & Mills, G. (2013). Terrestrial mosses as biomonitors of atmospheric POPs pollution: a review. *Environmental Pollution*, 173, 245-254.

Harris, B. J., Harrison, C. J., Hetherington, A. M., & Williams, T. A. (2020). Phylogenomic evidence for the monophyly of bryophytes and the reductive evolution of stomata. *Current Biology*, 30(11), 2001-2012.

He, X., He, K. S., & Hyvönen, J. (2016). Will bryophytes survive in a warming world?. *Perspectives in Plant Ecology, Evolution and Systematics*, 19, 49-60.

Hernández-Rodríguez, E., Escalera-Vázquez, L. H., García-Ávila, D., Montoro Girona, M., & Mendoza, E. (2021). Reduced-impact logging maintain high moss diversity in temperate forests. *Forests*, 12(4), 383.

Herrick, J. W. (1995). *Iroquois Medical Botany*. Syracuse University Press.

Hutsemékers, V., Mouton, L., Westenbohm, H., Collart, F., & Vanderpoorten, A. (2023). Disentangling climate change from air pollution effects on epiphytic bryophytes. *Global Change Biology*, 29(14), 3990-4000.

Jain, V., Ghorai, M., Das, T., & Dey, A. (2023). Anticancerous Compounds from Bryophytes: Recent Advances with Special Emphasis on Bis (bi) benzyls. *Bioactive Compounds in Bryophytes and Pteridophytes*, 91-115.

Karpiński, T. M., & Adamczak, A. (2017). Antibacterial activity of ethanolic extracts of some moss species. *Herba Polonica*, 63(3), 11-17.

Kollar, L. M., Kiel, S., James, A. J., Carnley, C. T., Scola, D. N., Clark, T. N., Khanal, T., Rosenstiel, T. N., Gall, E. T., Grieshop, K., & McDaniel, S. F. (2021). The genetic architecture of sexual dimorphism in the moss *Ceratodon purpureus*. *Proceedings of the Royal Society B*, 288(1946), 20202908.

Kulshrestha, S., Jibran, R., Van Klink, J. W., Zhou, Y., Brummell, D. A., Albert, N. W., Schwinn, K. E., Chagné, D., Landi, M., & Davies, K. M. (2022). Stress, senescence, and specialized metabolites in bryophytes. *Journal of Experimental Botany*, 73(13), 4396-4411.

Kumar, K., Nath, V., & Asthana, A. K. (2007). Concept of bryophytes in classical text of Indian ethnobotanical prospective. *Current Trends in Bryology*. Bishen Singh Mahendra Pal Singh. Dehra Dun, India, 215-220.

Li, F. W., Nishiyama, T., Waller, M., Frangedakis, E., Keller, J., Li, Z., Fernandez-Pozo, N., Barker, M. S., Bennet, T., Blázquez, M. A., Cheng, S., Cuming, A. C., de Vries, J., de Vries, S., Delaux, P. M., Diop, I. S., Harrison, C. J., Hauser, D., Hernández-García, J., Kirbis, A., Meeks, J. C., Monte, I., Mutte, S. K., Neubauer, A., Quandt, D., Robison, T., Shimamura, M., Rensing, S. A., Villarreal, J. C., Weijers, D., Wicke, S., Wong, G. K. S., Sakakibara, K., & Szövényi, P. (2020). Anthoceros genomes illuminate the origin of land plants and the unique biology of hornworts. *Nature plants*, 6(3), 259-272.

Lubaina, A. S., Pradeep, D. P., Aswathy, J. M., Remya Krishnan, M. K. V., & Murugan, K. (2014). Traditional knowledge of medicinal bryophytes by the kani tribes of Agasthiyarmalai biosphere reserve, southern western ghats. Indo *American Journal of Pharmaceutical Sciences (IAJPS)*, 4, 2116-2121.

Maresca, V., Bellini, E., Landi, S., Capasso, G., Cianciullo, P., Carraturo, F., Pirintsos, S., Sorbo, S., Sanità di Toppi, L., Esposito, S., & Basile, A. (2022). Biological responses to heavy metal stress in the moss *Leptodictyum riparium* (Hedw.) Warnst. *Ecotoxicology and Environmental Safety*, 229, 113078.

Marko, S., Aneta, B., & Dragoljub, G. (2001). Bryophytes as a potential source of medicinal compounds. *Pregl Rev*, 21(1), 17-29.

Martin, G. J. (2010). *Ethnobotany: a methods manual*. Routledge.

McCutcheon, S. C., & Jørgensen, S. E. (2008). Phytoremediation. *Encyclopedia of Ecology*, 2, 2751-2766.

McDaniel, S. F. (2005). Genetic correlations do not constrain the evolution of sexual dimorphism in the moss *Ceratodon purpureus*. *Evolution*, 59(11), 2353-2361.

Nandy, S., & Dey, A. (2020). Bibenzyls and bisbybenzyls of bryophytic origin as promising source of novel therapeutics: pharmacology, synthesis and structure-activity. *DARU Journal of Pharmaceutical Sciences*, 28, 701-734.

Ndochinwa, O. G., Wang, Q. Y., Amadi, O. C., Nwagu, T. N., Nnamchi, C. I., Okeke, E. S., & Moneke, A. N. (2024). Current status and emerging frontiers in enzyme engineering: An industrial perspective. *Heliyon*.

Nieto-Lugilde, M., Werner, O., McDaniel, S. F., Koutecký, P., Kučera, J., Rizk, S. M., & Ros, R. M. (2018). Peripatric speciation associated with genome expansion and female-biased sex ratios in the moss genus *Ceratodon*. *American Journal of Botany*, 105(6), 1009-1020.

Nirmala, L., & Sugathan, S. (2022). Role of lower plants in the remediation of polluted systems. *Biological Approaches to Controlling Pollutants* (pp. 307-320). Woodhead Publishing.

Nordström, U. (2019). *Moss*. Penguin UK.

Norrell, T. E., Jones, K. S., Payton, A. C., & McDaniel, S. F. (2014). Meiotic sex ratio variation in natural populations of *Ceratodon purpureus* (Ditrichaceae). *American Journal of Botany*, 101(9), 1572-1576.

Oberndorfer, E., Lundholm, J., Bass, B., Coffman, R. R., Doshi, H., Dunnett, N., Gaffin, S., Köhler, M., Liu, K. K. Y., & Rowe, B. (2007). Green roofs as urban ecosystems: ecological structures, functions, and services. *BioScience*, 57(10), 823-833.

Ozturk, M., Gökler, İ., & Altay, V. (2018). Medicinal bryophytes distributed in Turkey. *Plant and Human Health, Volume 1: Ethnobotany and Physiology*, 323-348.

Pescott, O. L., Simkin, J. M., August, T. A., Randle, Z., Dore, A. J., & Botham, M. S. (2015). Air pollution and its effects on lichens, bryophytes, and lichen-feeding Lepidoptera: review and evidence from biological records. *Biological Journal of the Linnean Society*, 115(3), 611-635.

Peters, K., Treutler, H., Döll, S., Kindt, A. S., Hankemeier, T., & Neumann, S. (2019). Chemical diversity and classification of secondary metabolites in nine bryophyte species. *Metabolites*, 9(10), 222.

Pilon-Smits, E. (2005). Phytoremediation. *Annual Review of Plant Biology*, 56(1), 15-39.

Pitkämäki, T. (2015). Bryophyte diversity in semi-natural grasslands: the effects of habitat quantity and quality (Master's thesis).

Proctor, M. C. F. (1990). The physiological basis of bryophyte production. Botanical Journal of the Linnean Society, 104(1-3), 61-77.

Radu, D. M., Trautz, D., & Cantor, M. (2015). Moss: Decorative and Ecological Potential in Landscape. *ProEnvironment Promediu*, 8(21).

Rao, S. A. (2021). Structural information of natural product metabolites in bryophytes. *Evolutionary Diversity as a Source for Anticancer Molecules* (pp. 209-231). Academic Press.

Remesh, M., & Manju, C. N. (2009). Ethnobryological notes from Western Ghats, India. *The Bryologist*, 532-537.

Rensing, S. A., Goffinet, B., Meyberg, R., Wu, S. Z., & Bezanilla, M. (2020). The moss *Physcomitrium* (*Physcomitrella*) *patens*: a model organism for non-seed plants. *The Plant Cell*, 32(5), 1361-1376.

Rensing, S. A., Lang, D., Zimmer, A. D., Terry, A., Salamov, A., Shapiro, H., ... & Boore, J. L. (2008). The *Physcomitrella* genome reveals evolutionary insights into the conquest of land by plants. *Science*, 319(5859), 64-69.

Ruklani, S., Rubasinghe, S. C., & Jayasuriya, G. (2021). A review of frameworks for using bryophytes as indicators of climate change with special emphasis on Sri Lankan bryoflora. *Environmental Science and Pollution Research*, 1-13.

Sabharwal, H., Shukla, G., Kondepudi, K. K., Maurya, R., Kapila, S., & Dogra, N. (2023). Phytochemical Analysis and In Vitro Assessment of Extracts of *Rhodobryum roseum* for Antioxidant, Antibacterial and Anti-Inflammatory Activities. *Journal of Herbs, Spices & Medicinal Plants*, 29(4), 419-437.

Sabovljević, A., Soković, M., Glamočlija, J., Ćirić, A., Vujičić, M., Pejin, B., & Sabovljević, M. (2011). Bio-activities of extracts from some axenically farmed and naturally grown bryophytes. *Journal of Medicinal Plants Research*, 5(4), 565-571.

Sarkar, P., Bosneaga, E., & Auer, M. (2009). Plant cell walls throughout evolution: towards a molecular understanding of their design principles. *Journal of Experimental Botany*, 60(13), 3615-3635.

Saxena, D. K., & Harinder. (2004). Uses of bryophytes. *Resonance Article*, 56-65.

Schaefer, D. G., & Zrÿd, J. P. (1997). Efficient gene targeting in the moss *Physcomitrella patens*. *The Plant Journal*, 11(6), 1195-1206.

Sen, K., Khan, M. I., Paul, R., Ghoshal, U., & Asakawa, Y. (2023). Recent advances in the phytochemistry of Bryophytes: Distribution, structures and biological activity of Bibenzyl and Bisbibenzyl compounds. *Plants*, 12(24), 4173.

Shaw, J., & Beer, S. C. (1999). Life history variation in gametophyte populations of the moss *Ceratodon purpureus* (Ditrichaceae). *American Journal of Botany*, 86(4), 512-521.

Singh, M., Singh, S., Nath, V., Sahu, V., & Singh Rawat, A. K. (2011). Antibacterial activity of some bryophytes used traditionally for the treatment of burn infections. *Pharmaceutical Biology*, 49(5), 526-530.

Sinha, S., Singh, A., Sinha, D., & Chatterjee, R. (2021). A review on bryophytes as key bio-indicators to monitor heavy metals in the atmosphere. *International Journal of Plant and Environment*, 7(01), 49-62.

Sirohi, S., Yadav, C., & Banerjee, D. (2019). Biofuel from Bryophyta as an Alternative Fuel for Future. *Nature Environment & Pollution Technology*, 18(3).

Spangler, K. (2021). Bryophyte ecosystem services: how bryophytes impact ecosystem processes and their use in urban systems. (Bachelor of Science Thesis)

Spitale, D. (2017). Forest and substrate type drive bryophyte distribution in the Alps. *Journal of Bryology*, 39(2), 128-140.

Táborská, M., Kovács, B., Németh, C., & Ódor, P. (2020). The relationship between epixylic bryophyte communities and microclimate. *Journal of Vegetation Science*, 31(6), 1168-1180.3

Taeprayoon, P., Printarakul, N., Somtrakoon, K., Chunwichit, S., Yooma, K., Wiangdao, S., Avakul, P., & Meeinkuirt, W. (2023). Potentially toxic element accumulation of bryophyte taxa in contaminated soils at Tak Province, Thailand. *Ecological Indicators*, 147, 109971.

Takezawa, D. (2018). Mechanisms Underlying Freezing and Desiccation Tolerance in Bryophytes. In: Iwaya-Inoue, M., Sakurai, M., Uemura, M. (eds) Survival

Strategies in Extreme Cold and Desiccation. Advances in Experimental Medicine and Biology, vol 1081. Springer, Singapore.
Taoda, H. (1972). Mapping of atmospheric pollution in Tokyo based upon epiphytic bryophytes. *Japanese Journal of Ecology*, 22(3), 125-133.
Tesser, T. T., Bordin, J., Da Rocha, C. M., & Da Silva, A. (2021). Application of the dry and wet biomass of bryophytes for phytoremediation of metals: batch experiments. *Environmental Challenges*, 5, 100382.
The World Flora Online Plant List (WFO) (2024). https://wfoplantlist.org/ (Access date: 01.09.2024)
Thornton, L. E., Keren, N., Ohad, I., & Pakrasi, H. B. (2005). *Physcomitrella patens* and *Ceratodon purpureus*, mosses as model organisms in photosynthesis studies. *Photosynthesis Research*, 83, 87-96.
Vásquez, C., Calva, J., Morocho, R., Donoso, D. A., & Benítez, Á. (2019). Bryophyte communities along a tropical urban river respond to heavy metal and arsenic pollution. *Water*, 11(4), 813.
Walsh SK, Wolkis D, Maunder M. (2024). Plant conservation. Samuel, M.S. (Ed.) *Encyclopedia of Biodiversity*. Elsevier, Oxford, 690–706.
Wood, A. J., Oliver, M. J., & Cove, D. J. (2000). Bryophytes as model systems. *The Bryologist*, 103(1), 128-133.
Wu, P. C. (1982). Some uses of mosses in China. *Bryological Times*, 13(5), 5-7.
Yadav, S., Basu, S., Srivastava, A., Biswas, S., Mondal, R., Jha, V. K., Singh, S.K., & Mishra, Y. (2023). Bryophytes as modern model plants: an overview of their development, contributions, and future prospects. *Journal of Plant Growth Regulation*, 42(11), 6933-6950.
Younis, A., Ahsan, M., Akram, A., Lim, K. B., Zulfiqar, F., & Tariq, U. (2022). Use of organic substrates in sustainable horticulture. Hasanuzzaman, M., Hawrylak Nowak, B., Islam, T. and Fujita M. (Ed.) *Biostimulants for Crop Production and Sustainable Agriculture*, 122-138.
Zhang, J., Fu, X. X., Li, R. Q., Zhao, X., Liu, Y., Li, M. H., Zwaenepoel, A., Ma, H., Goffinet, B., Guan, Y. L., Xue, J. Y., Liao, Y. Y., Wang, Q. F., Wang, Q. H., Wang, J. Y., Zhang, G. Q., Wang, Z. W., Jia, Y., Wang, M. Z., Dong, S. S., Yang, J. F., Jiao, Y. N., Guo, Y. L., Kong, H. Z., Lu, A. M. Yang, H. M., Zhang, S. S., de Peer, Y. V., & Chen, Z. D. (2020). The hornwort genome and early land plant evolution. *Nature Plants*, 6(2), 107-118.

MOLECULAR DOCKING: A POWERFUL TOOL FOR BIOTECHNOLOGY RESEARCH

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Introduction

The present century is commonly named as the Computer Age due to the rapid advancements that have occurred in development of computer technologies over the past fifty years. During this period, computers made a rapid entry into the societies and began to provide profitable services in many areas such as finance, health, and education. Scientific research studies have been also heavily influenced by this trend and a great amount of research approaches has been integrated by the computer aided calculations (Vivona et al., 2008; Liu et al., 2015; Oliveira, 2019).

In biotechnological research, computers are used not only to statistically evaluate in vitro and in vivo experimental results, but also to predict and model the potential effects of a new biotechnological product before experimental set up. In this regard, the Computer-Aided Drug Design (CADD) has been the most effective and profitable approach in medicinal, pharmaceutical and biotechnological research studies (Wooley, 1989; Hassan Baig et al., 2016; Bassani & Moro, 2023; Gu et al., 2023).

CADD uses *in silico* computational tools and the knowledge from biology, chemistry, pharmaceutical diciplines and identifies potential drug candidates for design of further *in vitro* and *in vivo* research studies (Figure 1). It mainly divided into two main workflow models named as Ligand-Based Drug Design (LBDD) and Structure-Based Drug Design (SBDD). The latter, SBDD, is one of the most common approach of CADD. It generally starts with Molecular Docking and flows to Homology Modeling, Calculations of molecular dynamics and De novo drug design. Among these, molecular docking is the most crucial step in the SBDD and a great majority of recent research has focused on it (Yu & MacKerell, 2017; Bharatam, 2021; Niazi & Mariam, 2023).

Thus, *in silico* tools of biotechnology research, recent use perspectives of molecular docking approach, current versions of docking softwares, databases and assistive tools of docking applications discussed in this book chapter. Moreover, the topic was supported with molecular docking examples.

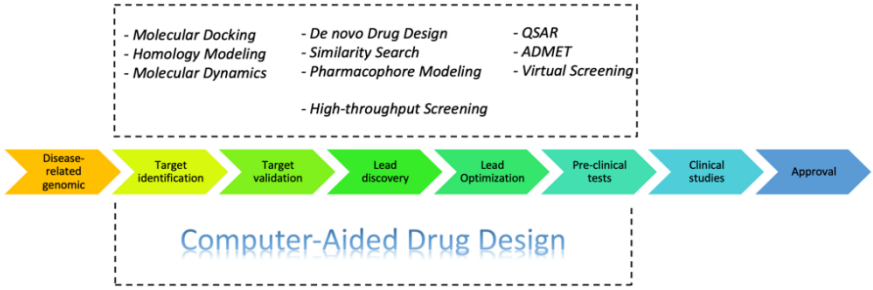

Figure 1. A typical workflow of the Computer-Aided Drug Design approach for identifying new drug candidates.

In silico Tools of Biotechnology Research

Although the number of computational tools used in recent biotechnological research is quite high, the most common *in silico* research tools encountered in the literature are belong to the CADD approach (Figure 2).

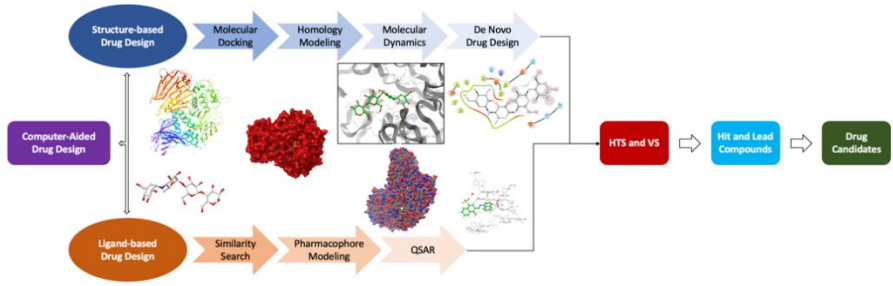

Figure 2. *In silico* research tools of the CADD approach.

Molecular Docking

Molecular docking is a computational method mainly used in the Structure-Based Drug Design workflow that focuses on 3D geometric and structural information of drug candidates and associated target biomolecules (Stanzione et al., 2021; Raval & Ganatra, 2022). Generally, the SBDD workflow starts with molecular docking studies to simulate possible molecular interactions between drug candidates called as ligands and target biomolecules

called as receptors. Therefore, it is considered a key component of *in silico* drug development studies and discussed in more detail in the next section of the chapter.

Homology Modeling

The 3D molecular structures of receptor proteins required for the molecular docking approach in elucidating receptor-ligand interactions are generally identified by X-ray crystallography or NMR (Vyas et al., 2012). However, the bottleneck encountered in cases where spectroscopic data of the target protein is not available can be overcome by the *in silico* homology modeling method. For this, the homology modeling uses an evolutionarily close protein as a model and its amino acid sequence to build predicted 3D molecular structure of the target receptor (Yu &MacKerell, 2017; Chahal et al., 2019).

Molecular Dynamics

Molecular dynamics (MD) is useful tool focusing on the ligand binding pockets and their possible conformational diversity. In this regard, MD simulations calculate ligand binding and protein folding parameters by using physical and atomic models (Hollingsworth & Dror, 2018).

De novo Drug Design

De novo drug design is another valuable tool of the SBDD approach and it includes a set of in silico computational methods to design novel drug candidates without any former information (Mouchlis et al., 2021; Atz et al., 2024).

Similarity Search

Similarity search is one of the effective tools of the Ligand-Based Drug Design that focuses on the information on the known bioactive molecules and uses it to calculate and simulate potentials of new drug candidates (Bacilieri & Moro, 2006; Acharya et al., 2011). Similarity search uses computer softwares and online databases to collect information on the studied drug structure and gives valuable predictions independent of any information about the potential targets (Paswan et al., 2021).

Pharmacophore Modeling

Pharmacophore modeling is a tool closely associated with other CADD *in silico* tools including virtual screening, molecular docking, drug target fishing, ligand profiling, and ADMET prediction. It mainly reveals the interactions between various types of ligands and a selected receptor site. The model development stages generally involve selecting a training set of ligands, conformational analysis, molecular superimposition, abstraction and validation steps (Yang, 2010; Schaller et al., 2020; Muhammed et al., 2021; Tyagi et al., 2022).

QSAR

Quantitative structure–activity relationship (QSAR) is another *in silico* tool of the LBDD approach. It is one of the first computer-aided methods and dates bact to the 19th century. QSAR analysis focuses physicochemical parameters such as hydrophobicity, topology, electronic properties, and steric effects and predicts possible biological activities of test compunds by using structural and molecular variations of the target molecule (Isarankura-Na-Ayudhya et al., 2009; Bastikar et al., 2022).

ADMET

ADMET is a powerful *in silico* approach that evaluates the drug potential of candidate molecules whose bioactivities have been revealed by in silico and/or in vitro experiments in terms of absorption, distribution, metabolism, excretion, and toxicology parameters (Dong et al., 2018; Ferreira & Andricopulo, 2019).

High-throughput Screening

High throughput screening (HTS) is a computational method that enables screening of thousands to millions of molecules for determination of their drug potential. By this way, giant molecule libraries can be examined in detail in an automated manner according to predetermined criteria (Wildey et al., 2017; Lloyd, 2020; Zeng et al., 2020).

Virtual Screening

Virtual screening is an advanced molecular docking method developed to examine and simulate possible molecular interactions of ligands in large molecule libraries with a specific target receptor. It stands out in drug development studies by allowing researchers to test a large number of molecules in a short time (Cosconati et al., 2010; Gimeno et al., 2019; Oliveira et al., 2023).

In silico Molecular Docking

Molecular docking has been one of the most used *in silico* tools in the computer aided drug design and discovery studies due to its high accurate predictions that reflect possible molecular and structural interactions between drug candidates and target biomolecules (Stanzione et al., 2021; Raval & Ganatra, 2022).

Principally, a molecular docking study starts with selection of ligands and receptors. In this regard, drug candidate molecules are referred as ligands and the target biomolecules are referred as receptors. Then, 3D molecular structures of ligands and receptors were obtained from experimental spectroscopic data or international online databases such as RCSB Protein Data Bank (RCSB PDB), UniProt, AlphaFold Protein Structure Database, ZINC database, DrugBank Online and NCBI PubChem database.

Among these RCSB Protein Data Bank, UniProt, AlphaFold Protein Structure Database are the most validated and used databases for obtaining 3D structures of target receptors. On the other hand, ZINC database, DrugBank Online and NCBI PubChem database are the well-known sources for obtaining of validated ligand structures. The user interfaces of these databases are shown in the Figures 3-8. After the obtaining the 3D molecular structures of ligands and receptors, the preparation steps are applied to the structures. In this regards, energy minimization of molecules is done, water molecules are removed, charges are added, missing atoms are added and polar hydrogens are added in this stage. Then, grid positions of the receptors are determined and docking procedure is performed. After molecular docking process is done, the results are visualized and evaluated (Figure 9).

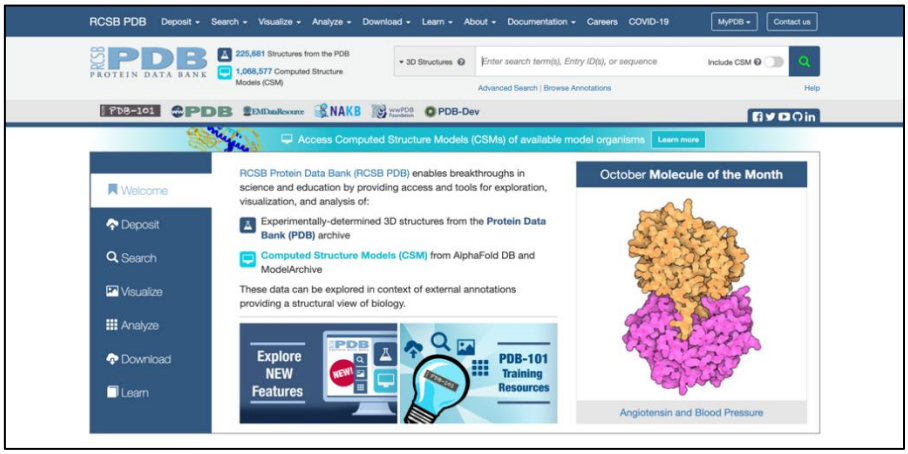

Figure 3. The user interface of RCSB Protein Data Bank (https://www.rcsb.org/).

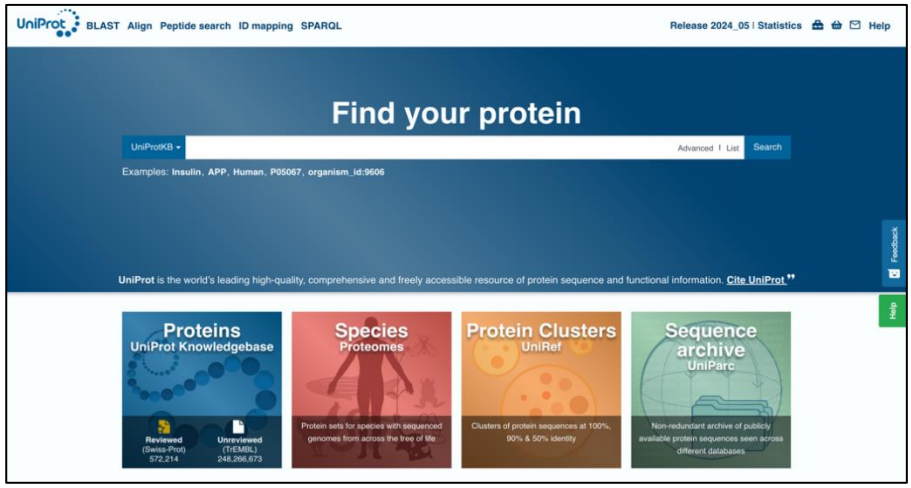

Figure 4. The user interface of UniProt Database (https://www.uniprot.org/).

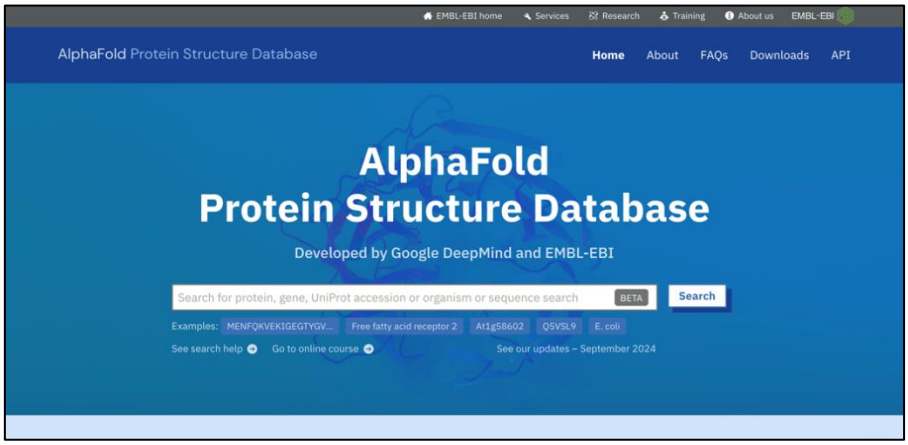

Figure 5. The user interface of AlphaFold Protein Structure Database (https://alphafold.ebi.ac.uk/).

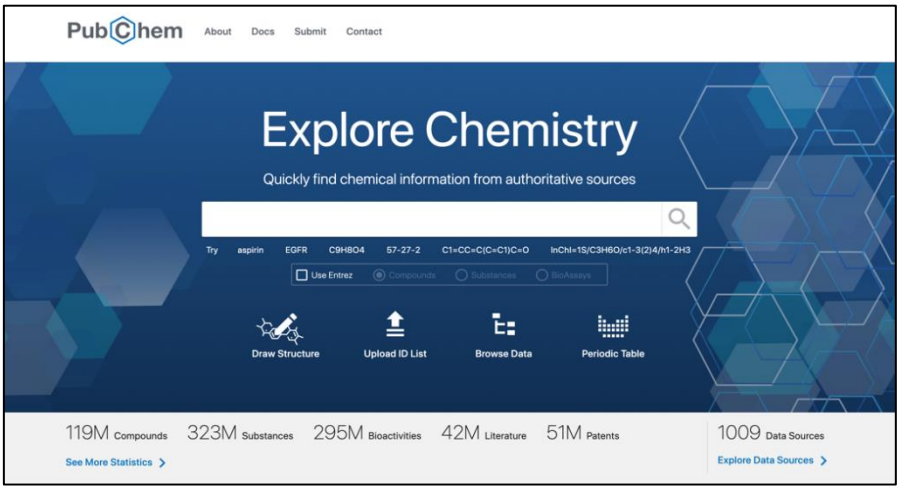

Figure 6. The user interface of NCBI PubChem Database (https://pubchem.ncbi.nlm.nih.gov/).

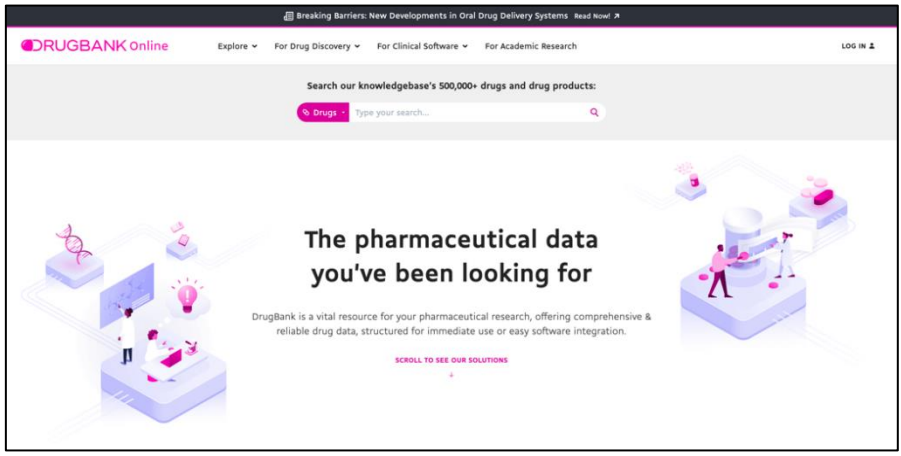

Figure 7. The user interface of DrugBank Online (https://go.drugbank.com/).

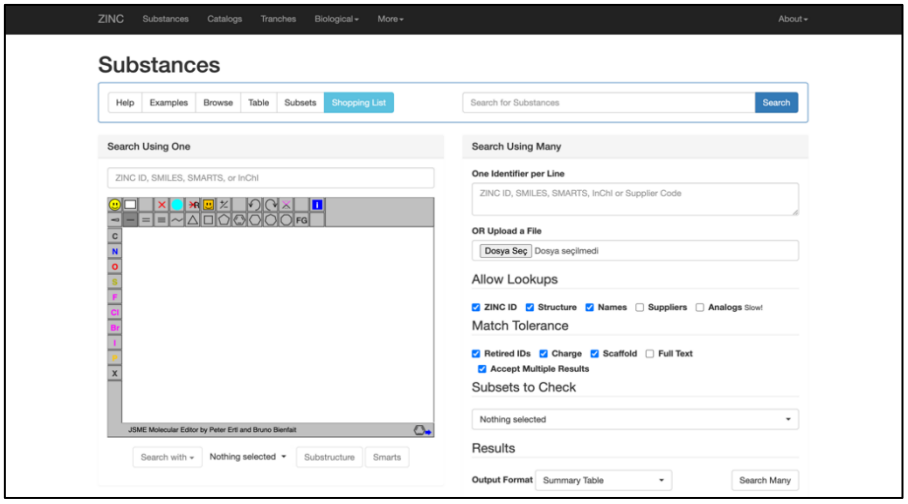

Figure 8. The user interface of ZINC database (https://zinc.docking.org/).

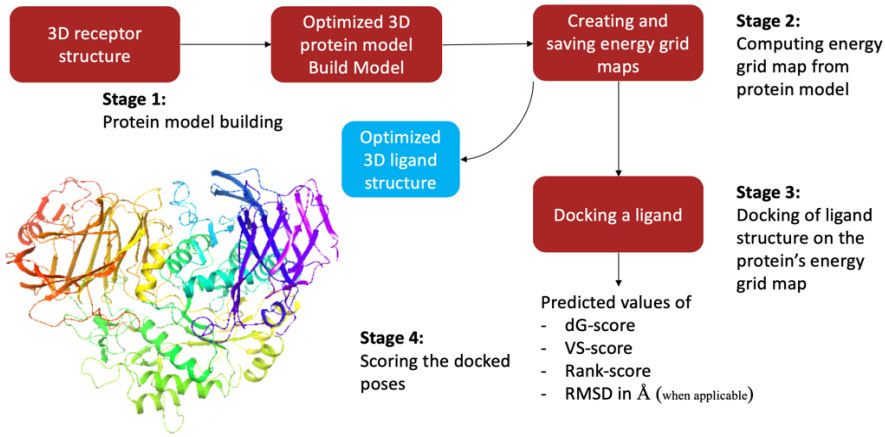

Figure 9. A principal workflow for a molecular docking study.

Common Molecular Docking Algorithms and Softwares

Although many kind of molecular docking algorithms and associated softwares have been used and reviewed in the literature, there is no one protocol for all studies. Most of the time, scientists have to choose appropriate algorithms and softwares depending on the nature of their study. In this regard, most popular molecular docking algorithms and softwares are summarized in the Table 1.

Example Molecular Docking Studies

The last section of the chapter presents four moulecular docking examples including standard antiviral, antibacterial, anticancer and antidiabetic drugs. All the drug structures were obtained from DrugBank Online database and the receptors were obtained from RCSB Protein Data Bank. The preparation of obtained molecular structures were done by using LigPrep, Protein Preparation Workflow and Receptor Grid Generation tools of Schrödinger. Then, docking studies were performed by Schrödinger Glide and visualized by Schrödinger Maestro version 13.9.138.

In this regard, the antiviral agent Nirmatrelvir was docked with COVID-19 main protease (PDB ID: 5R82), the antibacterial agent Gentamicin was docked with aquaporin-z (PDB ID: 1RC2), the anticancer agent Tamoxifen was docked with the crystal structure of CDK4, and the antidiabetic agent Acarbose was docked with the crystral structure of the N-terminal subunit of human maltase-glucoamylase (PDB ID: 2QMJ).

The visualized results of the example studies is shown in Figures 10-13. In addition, detailed interactions revealed by molecular docking approach are summarized Table 2.

Table 1. Common molecular docking algorithms and softwares (Hasan et al., 2022).

Programs	**Application**	**Accessibility**
AutoDock	Molecular docking software determines the binding capacity of a ligand to target protein	https://autodock.scripps.edu/
LPCCSU	Based on a comprehensive investigation of interatomic interactions and interface complementarity	https://oca.weizmann.ac.il/oca-bin/lpccsu
PatchDock	The method performs rigid docking, with surface variability	https://bioinfo3d.cs.tau.ac.il/PatchDock/php.php
Glide Schrodinger	Comprehensive molecular modeling and computer-aided drug development (CADD) tool	https://www.schrodinger.com/
Molecular operating environment	Comprehensive molecular modeling and computer-aided drug development (CADD) tool	https://www.chemcomp.com/
DockingServer	A user-friendly web-based interface that manages all elements of molecular docking.	https://www.dockingserver.com/web
SwissDock	A web service for predicting a protein's association with a small molecule ligand.	http://www.swissdock.ch/
LeDock	A molecular docking program for docking ligands with protein targets	http://www.lephar.com/software.htm
Molegro Virtual Docker	Based on a novel heuristic search method that integrates differential evolution and a cavity prediction algorithm	http://molexus.io/molegro-virtual-docker/
MOLS 2.0	Using mutually orthogonal Latin squares, induced-fit peptide protein, and small molecule–protein docking	https://sourceforge.net/projects/mols2-0/
ParaDockS	Metaheuristics for population-based molecular docking	http://www.paradocks.at/

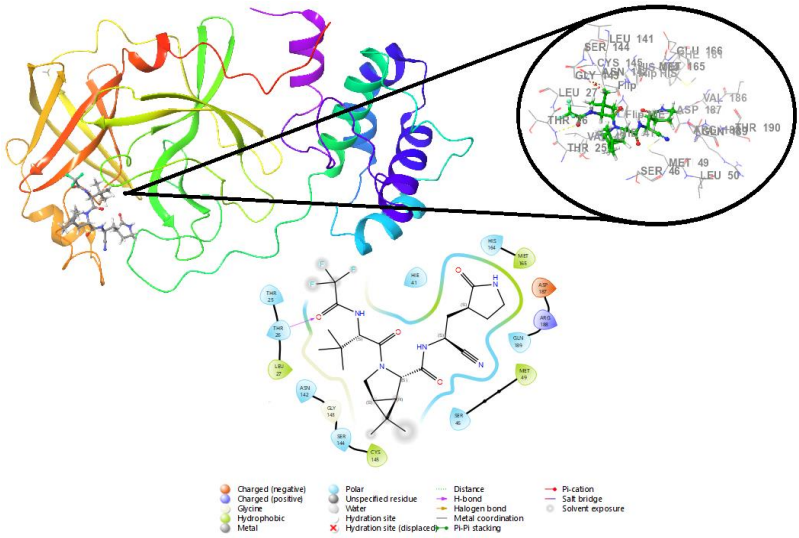

Figure 10. Molecular docking interactions of the antiviral agent Nirmatrelvir and the target receptor crystal structure of COVID-19 main protease (PDB ID: 5R82).

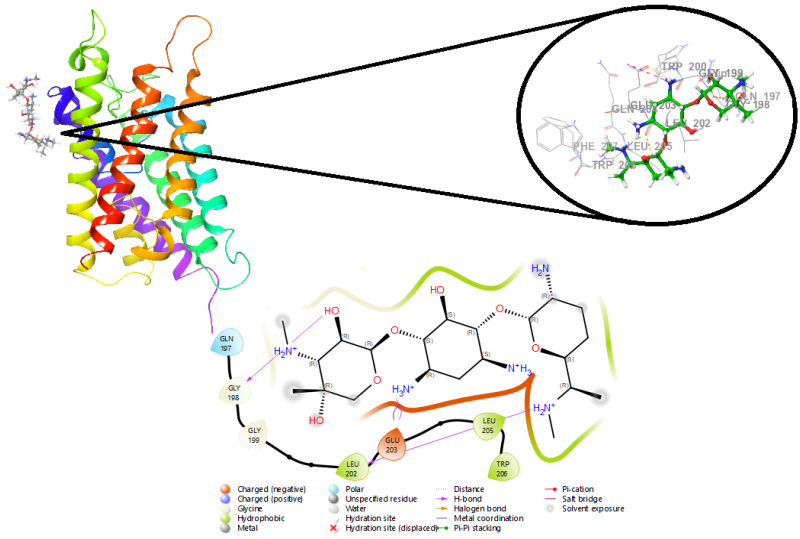

Figure 11. Molecular docking interactions of the antibacterial agent Gentamicin and the target receptor structure of aquaporin-z (PDB ID: 1RC2).

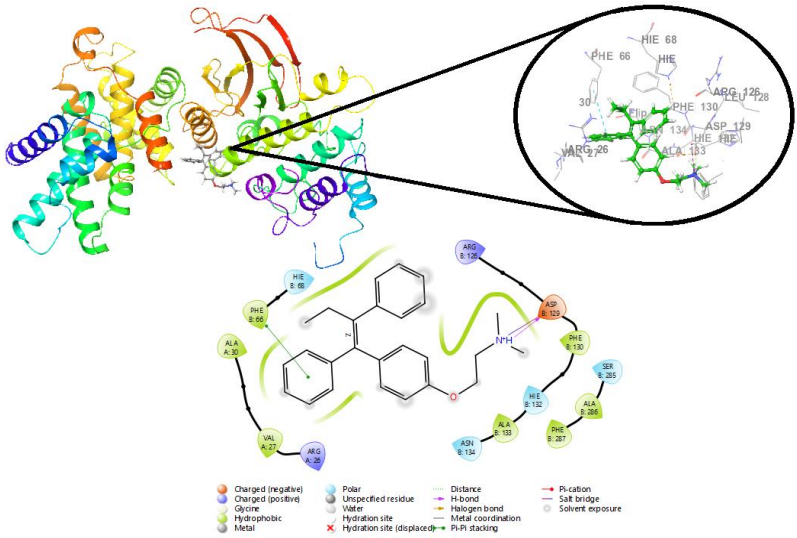

Figure 12. Molecular docking interactions of the anticancer agent Tamoxifen and the target receptor crystal structure of CDK4 (PDB ID: 2W96).

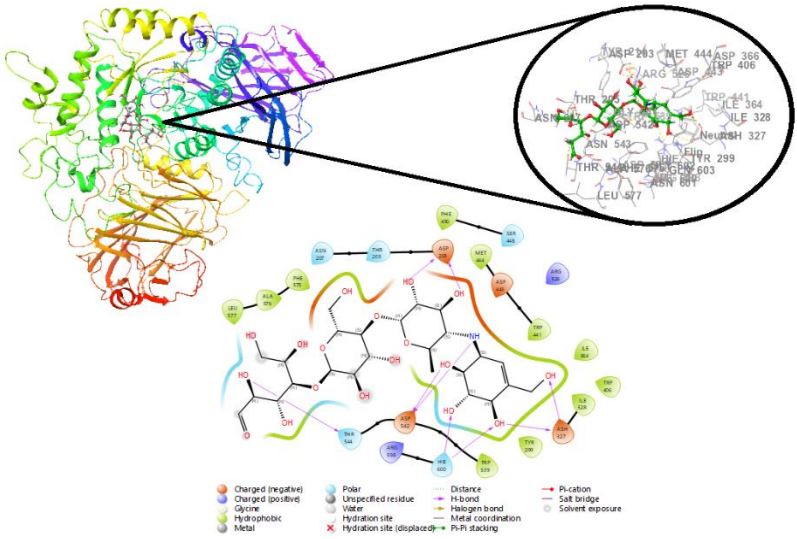

Figure 13. Molecular docking interactions of the antidiabetic agent Acarbose and the target receptor crystral structure of the N-terminal subunit of human maltase-glucoamylase (PDB ID: 2QMJ).

Table 2. Molecular interactions between selected ligands and related receptors.

Receptor ID	Ligand	Interacting Residues	Distance (Å)	Interaction	Glide Score (kcal/mol)
5R82	Nirmatrelvir	THR26	2.45	H-Bond	-4.699
1RC2	Gentamicin	GLY198 LEU202 GLU203 GLU203	1.92 1.80 1.90 5.00	H-Bond H-Bond H-Bond Salt Bridge	-3.937
2W96	Tamoxifen	ASP B129 ASP B129 PHE B66	1.65 4.75 5.34	H-Bond Salt Bridge π-π stacking	-3.404
2QMJ	Acarbose	THR544 ASP542 ASP542 ASP203 ASP203 ASH327 ASH327 HIE600 HIE600	2.40 2.03 2.40 1.90 2.21 1.76 2.15 2.12 2.64	H-Bond H-Bond H-Bond H-Bond H-Bond H-Bond H-Bond H-Bond H-Bond	-7.520

In conclusion, the molecular docking approach provides scientists a powerful *in silico* tool, especially for the discovery and development of new drugs. The use of this tool is increasing with the developments in the fields of biotechnology and computer sciences. In this way, promising candidate compounds to be carried to high-cost in vitro and in vivo experiments can be determined with high accuracy.

REFERENCES

Acharya, C., Coop, A., E Polli, J., & D MacKerell, A. (2011). Recent advances in ligand-based drug design: relevance and utility of the conformationally sampled pharmacophore approach. *Current Computer-Aided Drug Design*, *7(1)*, 10–22.

Ahmed, M., Maldonado, A. M., & Durrant, J. D. (2023). From byte to bench to bedside: molecular dynamics simulations and drug discovery. *BMC Biology*, *21(1)*, 299.

Atz, K., Cotos, L., Isert, C., Håkansson, M., Focht, D., Hilleke, M., Nippa, D.F., Iff, M., Schiebroek, C.C.G., Romeo, V., Hiss, J.A., Merk, D., Schneide, P., Kuhn, B., Grether, U., & Schneider, G. (2024). Prospective de novo drug design with deep interactome learning. *Nature Communications*, *15(1)*, 3408.

Bacilieri, M., & Moro, S. (2006). Ligand-based drug design methodologies in drug discovery process: an overview. *Current Drug Discovery Technologies*, *3(3)*, 155–165.

Bassani, D., & Moro, S. (2023). Past, present, and future perspectives on computer-aided drug design methodologies. *Molecules*, *28(9)*, 3906.

Bastikar, V., Bastikar, A., & Gupta, P. (2022). Quantitative structure–activity relationship-based computational approaches. In *Computational Approaches for Novel Therapeutic and Diagnostic Designing to Mitigate SARS-CoV-2 Infection*, 191–205. Academic Press.

Bharatam, P.V. (2021). Computer-aided drug design. *Drug discovery and development: From targets and molecules to medicines*, 137–210.

Chahal, V., Nirwan, S., & Kakkar, R. (2019). Combined approach of homology modeling, molecular dynamics, and docking: computer-aided drug discovery. *Physical Sciences Reviews*, *4(10)*, 20190066.

Cosconati, S., Forli, S., Perryman, A.L., Harris, R., Goodsell, D. S., & Olson, A.J. (2010). Virtual screening with AutoDock: theory and practice. *Expert Opinion on Drug Discovery*, *5(6)*, 597–607.

Dong, J., Wang, N.N., Yao, Z. J., Zhang, L., Cheng, Y., Ouyang, D., Lu, A.P., & Cao, D.S. (2018). ADMETlab: a platform for systematic ADMET evaluation based on a comprehensively collected ADMET database. *Journal of Cheminformatics*, *10*, 1–11.

Ferreira, L.L., & Andricopulo, A.D. (2019). ADMET modeling approaches in drug discovery. *Drug Discovery Today*, *24(5)*, 1157–1165.

Gimeno, A., Ojeda-Montes, M.J., Tomás-Hernández, S., Cereto-Massagué, A., Beltrán-Debón, R., Mulero, M., Pujadas, G., & Garcia-Vallvé, S. (2019). The light and dark sides of virtual screening: what is there to know?. *International Journal of Molecular Sciences*, *20(6)*, 1375.

Gu, R., Wu, F., & Huang, Z. (2023). Role of computer-aided drug design in drug development. *Molecules*, *28(20)*, 7160.

Hasan, M.R., Alsaiari, A.A., Fakhurji, B.Z., Molla, M.H.R., Asseri, A.H., Sumon, M.A.A., Park, M.N., Ahammad, F., & Kim, B. (2022). Application of mathematical modeling and computational tools in the modern drug design and development process. *Molecules*, *27*(*13*), 4169.

Hassan Baig, M., Ahmad, K., Roy, S., Mohammad Ashraf, J., Adil, M., Haris Siddiqui, M., Kamal, M., Provazník, I., & Choi, I. (2016). Computer aided drug design: success and limitations. *Current Pharmaceutical Design*, *22(5)*, 572–581.

Hollingsworth, S. A., & Dror, R. O. (2018). Molecular dynamics simulation for all. *Neuron*, *99(6)*, 1129–1143.

Liu, D., Valdiviezo-Díaz, P., Riofrio, G., Sun, Y. M., & Barba, R. (2015). Integration of virtual labs into science e-learning. *Procedia Computer Science*, *75*, 95–102.

Lloyd, M.D. (2020). High-throughput screening for the discovery of enzyme inhibitors. *Journal of Medicinal Chemistry*, *63(19)*, 10742–10772.

Mouchlis, V.D., Afantitis, A., Serra, A., Fratello, M., Papadiamantis, A.G., Aidinis, V., Lynch, I., Greco, D., & Melagraki, G. (2021). Advances in de novo drug design: from conventional to machine learning methods. *International Journal of Molecular Sciences*, *22(4)*, 1676.

Muhammed, M.T., & Akı Yalcın, E. (2021). Pharmacophore modeling in drug discovery: methodology and current status. *Journal of the Turkish Chemical Society Section A: Chemistry*, *8(3)*, 749–762.

Nantasenamat, C., Isarankura-Na-Ayudhya, C., Naenna, T., & Prachayasittikul, V. (2009). A practical overview of quantitative structure-activity relationship. *EXCLI Journal*, *8*, 74–88.

Niazi, S.K., & Mariam, Z. (2023). Computer-aided drug design and drug discovery: a prospective analysis. *Pharmaceuticals*, *17(1)*, 22.

Oliveira, A. L. (2019). Biotechnology, big data and artificial intelligence. *Biotechnology Journal*, *14(8)*, 1800613.

Oliveira, T.A.D., Silva, M.P.D., Maia, E.H.B., Silva, A.M.D., & Taranto, A.G. (2023). Virtual screening algorithms in drug discovery: A review focused on machine and deep learning methods. *Drugs and Drug Candidates*, *2(2)*, 311–334.

Paswan, S. K., Nath, V., Verma, P., Sikarwar, A. P., & Verma, S. K. (2021). Hydroxychloroquine: Similarity Search and Structure-Based Virtual Screening for Identification of Potential Hits for Chemoprophylaxis Against SARS-CoV-2. *FABAD Journal of Pharmaceutical Sciences*, *46(2)*, 147–158.

Raval, K., & Ganatra, T. (2022). Basics, types and applications of molecular docking: A review. *IP International Journal of Comprehensive and Advanced Pharmacology*, *7(1)*, 12–16.

Schaller, D., Šribar, D., Noonan, T., Deng, L., Nguyen, T.N., Pach, S., Machalz, D., Bermudez, M., & Wolber, G. (2020). Next generation 3D pharmacophore modeling. *Wiley Interdisciplinary Reviews: Computational Molecular Science*, *10(4)*, e1468.

Stanzione, F., Giangreco, I., & Cole, J.C. (2021). Use of molecular docking computational tools in drug discovery. *Progress in Medicinal Chemistry*, *60*, 273–343.

Tyagi, R., Singh, A., Chaudhary, K.K., & Yadav, M.K. (2022). Pharmacophore modeling and its applications. In *Bioinformatics*, 269–289. Academic Press.

Vivona, S., Gardy, J.L., Ramachandran, S., Brinkman, F. S., Raghava, G. P. S., Flower, D. R., & Filippini, F. (2008). Computer-aided biotechnology: from immuno-informatics to reverse vaccinology. *Trends in Biotechnology*, *26(4)*, 190–200.

Vyas, V. K., Ukawala, R.D., Ghate, M., & Chintha, C. (2012). Homology modeling a fast tool for drug discovery: current perspectives. *Indian Journal of Pharmaceutical Sciences*, *74(1)*, 1.

Wildey, M.J., Haunso, A., Tudor, M., Webb, M., & Connick, J.H. (2017). High-throughput screening. *Annual Reports in Medicinal Chemistry*, *50*, 149–195.

Wooley, J.C. (1989). Computational biology for biotechnology: Part II: Applications of scientific computing in biotechnology. *Trends in Biotechnology*, *7(5)*, 126–132.

Yang, S.Y. (2010). Pharmacophore modeling and applications in drug discovery: challenges and recent advances. *Drug Discovery Today*, *15(11-12)*, 444–450.

Yu, W., & MacKerell, A.D. (2017). Computer-aided drug design methods. *Antibiotics: Methods and Protocols*, 85–106.

Zeng, W., Guo, L., Xu, S., Chen, J., & Zhou, J. (2020). High-throughput screening technology in industrial biotechnology. *Trends in Biotechnology*, *38(8)*, 888–906.

THE LATEST TRENDS IN BACTERIOCINS IN BIOTECHNOLOGY: INNOVATIVE APPLICATIONS AND THE FUTURE

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Introduction

Lactic acid bacteria, widely used in the food industry, play a significant role in the development of distinctive characteristics such as taste, texture, aroma, and smell in food products derived from various raw materials, including meat, dairy, fruits, vegetables, and grains (Biler, 2009; Özlü, 2014; González-González et al., 2022). These bacteria guide the controlled fermentation process in the production of many food products, and the organic acids produced during this fermentation extend the shelf life of the products. Moreover, lactic acid bacteria also produce antimicrobial peptides, which are of great importance to the food industry. Among these peptides are bacteriocins, ammonia, free fatty acids, and hydrogen peroxide, which exhibit antagonistic effects against other microorganisms (Biler, 2009; Özlü, 2014; Karahançer, 2018; Bourdichon et al., 2021; González-González et al., 2022).

Antimicrobial peptides are known as an evolutionarily ancient host defense mechanism and play significant roles in various areas of modern medicine. In recent years, the misuse or overuse of antibiotics has led to the emergence of antibiotic resistance, which has become a serious global health threat. The rapid increase and spread of antibiotic-resistant bacteria have severely diminished the effectiveness of the antimicrobial and therapeutic agents currently available on the market. These alarming developments have heightened the need for new treatment methods and significantly increased research and interest in antimicrobial peptides. Due to their potential to replace traditional antibiotics, these peptides are seen as a promising alternative, particularly in the fields of infectious diseases and biomedical applications (Reuben et al., 2019, 2020; Wang et al., 2020; Anyaegbunam et al., 2022; Mba & Nweze, 2022; Field et al., 2023; Ye et al., 2023; Baquero et al., 2024; Reuben & Torres, 2024).

Bacteriocins are antimicrobial peptides (AMPs) synthesized by almost all bacteria, especially lactic acid bacteria, through ribosomal processes. These potent AMPs have the capacity to kill or inhibit the growth of other bacterial strains, both in the immediate vicinity and in more distant environments of the bacteriocinogenic strains—that is, the bacterial species that produce bacteriocins (Montalbán-López et al., 2011; Ladjouzi et al., 2023). The strong antimicrobial properties of bacteriocins make them valuable tools in the control and treatment of a wide range of bacterial infections.

Bacteria that produce bacteriocins are not harmed by these toxic molecules; this protection is ensured by their own immunity systems. These immunity systems safeguard the producer bacteria from the destructive effects of their own bacteriocins, allowing these bacteriocins to effectively target and neutralize potential pathogens in the environment. This characteristic makes bacteriocins highly significant not only in natural ecosystems but also in the food industry and medical applications. With a wide range of uses, from preserving food products to preventing harmful bacteria, bacteriocins hold the potential to offer innovative solutions in the fight against the growing resistance to antimicrobial agents (Yang et al., 2014; Teso-Pérez et al., 2021; González-González et al., 2022; Ladjouzi et al., 2023).

Bacteriocin

Bacteriocins are primary metabolites composed of proteins, polypeptides, and protein complexes, which have been used as alternatives to antibiotics in the food industry for many years. These compounds exhibit antagonistic activity either within a narrow genetic spectrum against closely related species or within a broader spectrum against species of the same genus. Bacteriocins have no adverse effects on human health or the environment; on the contrary, they provide strong protection against pathogenic microorganisms that can cause food spoilage or foodborne diseases (Sharma et al., 2022; Doğan, 2024). Due to these properties, bacteriocins enhance food safety by extending the shelf life of food products and reducing food waste. Additionally, their natural and biodegradable nature offers a solution that supports environmental sustainability. In the food industry, bacteriocins play a critical role in preserving the microbial stability and quality of various products, including meat, dairy products, fruits, vegetables, and grains. This versatile protection mechanism makes bacteriocins extremely valuable for both food safety and sustainability (Biler, 2009; Fernandes & Jobby, 2022; Sharma et al., 2022; Doğan, 2024).

The effective use of bacteriocins in the food industry offers an innovative approach that minimizes environmental impact by reducing dependency on antibiotics. These peptides stand out as solutions that not only protect consumer health but also support the sustainability of the food supply chain. Bacteriocins are antimicrobial peptides encoded by plasmid DNA, chromosomes, and other mobile genetic elements, synthesized via ribosomes.

They exert their antimicrobial effects by binding to specific receptors on target cells.

Bacteriocins, which form a highly heterogeneous group of peptides in terms of structure, size, shape, and mechanisms of action, vary depending on the cell receptors they target. They are typically produced by Gram-positive bacteria, though there are also bacteriocins known as microcins that are synthesized by Gram-negative bacteria. In the context of the food industry, bacteriocins exhibit strong antimicrobial activity, particularly against dangerous pathogenic microorganisms such as *Staphylococcus aureus*, *Clostridium botulinum*, and *Listeria monocytogenes*. These antimicrobial properties make bacteriocins extremely valuable for enhancing food safety and extending shelf life (Zacharof & Lovitt, 2013; Demir, 2014; Özlü, 2015; Kumar et al., 2018; Arsoy, 2019; Negash & Tsehai 2020; Simons et al., 2020; Abacı, 2022; Lei et al., 2022; Sharma et al., 2022; Kaya-Şen & Katı, 2023). The ability of bacteriocins to be used both as a preservative and as a quality-enhancing component stems from their versatile properties. Their natural origin and biodegradability are key factors that support environmental sustainability. For this reason, bacteriocins will continue to play a crucial role in future food safety strategies, ensuring the microbial stability of food products and extending their shelf life (Lei et al., 2022; Sharma et al., 2022; Kaya-Şen & Katı, 2023).

The scientific development of bacteriocins began in 1877 with Pasteur and Joubert's discovery of antimicrobial interactions between bacteria, a finding that led to the recognition of bacteriocins in the scientific community (Figure 1). This significant discovery laid the groundwork for future research on antimicrobial peptides and sparked intensive studies on the potential applications of bacteriocins. Since then, research into the effects of bacteriocins on various bacteria and their wide range of applications has continued unabated. Today, bacteriocins play a vital role in numerous fields, including food safety, medicine, and biotechnology (Pasteur & Jouber, 1877; Gratia, 1925; Gratia & Frederiq, 1946; Biler, 2008; Sharma et al., 2022; Kaya-Şen & Katı, 2023). This historical progression has established bacteriocins as significant not only in the food industry but also in other scientific and industrial fields. In today's context of rising antimicrobial resistance issues, the natural and effective solutions offered by bacteriocins will ensure that these peptides become increasingly prominent in future research and applications.

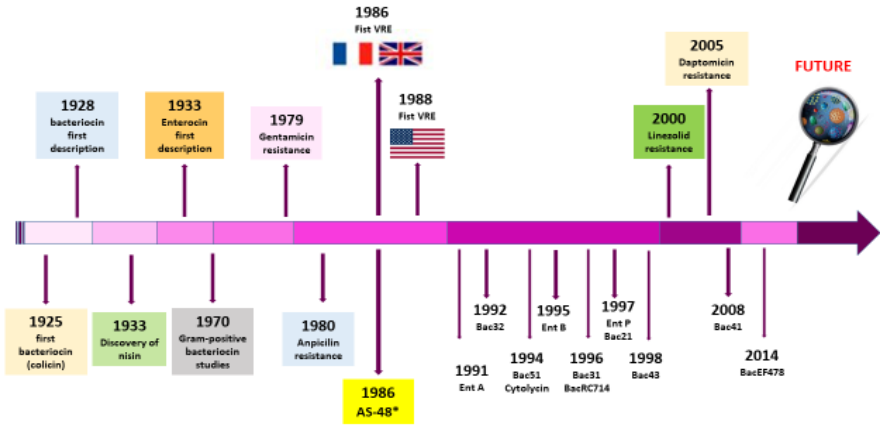

Figure 1. Timeline of important events in the history of bacteriocins (adapted from Almeida-Santos et al., 2021).

Classification of Bacteriocins

Bacteriocins are antimicrobial peptides produced by both Gram-positive and Gram-negative bacteria. Notably, the bacteriocins produced by Gram-positive lactic acid bacteria (LAB) are of great importance to the food industry, as many of them are considered GRAS (Generally Recognized As Safe). Bacteriocins produced by Gram-positive bacteria are proteins or proteinaceous peptides that exhibit high physicochemical heterogeneity, synthesized by genes encoded on plasmid or chromosomal DNA, or other mobile genetic elements. These molecules can exert inhibitory effects not only on phylogenetically closely related species but also on more distantly related species at nanomolar or picomolar concentrations (Estévez-Moreno & Lama, 2022; Choi, 2022; Smaoui et al., 2024). Due to their broad spectrum of activity, bacteriocins are considered part of the host defense mechanisms, alongside classical antibiotics, lytic agents, metabolic byproducts, and protein exotoxins. These defense peptides are produced not only by bacteria but also by a wide range of organisms, including plants, animals, fungi, and archaea. Bacteriocins serve as critical biomolecules for microorganisms, acting as a defense mechanism by inhibiting the growth of pathogens, thus supporting their survival in various environmental and ecological niches. These properties make bacteriocins valuable antimicrobial agents, with significant applications in both healthcare and food preservation strategies (Jack et al., 1995; Estévez-Moreno & Lama, 2022; Choi, 2022; Smaoui et al., 2024).

Bacteriocins produced by Gram-positive bacteria are categorized based on their diverse biological activities and structural characteristics. This classification is determined by the bacteriocins' intrinsic functions, molecular weights, amino acid sequence structures, modes of action, microbial targets, and immunity mechanisms. In general, these antimicrobial peptides are classified into four major groups: Class I, II, III, and IV (Smaoui et al., 2024).

Class I bacteriocins are small peptides with low molecular weight that become active through post-translational modifications. This class includes lantibiotics, which are antimicrobial compounds containing lanthionine. Class II bacteriocins, on the other hand, do not undergo post-translational modifications, are less than 10 kDa in size, heat-resistant, and usually consist of one or two peptide chains. Class III bacteriocins are heat-sensitive proteins with high molecular weight and are known as hydrolytic polypeptides. This class of bacteriocins typically induces cell lysis by breaking down the cell walls of target microorganisms. Class IV bacteriocins consist of proteins complexed with large macromolecules such as carbohydrates, lipids, or other components. However, in recent years, with advancing research and evolving classification criteria, the large molecular structures and protein complexes previously categorized as Class IV have been excluded from the LAB bacteriocin classification. It has been recognized that these structures share more characteristics with bacteriolysins, which are hydrolytic polypeptides now classified under Class III. As a result, large complex molecules previously grouped in Class IV are now referred to as bacteriolysins and included as a subclass within Class III, which is characterized by lytic activity targeting the cell wall (Klaenhammer, 1993; Alvarez-Sieiro et al., 2016; Timothy et al., 2021; Zinina et al., 2022; Smaoui et al., 2024).

These updated classifications are essential for better understanding the biological functions of bacteriocins and assessing their potential applications in food preservation, medical treatments, and biotechnological innovations more effectively.

Class I Bacteriocins (Lantibiotics)

Class I bacteriocins, also known as lantibiotics, are post-translationally modified peptides, a feature that makes them particularly distinctive. These bacteriocins contain rare dehydrated amino acids, such as lanthionine, methyllanthionine, dehydroalanine, and dehydrobutyrine. Typically, smaller than 5 kDa, these peptides are characterized by their thermal and proteolytic

resistance. The modified residues within these ribosomally synthesized peptides include 2,3-didehydrobutyrine (Dhb) and α-β-didehydroalanine (Dha), which contribute to their unusual and complex chemical structures (Klaenhammer, 1993; da Silva Sabo et al., 2014; Jamaluddin et al., 2018; Kumariya et al., 2019; Negash & Tsehai, 2022; Yu et al., 2023).

Class I bacteriocins can be divided into subgroups ranging from Ia to If, based on the biosynthetic enzymes encoded by operons involved in their production and modification processes. Additionally, these bacteriocins are classified into two main groups, Type A (more specifically AI and AII) and Type B, based on their structural features, charge, functional differences, and antimicrobial activities (Akkoç et al., 2009; Alvarez-Sieiro et al., 2016; Meade & Garvey, 2020; Huang et al., 2022; Smaoui et al., 2024).

Type A lantibiotics, which consist of 21-38 amino acids and weigh between 2-4 kDa, are positively charged peptides. They typically have a helical structure and exert their antimicrobial effects by depolarizing the cytoplasmic membrane of the target cell. Nisin, produced by Lactococcus lactis, is the most well-known member of this group. Approved as GRAS (Generally Recognized as Safe) by the FDA in 1988, nisin is widely used in the food industry due to its broad-spectrum antibacterial activity against Gram-positive bacteria. In contrast, Type B lantibiotics are smaller, rigid peptides, usually composed of fewer than 19 amino acids and weighing between 2-3 kDa. They are neutral or negatively charged and act by inhibiting host cell enzymes to exert their antimicrobial effects. Their compact and globular structure enhances their durability and biological functionality (Zimina et al., 2020; El-Saber Batiha et al., 2021; Kaur & Kaur, 2021; Smaoui et al., 2024).

Class II bacteriocins (non-lantibiotics)

Class II bacteriocins, though similar to class I bacteriocins in their small size and structural characteristics, exhibit significant differences. Class II bacteriocins are peptides composed of approximately 30-60 amino acids, with a molecular weight of less than 10 kDa. One of their key features is the absence of lanthionine, which differentiates them structurally from lantibiotics. These bacteriocins are resistant to temperature and pH fluctuations, and they are cationic, hydrophobic peptides with isoelectric points ranging from 8.3 to 10.0. Unlike class I bacteriocins, class II bacteriocins do not undergo post-translational modifications during their

biosynthesis, meaning they do not require modification enzymes for maturation. As a result, they lack specific amino acids such as lanthionine (Lan) or methyllanthionine (MeLan) that are present in lantibiotics (Pérez-Ramos et al., 2021; Bhattacharya et al., 2022; Mehenktaş, 2022).

Class II bacteriocins are divided into four main subgroups based on their wide structural diversity: IIa (pediocin-like bacteriocins), IIb (two-peptide bacteriocins), IIc (leaderless bacteriocins), and IId (non-pediocin-like, single-peptide bacteriocins). These bacteriocins are characterized by the presence of a hydrophilic N-terminal consensus sequence in the form of Tyr-Gly-Asn-Gly-Val-Xaa-Cys. This conserved sequence plays a critical role in determining their biological activity and impact on target cells (Akkoç et al., 2009; Mokoena, 2017; Smaoui et al., 2024).

Class IIa Bacteriocins: The largest subgroup, class IIa bacteriocins, also known as pediocin-like bacteriocins, contain a conserved amino-terminal sequence (-Tyr-Gly-Asn-Gly-Val-Xaa-Cys) and are particularly effective against pathogens such as *Listeria monocytogenes*. These bacteriocins exert their antimicrobial effect by forming pores in the cytoplasmic membrane, ultimately leading to cell destruction. Due to their significant role in food safety, class IIa bacteriocins are widely used as protective agents in the food industry. Well-known examples include pediocin PA1, sakacin P, leucocin A, and acidocin A (Akkoç et al., 2009; Mokoena, 2017; Smaoui et al., 2024).

Class IIb Bacteriocins: Class IIb bacteriocins are distinguished by their structure, consisting of two distinct peptide molecules. As a result, they are referred to as "two-peptide bacteriocins" or "dipeptide bacteriocins." The two peptides combine to form pores in the cell membrane, through which they exert their antimicrobial effects. Their dual-peptide structure provides a broader antimicrobial spectrum, effectively targeting cell membrane permeability and leading to the destruction of target cells (Akkoç et al., 2009; Alvarez-Sieiro et al., 2016).

Class IIc Bacteriocins: Known as circular bacteriocins, class IIc bacteriocins are classified as leaderless bacteriocins. Their unique feature is the absence of an N-terminal leader peptide, allowing them to be secreted without the need for a typical secretion system. This characteristic sets them apart from other bacteriocins. Their circular structure enhances their stability and prolongs their antimicrobial effects, making them more resilient to external environmental factors (Akkoç et al., 2009; Meng et al., 2021; Yu et al., 2023).

Class IId Bacteriocins: This subclass comprises structurally unrelated peptides that are typically linear and simple in structure. Class IId bacteriocins display a wide variety of mechanisms of action and secretion systems. Despite their structural differences, these peptides exhibit notable antimicrobial activities. Each peptide within this group possesses unique structural and functional properties, contributing to the vast biological diversity observed in this class (Akkoç et al., 2009; Meng et al., 2021; Yu et al., 2023).

In conclusion, class II bacteriocins represent a broad range of structurally and functionally diverse antimicrobial peptides. Their significant role in food safety and pathogen control, combined with their biological diversity, presents promising potential for future biotechnological applications.

Class III bacteriocins

Unlike Class I and Class II bacteriocins, Class III bacteriocins consist of larger protein molecules that are heat-sensitive and typically have a molecular weight greater than 30 kDa. This characteristic sets them apart from other bacteriocins that have smaller peptide structures. Due to their polypeptide composition and size, some researchers have classified Class III bacteriocins outside the conventional LAB (lactic acid bacteria) bacteriocin classification. These scientists regard Class III bacteriocins as enzymes due to their large molecular size and have reclassified them as "bacteriolysins." However, in much of the existing literature, these proteins are still referred to as "Class III bacteriocins" (Wang et al., 2019; Simons et al., 2020; Hernández-González et al., 2021; Mercado & Olmos, 2022; Sharma et al., 2022).

Class III bacteriocins are divided into two main subgroups based on their hydrolytic activity: Class IIIa and Class IIIb. Class IIIa bacteriocins, also known as bacteriolysins, exhibit hydrolase-like activity, breaking down the components of the bacterial cell wall. This action disrupts the target bacteria's cell wall, leading to lysis, which means the cell wall is destroyed, causing bacterial cell death. Bacteriolysins are significant proteins with strong antimicrobial activity against pathogenic bacteria, particularly effective against Gram-positive bacteria. They are considered potential biopreservative agents in the food industry. Class IIIb bacteriocins, on the other hand, consist of proteins that do not exhibit hydrolytic or lytic activity. These bacteriocins exert their antimicrobial effects through non-lytic mechanisms. Such mechanisms often include disrupting metabolic processes within the target

cell, inhibiting protein synthesis, or altering the permeability of the cell membrane. Despite their structural differences, Class IIIb bacteriocins are also effective across a broad spectrum, especially against Gram-positive bacteria (Wang et al., 2019; Simons et al., 2020).

When compared to other bacteriocin classes, the heat sensitivity of Class III bacteriocins can be a limiting factor in their applications. However, their antimicrobial effects, primarily due to their ability to disrupt cell walls, make them valuable in various industries and biotechnological applications. These large-molecule bacteriocins hold significant potential as natural antimicrobials, providing alternative solutions for pathogen control and biopreservation strategies in the food industry (Wang et al., 2019; Simons et al., 2020; Sharma et al., 2022).

In conclusion, Class III bacteriocins differ from other classes due to their large molecular structure and hydrolase activity, offering broad-spectrum antimicrobial effects. They play a crucial role in pathogen control and biopreservation in the food industry. Both bacteriolysins and non-lytic mechanisms contribute to their potential impact in future biotechnological developments.

Potential Industrial Application Areas of Bacteriocins

1. Food Industry

In recent years, the use of bacteriocins in the food industry has increased significantly. These antimicrobial peptides play critical roles not only in extending the shelf life of food products but also in maintaining product quality, preventing microbial contamination, and mitigating foodborne illnesses. Moreover, research on bacteriocins has revealed many new potential applications beyond their traditional uses. Studies aimed at enhancing food safety and expanding industrial applications have further explored the benefits that bacteriocins can provide in various food products and processes. One of the most significant and trending applications in this field is the use of bacteriocins in packaging technologies. The integration of bacteriocins into modern packaging solutions is attracting substantial scientific and industrial interest and is becoming a determinant of future trends in the food industry (Biler, 2008; Doğan, 2024).

Traditional packaging materials are typically made from plastics such as polyethylene (PE), polystyrene (PS), polypropylene (PP), and polyethylene terephthalate (PET), which, while protecting food from external factors like oxygen and moisture, pose health risks and are not biodegradable (Duda-Chodak et al., 2023; Correia et al., 2024). Alternative materials like glass, paper, cardboard, and metal are also commonly used in packaging. Although these materials offer significant advantages such as temperature resistance and mechanical strength, they create serious environmental sustainability issues due to their non-biodegradability and contribution to increased waste. For these reasons, there is a growing emphasis on developing new packaging and protection methods using environmentally friendly, sustainable, and ecological materials to replace traditional packaging (Mustafa & Andreescu, 2020; Correia et al., 2024).

In this context, the integration of bacteriocins into packaging materials to prevent or neutralize microbial contamination and extend the shelf life of food products stands out as a noteworthy method. Bacteriocins function as protein-based antagonistic preservatives, enhancing the microbial safety of packaged foods and helping to maintain product freshness for a longer period (Velasco et al., 2022; Kaynakçı & Pekşen 2023).

A notable trend in packaging technologies that utilize bacteriocins is the development of edible films and coatings. Edible coatings are thin layers made from edible materials that are applied to the surface of food products (Güler & Özbey, 2024). These coatings protect the external surface of food products, enhancing freshness and microbial safety. On the other hand, edible films are pre-formed layers made from natural materials, which are placed on or between food products to provide protection and extend shelf life (Bourtoom, 2008; Guimaraes et al., 2018). All of these packaging methods and materials, when integrated with bacteriocins, offer an innovative approach to preventing microbial contamination and enhancing food safety.

2. Health

Bacteriocins emerge as important biomolecules used in various fields within healthcare, including antimicrobial, antiviral, anticancer, and antibiofilm.

Anticancer: Bacteriocins are considered potential candidates for anticancer drug development due to their distinct biological activities against

various cancer cells. Specifically, it has been reported that bacteriocins exhibit different cytotoxicity towards cancer cells compared to normal cells. Some bacteriocins have been observed to demonstrate effective anticancer activities by showing high selectivity towards cancer cells (Baindara et al., 2018). Additionally, the numerous microvilli on the membranes of cancer cells facilitate the binding and entry of bacteriocin molecules into these cells. These characteristics support the idea that bacteriocins could potentially serve as new and effective cancer therapeutic agents (Kaur & Kaur, 2015; Fidan & Uğraş, 2022).

Antiviral: Viruses, which are significant microorganisms in the field of health, include many well-known primary pathogens and are structures capable of reproducing by infecting a host organism. Given the pandemic caused by the SARS-CoV-2 virus, the development of alternative methods to combat viruses is of great importance. In this context, studies on the inhibitory effects of bacteriocins produced by lactic acid bacteria (LAB) against various viruses are noteworthy. Many studies in this area have detailed the mechanisms of action of bacteriocins, their activities at different viral stages, and their synergistic or antagonistic effects (Wachsman et al., 2003; Minor et al., 2016; Fidan & Uğraş, 2022). As a result, bacteriocins are considered promising candidates for antiviral therapies.

Antibiofilm: Biofilms are microbial communities formed by certain microorganisms to protect themselves from external factors and to survive. These structures provide microorganisms with significant advantages by shielding them from host defenses or antibiotics. Antibiofilm refers to efforts aimed at developing effective therapeutic strategies against biofilms; these strategies often focus on core detection, adhesion inhibition, distribution enhancement, or phage-based treatments (Li et al., 2002; Temel & Eraç, 2018).

Currently, bacteriocins are also being evaluated as a potential strategy for reducing biofilm-related resistance. Bacteriocins can support antibiofilm activities by preventing biofilm formation or offering effective combat against existing biofilms. These antimicrobial agents are emerging as a promising solution for reducing resistance to antibiotics by helping to prevent or delay biofilm resistance (Yamakami et al., 2013; Fidan & Uğraş, 2022).

Infection treatment: De Kwaadsteniet et al. (2005) investigated the efficacy of F10-nisin F, a bacteriocin produced by *Lactococcus lactis*, in treating *Staphylococcus aureus* infections, a key pathogen responsible for respiratory infections. The study evaluated the therapeutic effects of F10-nisin F in immunocompromised and non-immunocompromised rats suffering from respiratory infections. The results demonstrated that strains infected with *S. aureus* were successfully treated with 8192 units (AU) of F10-nisin F. Similarly, De Mello and colleagues managed to treat mice infected with an intraperitoneal strain of *S. aureus xen 36*, which exhibits bioluminescent activity, using 2139 units (AU) of nisin (Mathews et al., 2015).

In another study, the bacteriocin-producing strain *Streptococcus salivarius* K12 was found to be effective in reducing respiratory infections caused by *Streptococcus pyogenes* and *Streptococcus pneumoniae*, as well as dental caries caused by *Streptococcus mutans*. The K12 strain produces salivaricin A and B bacteriocins, which demonstrated significant antibiofilm activity against key pathogenic bacteria. This antibiofilm effect has been shown to help prevent severe inflammations, particularly in elderly individuals and those with weakened immune systems. In vivo and in vitro studies using a mouse oral cavity model evaluated the effects of *S. salivarius*, revealing that it could prevent the colonization of the pathogen *Candida albicans*, further highlighting its potential for promoting oral health (Tamura et al., 2009; Ishijima et al., 2012).

These studies underscore the effectiveness of bacteriocins as biological tools for both treating and preventing infections caused by pathogenic bacteria. The antimicrobial and antibiofilm activities of different bacteriocin types against specific pathogens hold significant potential, particularly for infection control in immunocompromised individuals.

Population control: The potential role of bacteriocins in population control is attributed to their ability to affect sperm motility, which raises the possibility of using them as natural spermicidal agents. This approach could prevent the adverse effects commonly associated with chemical spermicides, such as the disruption of vaginal flora and an increased risk of urinary tract infections. Bacteriocins, as a more natural alternative, could minimize these negative outcomes, offering a safer solution. In this context, studies on the inhibitory effects of various bacteriocins against significant vaginal pathogens are particularly noteworthy. For instance, bacteriocins such as SB83 produced by *Pediococcus pentosaceus* SB83, enterocin 62-6 from *Enterococcus*

faecalis, lactocin 160 from *Lactobacillus rhamnosus*, and fermenticin HV6b have demonstrated effectiveness against pathogenic bacteria that cause vaginal infections. Multiple studies have shown that these bacteriocins inhibit the growth of key pathogens, including *Gardnerella vaginalis*, one of the main culprits behind vaginal infections (Silkin et al., 2008; Abacı, 2022).

These findings suggest that bacteriocins could provide a natural, safe, and effective solution for maintaining vaginal health and controlling population. Their potential to reduce the risk of vaginal infections and serve as spermicidal agents highlights their promising future in biotechnological applications.

Immune Support Against Viruses and in the Treatment of Viral Outbreaks like COVID-19

Viral infections, affecting various tissues and cells such as leukocytes, the spinal cord, liver, colon, and vascular endothelial cells, are major causes of numerous diseases and deaths, particularly impacting the upper respiratory tract and lungs (Dreyer et al., 2019; Weeks & Chikindas, 2019; Foysal et al., 2020; Jenab et al., 2020; Villena et al., 2020). Much of the research aimed at treating these infections has focused on the use of antimicrobial agents derived from plants. In addition to plant-based antiviral drugs as therapeutic targets, gene therapies such as pro- and anti-inflammatory cytokines, ribavirin application, pegylated interferon-α (IFN-α), recombinant human tumor necrosis factor-alpha (TNF-α) antagonist therapy, and antiretroviral treatments are used either alone or in combination for treating various viral infections (Muhammad et al., 2018; Lehtoranta et al., 2020; Umair et al., 2020; Lopez-Santamarina et al., 2021; Riaz Rajoka et al., 2021; Senan et al., 2021; Youssef et al., 2021; Rajoka et al., 2022; Umair et al. 2022). However, the potential for mutations in the viral genome to cause fatal effects or develop resistance to antiviral treatments has highlighted the need for new, preventive, or complementary approaches to treating these infections. This situation underscores the necessity for exploring broader and innovative strategies to handle future viral outbreaks and enhance the effectiveness of existing treatments (Umair et al., 2022).

During the COVID-19 pandemic, research on the prevention of viral infections has revealed a strong interaction between the gut microbiota and viral infections. During the pandemic, a significant reduction in beneficial

microorganisms such as *Bifidobacterium* and *Lactobacillus* was observed in the intestines of individuals infected with COVID-19 (Chen et al., 2020). This finding indicates that adequate intake of probiotics, which are important for gut health, has the potential to improve gut microbiota, treat dysbiosis, and prevent viral infections (Hill et al., 2014; Minj et al., 2021). Specifically, antimicrobial peptides synthesized by *Lactobacillus* species, known as bacteriocins, have the ability to regulate the gut microbiota and protect the organism against various viral infections. In this context, the evaluation of bacteriocins as potential biotherapeutic agents for both improving gut health and preventing viral infections holds significant importance for future research and treatment strategies.

3. Veterinary

Mastitis, a common disease in cattle in veterinary medicine, has become a significant clinical issue today due to the widespread development of antibiotic resistance and treatment failures. The reliance on often costly and limitedly effective antibiotic treatments for managing chronic infections poses a major obstacle in the treatment process. This situation has necessitated the exploration of alternative treatment approaches to antibiotics. In this context, research on bacteriocins has revealed that these biomolecules have the potential to inhibit or eliminate pathogens causing mastitis, indicating that bacteriocins could be a promising alternative for the treatment of bovine mastitis (Ceotto-Vigoder et al., 2016; Cunha et al., 2018; Ali et al., 2019; Deliktaş & Gökbulut, 2023).

In recent years, another area of interest in veterinary science has been the use of bacteriocins as feed additives. The ban on the use of antibiotics at sub-therapeutic doses for growth promotion in many countries has led to increased research in this area. The antimicrobial properties of bacteriocins, their narrow spectrum of activity, and their ability to target specific harmful bacterial species highlight their potential as feed additives. Studies have shown that bacteriocins help control and eliminate harmful bacteria by increasing the proportion of beneficial bacteria in the gastrointestinal system. This results in improved growth rates, reduced mortality, and enhanced reproductive performance in animals. These effects make bacteriocins a strong alternative for supporting animal health and performance (Kierończyk et al., 2017; Vieco-Saiz et al., 2019; Deliktaş & Gökbulut, 2023).

Conclusion

Bacteriocins offer extensive application potential beyond traditional food preservation practices, spanning various sectors including healthcare, veterinary medicine, pharmaceuticals, agriculture, and cosmetics. In the food industry, the integration of bacteriocins into packaging materials or their use in producing edible films and coatings has garnered significant interest in both scientific and industrial communities. These modern packaging solutions not only enhance food safety but also influence future industry trends.

Therapeutic use of bacteriocins has demonstrated effective results in areas such as regulating gut microbiota and preventing viral infections responsible for large-scale pandemics. Additionally, the combination of these peptides with antimicrobial agents holds significant potential in reducing the likelihood of pathogen resistance development. Such combination strategies offer innovative solutions in combating antibiotic resistance, contributing substantially to public health and treatment methods.

Recent research in veterinary medicine has also shown promising results regarding the use of bacteriocins in treating bovine mastitis and as feed additives to promote animal growth. These findings suggest that bacteriocins should be considered a valuable tool in pathogen control and enhancing animal health in veterinary practice.

In conclusion, the broad-spectrum antimicrobial activities of bacteriocins make them valuable biological agents not only for the food industry but also for a wide range of sectors. Future research should aim to further expand the potential applications of these peptides and focus on maximizing the opportunities provided by these innovative biomolecules.

REFERENCES

Abacı, D. (2022). Screening of the antimicrobial effects of cell-free supernatants of thermophilic Geobacillus members and partial characterization of a potential bacteriocin-like thermostable peptide (Master's thesis, Institute of Science, Ankara University).

Akkoç, N., Şanlıbaba, P., & Akçelik, M. (2009). Bacteriocıns: Alternative Food Preservatives. Erciyes University, Institute of Science, Journal of Science, 25(1), 59-70.

Ali, Z. I., Saudi, A. M., Albrecht, R., & Talaat, A. M. (2019). The inhibitory effect of nisin on *Mycobacterium avium* ssp. *paratuberculosis* and its effect on mycobacterial cell wall. *Journal of Dairy Science*, 102(6), 4935-4944.

Almeida-Santos, A. C., Novais, C., Peixe, L., & Freitas, A. R. (2021). *Enterococcus* spp. as a producer and target of bacteriocins: a double-edged sword in the antimicrobial resistance crisis context. Antibiotics, 10(10), 1215.

Alvarez-Sieiro, P., Montalbán-López, M., Mu, D., & Kuipers, O. P. (2016). Bacteriocins of lactic acid bacteria: extending the family. *Applied Microbiology and Biotechnology*, 100, 2939-2951.

Anyaegbunam, N. J., Anekpo, C. C., Anyaegbunam, Z. K. G., Doowuese, Y., Chinaka, C. B., Odo, O. J., Sharndama, H. C., Okeke, O. P., Mba, I. E. (2022). The resurgence of phage-based therapy in the era of increasing antibiotic resistance: from research progress to challenges and prospects. *Microbiological Research*, 264, 127155.

Arsoy-Saraç, E., 2019. Determination of antifungal activity of sourdough lactic acid bacteria. Doctoral Thesis, Ondokuz Mayıs University, Institute of Science and Technology, Samsun.

Baindara, P., Korpole, S., & Grover, V. (2018). Bacteriocins: perspective for the development of novel anticancer drugs. Applied Microbiology and Biotechnology, 102, 10393-10408.

Baquero, F., Beis, K., Craik, D. J., Li, Y., Link, A. J., Rebuffat, S., Salomon, R., Severinov, K., Zirah, S., Hegemann, J. D. (2024). The pearl jubilee of microcin J25: thirty years of research on an exceptional lasso peptide. *Natural Product Reports*, 3.

Batiha, G. E. S., Hussein, D. E., Algammal, A. M., George, T. T., Jeandet, P., Al-Snafi, A. E., & Cruz-Martins, N. (2021). Application of natural antimicrobials in food preservation: Recent views. *Food Control*, 126, 108066.

Bhattacharya, D., Nanda, P. K., Pateiro, M., Lorenzo, J. M., Dhar, P., & Das, A. K. (2022). Lactic acid bacteria and bacteriocins: novel biotechnological approach for biopreservation of meat and meat products. *Microorganisms*, 10(10), 2058.

Biler, B. (2009). Characterization and purification of bacteriocin produced by *Pediococcus acidilactici* PBF strain. Master's Thesis, Ankara University,

Institute of Science and Technology, Ankara.

Bourdichon, F., Arias, E., Babuchowski, A., Bückle, A., Bello, F. D., Dubois, A., Fontana, A., Fritz, D., Kemperman, R., Laulunud, S., Morelli, L. (2021). The forgotten role of food cultures. *FEMS Microbiology Letters*, 368(14), fnab085.

Bourtoom, T. (2008). Edible films and coatings: characteristics and properties. *International Food Research Journal*, 15(3), 237-248.

Ceotto-Vigoder, H., Marques, S. L. S., Santos, I. N. S., Alves, M. D. B., Barrias, E. S., Potter, A., Alviano, D. S., Bastos, M. C. F. (2016). Nisin and lysostaphin activity against preformed biofilm of *Staphylococcus aureus* involved in bovine mastitis. *Journal of Applied Microbiology*, 121(1), 101-114.

Chen, N., Zhou, M., Dong, X., Qu, J., Gong, F., Han, Y., Qui, Y., Wang, J., Liu, Y., Wei, Y., Xia, J., Yu, T., Zhamg, X., Zhang, L. (2020). Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *The Lancet*, 395(10223), 507-513.

Choi, G. H., Holzapfel, W. H., & Todorov, S. D. (2023). Diversity of the bacteriocins, their classification and potential applications in combat of antibiotic resistant and clinically relevant pathogens. *Critical Reviews in Microbiology*, 49(5), 578-597.

Correia, L. F., da Silva Pinho, G., da Cruz Neves, T. J., de Oliveira Vieira, K. C., Maddela, N. R., Prasad, R., & Winkelstroter, L. K. (2024). Nanotechnology innovation combined with bacteriocins as emerging strategy for the development of active and intelligent food packaging. *Sustainable Chemistry and Pharmacy*, 39, 101551.

Cunha, E., Trovão, T., Pinheiro, A., Nunes, T., Santos, R., Moreira da Silva, J., Braz, B. S., Tavares, L., Veiga, A. S., Oliveira, M. (2018). Potential of two delivery systems for nisin topical application to dental plaque biofilms in dogs. *BMC Veterinary Research*, 14, 1-10.

da Silva Sabo, S., Vitolo, M., González, J. M. D., & de Souza Oliveira, R. P. (2014). Overview of *Lactobacillus plantarum* as a promising bacteriocin producer among lactic acid bacteria. *Food Research International*, 64, 527-536.

Deliklitaş, V. H. K., & Gökbulut, C. (2023). Potential uses of bacteriocins in veterinary medicine, 4th International Cukurova Agriculture and Veterinary Congress, Adana, Türkiye.

Demir, E., (2014). Characterization of bacteriocin production in lactic acid bacteria isolated from fermented milk products. Master's Thesis, Adnan Menderes University, Institute of Science and Technology, Aydın.

Dogan, S., (2024). Isolation of bacterioccin- producting lacic acid bacteria from high commercial value local cheese of Easern Anatolia Region and partial purification of the bacteriocin. Phd. Thesis, Atatürk University, Institute of Science, Erzurum.

Dreyer, L., Smith, C., Deane, S. M., Dicks, L. M., & Van Staden, A. D. (2019). Migration of bacteriocins across gastrointestinal epithelial and vascular

endothelial cells, as determined using in vitro simulations. *Scientific Reports*, 9(1), 11481.

Duda-Chodak, A., Tarko, T., & Petka-Poniatowska, K. (2023). Antimicrobial compounds in food packaging. *International Journal of Molecular Sciences*, 24(3), 2457.

Estévez-Moreno, L. X., & Miranda-de la Lama, G. C. (2022). Meat consumption and consumer attitudes in México: Can persistence lead to change?. *Meat Science*, 193, 108943.

Fernandes, A., & Jobby, R. (2022). Bacteriocins from lactic acid bacteria and their potential clinical applications. *Applied Biochemistry and Biotechnology*, 194(10), 4377-4399.

Fidan, A., & Uğraş, S. (2022). Use of bacteriocins in health field. Journal of the Turkish Microbiology Society, 145.

Field, D., Fernandez de Ullivarri, M., Ross, R. P., & Hill, C. (2023). After a century of nisin research-where are we now?. *FEMS Microbiology Reviews*, 47(3), fuad023.

Foysal, M. J., Fotedar, R., Siddik, M. A., & Tay, A. (2020). *Lactobacillus acidophilus* and *L. plantarum* improve health status, modulate gut microbiota and innate immune response of marron (*Cherax cainii*). *Scientific Reports*, 10(1), 5916.

González-González, F., Delgado, S., Ruiz, L., Margolles, A., & Ruas-Madiedo, P. (2022). Functional bacterial cultures for dairy applications: Towards improving safety, quality, nutritional and health benefit aspects. *Journal of Applied Microbiology*, *133*(1), 212-229.

Gratia, A., (1925). Sur un remarquable exemple d'antagonisme entre deux souches de colibacille. Comptes rendus des seances de la socıete de bıologıe et de ses fılıales, 93, 1040-1042.

Gratia, A., & Fredericq, P. (1946). Diversité des souches antibiotiques de B. Coli et étendue variable de leur champ d'action. *Comptes Rendus des Seances de la Societe de Biologie et de ses Filiales*, 140.

Guimaraes, A., Abrunhosa, L., Pastrana, L.M., Cerqueira, M.A. (2018). Edible films and coatings: Characteristics and properties. *International Food Research Journal*, 15(3), 237-248.

Güler, E. A., & Özbey, E. (2024). Use of food additives in edible films and coatings, application methods and areas. *Özal Journal of Agriculture and Food Sciences*, 1(1), 44-60.

Hernández-González, J. C., Martínez-Tapia, A., Lazcano-Hernández, G., García-Pérez, B. E., & Castrejón-Jiménez, N. S. (2021). Bacteriocins from lactic acid bacteria. A powerful alternative as antimicrobials, probiotics, and immunomodulators in veterinary medicine. *Animals*, 11(4), 979.

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., Morelli, L., Canani, R. B., Flint, H. J., Salminen, S., Calder, P. C., Sanders, M. E. (2014). Activity of cecropin P1 and FA-LL-37 against urogenital microflora. *Nature Reviews Gastroenterology and Hepatology*, 11(8), 506.

Huang, Y., Yu, J., Yan, H., Zhang, C., Kang, W., Pan, L., ... & Gu, R. (2022). Screening of Lactobacillus from breast milk and infant feces and evaluation of their bile salt tolerance. *Quality Assurance and Safety of Crops & Foods*, 14(4), 115-124.

Ishijima, S. A., Hayama, K., Burton, J. P., Reid, G., Okada, M., Matsushita, Y., & Abe, S. (2012). Effect of *Streptococcus salivarius* K12 on the in vitro growth of *Candida albicans* and its protective effect in an oral candidiasis model. *Applied and Environmental Microbiology*, 78(7), 2190-2199.

Jack, R. W., Tagg, J. R., & Ray, B. (1995). Bacteriocins of gram-positive bacteria. *Microbiological Reviews*, 59(2), 171-200.

Jamaluddin, N., Stuckey, D. C., Ariff, A. B., & Faizal Wong, F. W. (2018). Novel approaches to purifying bacteriocin: A review. *Critical Reviews in Food Science and Nutrition*, 58(14), 2453-2465.

Jenab, A., Roghanian, R., & Emtiazi, G. (2020). Bacterial natural compounds with anti-inflammatory and immunomodulatory properties (mini review). *Drug Design, Development and Therapy*, 3787-3801.

Karahançer, H. (2018). Determination of the effects of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* used in its production on some properties of white cheese.

Kaur, S., & Kaur, S. (2015). Bacteriocins as potential anticancer agents. *Frontiers in Pharmacology*, 6, 272.

Kaur, R., & Kaur, L. (2021). Encapsulated natural antimicrobials: A promising way to reduce microbial growth in different food systems. *Food Control*, 123, 107678.

Kaya Şen, S., & Katı, H. (2023). Biochemical characterization and partial purification of a bacteriocin like-inhibitory substance produced from *Bacillus sp*. T68 strain. *Eskişehir Technical University Journal of Science & Technology A-Applied Sciences & Engineering*, 24(1).

Kaynaci, E., & Pekşen, İ. S. Natural Preservatives in Meat and Meat Products: Nisin and Chitosan. 49.

Kierończyk, B., Sassek, M., Pruszyńska-Oszmałek, E., Kołodziejski, P., Rawski, M., Świątkiewicz, S., & Józefiak, D. (2017). The physiological response of broiler chickens to the dietary supplementation of the bacteriocin nisin and ionophore coccidiostats. Poultry Science, 96(11), 4026-4037.

Klaenhammer, T. R. (1993). Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiology Reviews*, 12(1-3), 39-85.

Kumar, P., Nagarajan, A., & Uchil, P. D. (2018). Analysis of cell viability by the lactate dehydrogenase assay. *Cold Spring Harbor Protocols*, 2018(6), pdb-prot095497.

Kumariya, R., Garsa, A. K., Rajput, Y. S., Sood, S. K., Akhtar, N., & Patel, S. (2019). Bacteriocins: Classification, synthesis, mechanism of action and resistance development in food spoilage causing bacteria. *Microbial Pathogenesis*, 128, 171-177.

Ladjouzi, R., Dussert, E., Teiar, R., Belguesmia, Y., & Drider, D. (2023). A review on enterocin DD14, the leaderless two-peptide bacteriocin with multiple biological functions and unusual transport pathway. *Antibiotics*, 12(7), 1188.

Lehtoranta, L., Latvala, S., & Lehtinen, M. J. (2020). Role of probiotics in stimulating the immune system in viral respiratory tract infections: A narrative review. *Nutrients*, 12(10), 3163.

Lei, W., Hao, L., You, S., Yao, H., Liu, C., & Zhou, H. (2022). Partial purification and application of a bacteriocin produced by probiotic *Lactococcus lactis* C15 isolated from raw milk. *Lwt*, 169, 113917.

Li, Y. H., Tang, N., Aspiras, M. B., Lau, P. C., Lee, J. H., Ellen, R. P., & Cvitkovitch, D. G. (2002). A quorum-sensing signaling system essential for genetic competence in *Streptococcus mutans* is involved in biofilm formation. *Journal of Bacteriology*, 184(10), 2699-2708.

Lopez-Santamarina, A., Lamas, A., del Carmen Mondragón, A., Cardelle-Cobas, A., Regal, P., Rodriguez-Avila, J. A., Miranda, J. M., Franco, C. M., Cepeda, A. (2021). Probiotic effects against virus infections: new weapons for an old war. *Foods*, 10(1), 130.

Mathews, T. J., MacDorman, M. F., & Thoma, M. E. (2015). Infant mortality statistics from the 2013 period linked birth/infant death data set.

Mba, I. E., & Nweze, E. I. (2022). Application of nanotechnology in the treatment of infectious diseases: an overview. *Nanotechnology for Infectious Diseases*, 25-51.

Meade, E., Slattery, M. A., & Garvey, M. (2020). Bacteriocins, potent antimicrobial peptides and the fight against multi drug resistant species: resistance is futile?. *Antibiotics*, 9(1), 32.

Mehenktaş, C. (2022). Bacteriocins: Classification, mechanisms of action and use in food preservation. *Academic Food*, 20(2), 194-198.

Meng, F., Zhu, X., Zhao, H., Nie, T., Lu, F., Lu, Z., & Lu, Y. (2021). A class III bacteriocin with broad-spectrum antibacterial activity from Lactobacillus acidophilus NX2-6 and its preservation in milk and cheese. *Food Control*, 121, 107597.

Mercado, V., & Olmos, J. (2022). Bacteriocin production by Bacillus species: Isolation, characterization, and application. *Probiotics and Antimicrobial Proteins*, 14(6), 1151-1169.

Minj, J., Chandra, P., Paul, C., & Sharma, R. K. (2021). Bio-functional properties of probiotic *Lactobacillus*: current applications and research perspectives. *Critical Reviews in Food Science and Nutrition*, 61(13), 2207-2224.

Minor, P. D. (2016). An introduction to poliovirus: pathogenesis, vaccination, and the endgame for global eradication. *Poliovirus: Methods and Protocols*, 1-10.

Mokoena, M. P. (2017). Lactic acid bacteria and their bacteriocins: classification, biosynthesis and applications against uropathogens: a mini-review. *Molecules*, 22(8), 1255.

Montalbán-López, M., Sánchez-Hidalgo, M., Valdivia, E., Martínez-Bueno, M., &

Maqueda, M. (2011). Are bacteriocins underexploited? Novel applications for old antimicrobials. *Current Pharmaceutical Biotechnology*, 12(8), 1205-1220.

Muhammad, U., Zhu Xiaoyu, Z. X., Lu Zhaoxin, L. Z., Han Jinzhi, H. J., Sun Jing, S. J., Tayyaba, S., Abbasi, B., Siyal, F. A., Kuldeep Dhama, D. K., Jabbar Saqib, J. S. (2018). Effects of extraction variables on pharmacological activities of vine tea extract (*Ampelopsis grossedentata*), *CABI Databases*, 495-505.

Mustafa, F., & Andreescu, S. (2020). Nanotechnology-based approaches for food sensing and packaging applications. *RSC Advances*, 10(33), 19309-19336.

Negash, A. W., & Tsehai, B. A. (2020). Current applications of bacteriocin. *International Journal of Microbiology*, 2020(1), 4374891.

Özlü, H., (2015). Bacteriocin producing ability of lactic acid bacteria isolated from some cheeses, Doctoral Thesis, Atatürk University, Health Sciences Institute, Erzurum.

Pasteur, L., & Joubert, J. (1877). Charbon et septicémie. *Recueil de Médecine Vétérinaire*, 54(1), 753-770.

Pérez-Ramos, A., Madi-Moussa, D., Coucheney, F., & Drider, D. (2021). Current knowledge of the mode of action and immunity mechanisms of LAB-bacteriocins. *Microorganisms*, 9(10), 2107.

Rajoka, M. S. R., Mehwish, H. M., Kitazawa, H., Barba, F. J., Berthelot, L., Umair, M., Zhu, Q., He, Z., & Zhao, L. (2022). Techno-functional properties and immunomodulatory potential of exopolysaccharide from Lactiplantibacillus plantarum MM89 isolated from human breast milk. Food Chemistry, 377, 131954.

Reuben, R. C., Roy, P. C., Sarkar, S. L., Alam, R. U., & Jahid, I. K. (2019). Isolation, characterization, and assessment of lactic acid bacteria toward their selection as poultry probiotics. *BMC Microbiology*, 19, 1-20.

Reuben, R. C., Roy, P. C., Sarkar, S. L., Alam, A. R. U., & Jahid, I. K. (2020). Characterization and evaluation of lactic acid bacteria from indigenous raw milk for potential probiotic properties. *Journal of Dairy Science*, 103(2), 1223-1237.

Reuben, R. C., & Torres, C. (2024). Bacteriocins: potentials and prospects in health and agrifood systems. *Archives of Microbiology*, 206(5), 1-51.

Riaz Rajoka, M. S., Thirumdas, R., Mehwish, H. M., Umair, M., Khurshid, M., Hayat, H. F., ... & Barba, F. J. (2021). Role of food antioxidants in modulating gut microbial communities: Novel understandings in intestinal oxidative stress damage and their impact on host health. *Antioxidants*, 10(10), 1563.

Senan, A. M., Yin, B., Zhang, Y., Nasiru, M. M., Lyu, Y. M., Umair, M., Khurshid, M., Hayat, H. F., Phimolsiripol, Y., Pallarés, N., Martí-Quijal, F. J., & Liu, L. (2021). Efficient and selective catalytic hydroxylation of unsaturated plant oils: a novel method for producing anti-pathogens. *BMC Chemistry*, 15, 1-11.

Sharma, B. R., Halami, P. M., & Tamang, J. P. (2021). Novel pathways in bacteriocin synthesis by lactic acid bacteria with special reference to ethnic fermented

foods. *Food Science and Biotechnology*, 1-16.

Silkin, L., Hamza, S., Kaufman, S., Cobb, S. L., & Vederas, J. C. (2008). Spermicidal bacteriocins: lacticin 3147 and subtilosin A. *Bioorganic & Medicinal Chemistry Letters*, 18(10), 3103-3106.

Simons, A., Alhanout, K., & Duval, R. E. (2020). Bacteriocins, antimicrobial peptides from bacterial origin: overview of their biology and their impact against multidrug-resistant bacteria. *Microorganisms*, 8(5), 639.

Smaoui, S., Echegaray, N., Kumar, M., Chaari, M., D'Amore, T., Shariati, M. A., & Lorenzo, J. M. (2024). Beyond conventional meat preservation: saddling the control of bacteriocin and lactic acid bacteria for clean label and functional meat products. *Applied Biochemistry and Biotechnology*, 196(6), 3604-3635.

Tamura, K., Battistuzzi, F. U., Billing-Ross, P., Murillo, O., Filipski, A., & Kumar, S. (2012). Estimating divergence times in large molecular phylogenies. *Proceedings of the National Academy of Sciences*, 109(47), 19333-19338.

Temel, A., & Eraç, B. (2018). Bakteriyel biyofilmler: Saptama yöntemleri ve antibiyotik direncindeki rolü. *Türk Mikrobiyol Cemiyeti Dergisi*, 48(1), 1-13.

Teso-Pérez, C., Martínez-Bueno, M., Peralta-Sánchez, J. M., Valdivia, E., Maqueda, M., Fárez-Vidal, M. E., & Martín-Platero, A. M. (2021). Enterocin cross-resistance mediated by ABC transport systems. *Microorganisms*, 9(7), 1411.

Timothy, B., Iliyasu, A. H., & Anvikar, A. R. (2021). Bacteriocins of lactic acid bacteria and their industrial application. *Current Topics in Lactic Acid Bacteria and Probiotics*, 7(1), 1-13.

Umair, M., Jabbar, S., Sultana, T., Ayub, Z., Abdelgader, S. A., Xiaoyu, Z., ... & Zhaoxin, L. (2020). Chirality of the biomolecules enhanced its stereospecific action of dihydromyricetin enantiomers. *Food Science & Nutrition*, 8(9), 4843-4856.

Umair, M., Sultana, T., Xiaoyu, Z., Senan, A. M., Jabbar, S., Khan, L., Abid, M., Murtaza, M. A., Kurdeep, D., Al-Areqi, N. A. S., & Zhaoxin, L. (2022). LC-ESI-QTOF/MS characterization of antimicrobial compounds with their action mode extracted from vine tea (*Ampelopsis grossedentata*) leaves. *Food Science & Nutrition*, 10(2), 422-435.

Velasco, V., Sepúlveda, E., Williams, P., Rodríguez-Llamazares, S., Gutiérrez, C., & Valderrama, N. (2022). Starch-based composite foam for chicken meat packaging. *Journal of Food Science and Technology*, 59(12), 4594-4602.

Vieco-Saiz, N., Belguesmia, Y., Raspoet, R., Auclair, E., Gancel, F., Kempf, I., & Drider, D. (2019). Benefits and inputs from lactic acid bacteria and their bacteriocins as alternatives to antibiotic growth promoters during food-animal production. *Frontiers in Microbiology*, 10, 57.

Villena, J., Shimosato, T., Vizoso-Pinto, M. G., & Kitazawa, H. (2020). Nutrition, immunity and viral infections. *Frontiers in Nutrition*, 7, 125.

Wachsman, M. B., Castilla, V., de Ruiz Holgado, A. P., de Torres, R. A., Sesma, F., & Coto, C. E. (2003). Enterocin CRL35 inhibits late stages of HSV-1 and HSV-2 replication in vitro. *Antiviral Research*, 58(1), 17-24.

Wang, J., Zhang, S., Ouyang, Y., & Li, R. (2019). Current developments of bacteriocins, screening methods and their application in aquaculture and aquatic products. *Biocatalysis and Agricultural Biotechnology*, 22, 101395.

Wang, C. H., Hsieh, Y. H., Powers, Z. M., & Kao, C. Y. (2020). Defeating antibiotic-resistant bacteria: exploring alternative therapies for a post-antibiotic era. *International Journal of Molecular Sciences*, 21(3), 1061.

Weeks, R., & Chikindas, M. L. (2019). Biological Control of Food-Challenging Microorganisms. *Food Microbiology: Fundamentals and Frontiers*, 733-754.

Yamakami, K., Tsumori, H., Sakurai, Y., Shimizu, Y., Nagatoshi, K., & Sonomoto, K. (2013). Sustainable inhibition efficacy of liposome-encapsulated nisin on insoluble glucan-biofilm synthesis by *Streptococcus mutans*. *Pharmaceutical Biology*, 51(2), 267-270.

Yang, S. C., Lin, C. H., Sung, C. T., & Fang, J. Y. (2014). Antibacterial activities of bacteriocins: application in foods and pharmaceuticals. *Frontiers in Microbiology*, 5, 241.

Ye, K., Ke, Z., Zhang, X., Liang, K., Zhang, Q., & Huang, J. (2023). Bacterial peptides and bacteriocins as novel treatment for prostate cancer. *International Journal of Peptide Research and Therapeutics*, 29(5), 78.

Yu, W., Guo, J., Liu, Y., Xue, X., Wang, X., Wei, L., & Ma, J. (2023). Potential impact of combined inhibition by bacteriocins and chemical substances of foodborne pathogenic and spoilage bacteria: a review. *Foods*, 12(16), 3128.

Youssef, M., Ahmed, H.Y., Zongo, A., Korin, A., Zhan, F., Hady, E., ... & Li, B. (2021). Probiotic supplements: their strategies in the therapeutic and prophylactic of human life-threatening diseases. *International Journal of Molecular Sciences*, 22(20), 11290.

Zacharof, M.P. & Lovitt, R.W. (2013). Modelling and simulation of cell growth dynamics, substrate consumption, and lactic acid production kinetics of *Lactococcus lactis*. *Biotechnology and Bioprocess Engineering*, 18, 52-64.

Zimina, M., Babich, O., Prosekov, A., Sukhikh, S., Ivanova, S., Shevchenko, M., & Noskova, S. (2020). Overview of global trends in classification, methods of preparation and application of bacteriocins. *Antibiotics*, 9(9), 553.

Zinina, O., Merenkova, S., Rebezov, M., Galimov, D., Khayrullin, M., & Burkov, P. (2022). Physicochemical, functional, and technological properties of protein hydrolysates obtained by microbial fermentation of broiler chicken gizzards. *Fermentation*, 8(7), 317.

BIOSORPTION: AN EFFECTIVE AND SUSTAINABLE APPROACH FOR SYNTHETIC DYE REMOVAL

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Introduction

Water is one of the most basic needs for living things to maintain their lives. However, rapid population growth, anthropogenic causes, global warming, and the industrial revolution are seriously reducing water resources. The decrease in water resources is an important environmental problem (Aragaw & Bogale, 2021; Kausar et al., 2023).

The industrial revolution has played an important role in people's ability to live in prosperity. However, the industrial revolution has increased people's concerns due to the damage they have caused to the environment. Textile industries, one of these industries, use high amounts of toxic dyes. According to studies, more than 100,000 different types of dyes are used in textile industries and approximately 300,000 tons of these dyes contribute to environmental damage (Adeleke et al., 2024; Mehrez et al., 2024).

Synthetic Dye

Synthetic dyes consist of organic and inorganic compounds. These dyes are produced by modification of the chromophore having hetero atoms such as N, O and S in their structures (Kainth et al., 2024; Upadhyay et al., 2024). Synthetic dyes are divided into three groups according to their chemical structures: azo dyes, anthraquinone dyes, and triphenylmethane dyes (figure 1). Synthetic dyes are classified into 6 classes according to their application methods: acidic, basic, reactive, direct, dispersion, and vast dyes (Das et al., 2023). The application areas and toxic properties of these dyes are shown in Table 1 and the general properties of these dyes are shown in Table 2. Synthetic dyes are widely used in various industries such as wool, textile, cosmetics, and paint. Since these dyes consist of trace elements such as Pb, Zn, Cu, and Cr, they are stable against oxidation agents. Therefore, synthetic dyes seriously harm human health and the environment (Amalraj et al., 2021).

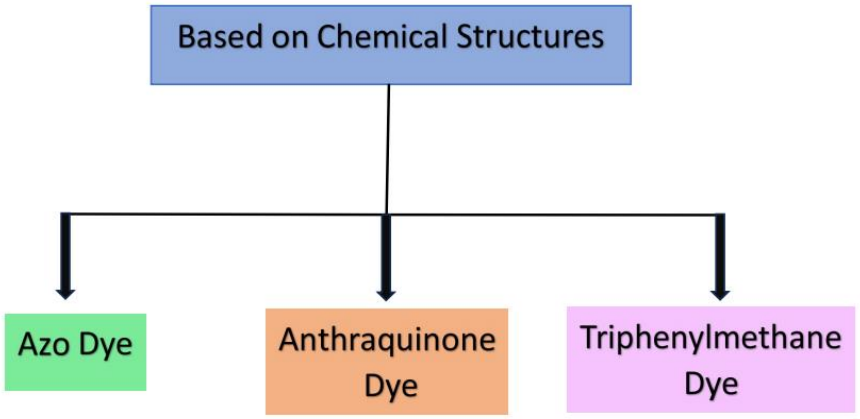


Figure 1. Classification of synthetic dyes according to their chemical structures.

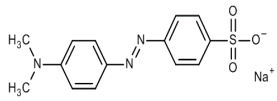
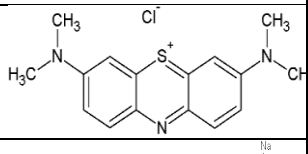
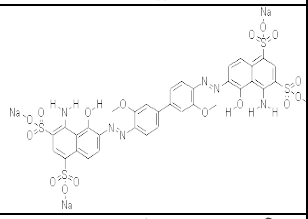
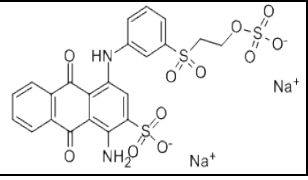
When the wastewater of synthetic dyes used in industries is released into receiving environments such as lakes, streams, and rivers, it has a negative impact on the environment. These wastewaters released into receiving environments cover the water surface and prevent the passage of sunlight. When they prevent the passage of sunlight, they prevent the living beings living in these receiving environments from photosynthesizing, causing eutrophication, which leads to a serious environmental problem. Therefore, synthetic dyes that harm the environment must be treated before being released into receiving environments (Martini et al., 2021; Saranya et al., 2023).

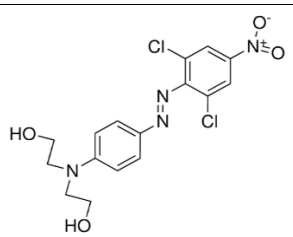
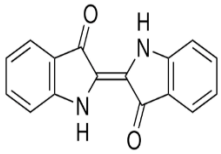
Table 1. Applications and toxic effects of synthetic dyes.

Dye Name	Example Dyes Name	Applications of Dye	Toxic Effects of Dye
Acid Dyes	Acid Red 57, Congo Red, Methyl Orange, Orange I	Wool, Silk, Cotton, Paper	Burns and skin, Carcinogens
Basic Dyes	Methylene Blue, Malachite Green, Crystal Violet	Cotton, Wool, Silk, Polyester	Carcinogens, skin allergic mutagenic,

Direct Dyes	Direct blue, Direct orange, Direct violet	Cellulose, Wool, Cotton	Carcinogens
Reactive Dyes	Reactive blue, Reactive yellow, Reactive black	Cellulosic fiber	Respiratory problem
Disperse Dyes	Disperse brown, Disperse yellow	Polyesters Nylon, PVC industry	Cancer, DNA damage
Vat Dyes	Vat blue, Indigo	Cellulose, Wool	Severe burns, Skin irritation

Table 2. General properties of dyes.

Dye Name	**Example Dye Name**	**Molecular Structure**	**Molecular Formula**	**Molecular Weight (g/mol)**
Acid Dyes	Methyl Orange		$C_{14}H_{14}N_3NaO_3S$	327.34
Basic Dyes	Methylene Blue		$C_{16}H_{18}ClN_3S$	319.85
Direct Dyes	Direct blue		$C_{34}H_{28}N_6O_{16}S_4$	904.9
Reactive Dyes	Reactive black		$C_{26}H_{21}N_5Na_4O_{19}S_6$	991.82

Dispers e Dyes	Disperse brown		$C_{16}H_{16}Cl_2N_4O_4$	399.236
Vat Dyes	Indigo		$C_{16}H_{10}N_2O_2$	262.26

Wastewater Treatment Methods

There are many wastewater treatment methods used in the removal of synthetic dyes from aqueous solutions. These wastewater treatment methods are divided into 3 groups: chemical treatment, biological treatment and physical treatment. The advantages and disadvantages of these treatment methods are shown in Table 3.

Chemical Treatment

Chemical treatment method is a process applied in the removal of organic pollutants using various chemical substances. Although coagulation/flocculation processes are mostly used in synthetic dye removal, in addition to this process, ozonation, Fenton and photochemical processes are other chemical treatment methods used in synthetic dye removal. These chemical treatment processes are effective methods in synthetic dye removal. However, coagulation/flocculation, Fenton and ozonation processes are high-cost processes. In addition, the addition of chemical substances, sludge formation and sedimentation processes increase operating costs (Rashid et al., 2020; Ungureanu et al., 2023).

Biological Treatment

Biological treatment is a process applied to remove pollutants that can be biodegraded by microorganisms. Aerobic, anaerobic, algae biodegradation processes are the processes used in biological treatment. These processes are easy and low-cost processes in synthetic dye removal. However, since it

cannot completely remove synthetic dyes from pollutants, biological treatment has a disadvantage in dye substance removal because it cannot provide effective treatment (Aruna et al., 2021).

Physical Treatment

Physical treatment is a process generally used to remove pollutants with the help of mass transfer, gravity and Van der Waals forces. The most commonly used physical treatment methods in synthetic dye removal are membrane separation, ion exchange, nanofiltration, and reverse osmosis processes. These processes have advantages such as easy operation and effective treatment. It is a treatment method that has disadvantages such as high cost, high energy consumption, and membrane fouling in the membrane separation process (Aragaw & Bogale, 2021). However, in recent years, the biosorption process, which is an effective treatment method for synthetic dye removal using various biosorbents, has come into prominence (Yaashikaa et al., 2021).

Table 3. Applications and toxic effects of synthetic dye.

Wastewater Treatment Methods	Advantages	Disadvantages
Chemical Treatment		
Coagulation/Flocculation	Effective in dye removal, Low capital cost, Simplicity of operation	High operational cost, Produce high sludge
Ozonation	Effective in dye removal, Ozone can be used in gas form, Not sludge production	High operational cost, Produces toxic by-products, Its half-life is shorter at twenty min.
Fenton	It breaks down all toxins in dye wastewater, Effective in dye removal	Reaction time is quite long, Produce sludge
Photochemical	Effective in dye removal, Not sludge production	Produces by-products

Biological Treatment		
Aerobic Biodegradation	Effective in dye removal, Low capital cost	Microorganisms grow due to appropriate environment
Anaerobic Biodegradation	By-products can be used as energy sources	More treatment is needed under aerobic environment and release H_2S and CH_4
Algae Biodegradation	Effective in dye removal, Ecofriendly, Low cost	Unstable system
Physical Treatment		
Membrane Filtration	Effective in dye removal	Membrane fouling, Produce sludge
Ion Exchange	Low cost, Reusing	Not effective in the treatment of disperse dyes
Nanofiltration	Used be to remove a lot of dyes	High energy consumption
Reverse Osmosis	Effective in dye removal	High operational cost,

Biosorpsion Process

Biosorption process is a new biotechnology treatment method using various biosorbents in the removal of synthetic dyes (Yaashika et al., 2021). Pollutants are removed with the help of physical-chemical mechanisms between dye molecules and biomass. Biosorbents used in the biosorption process have many advantages such as low cost, eco-friendly, and abundance in nature (Lima & Asencios, 2021). The advantages of the biosorption process are listed below:

- Low cost,
- High dye removal efficiency,
- No need for additional nutrients and chemicals,
- Reuse of biosorbents.

The biosorption mechanism occurs in two stages. In the first stage, the dye molecules move towards the surface of the biosorbents by means of film diffusion. In the second stage, the dye molecules are binding on the biosorbent surface by means of Van der Walls and electrostatic interactions between the dye molecules and the biosorbent. The biosorption mechanism is shown in Figure 2.

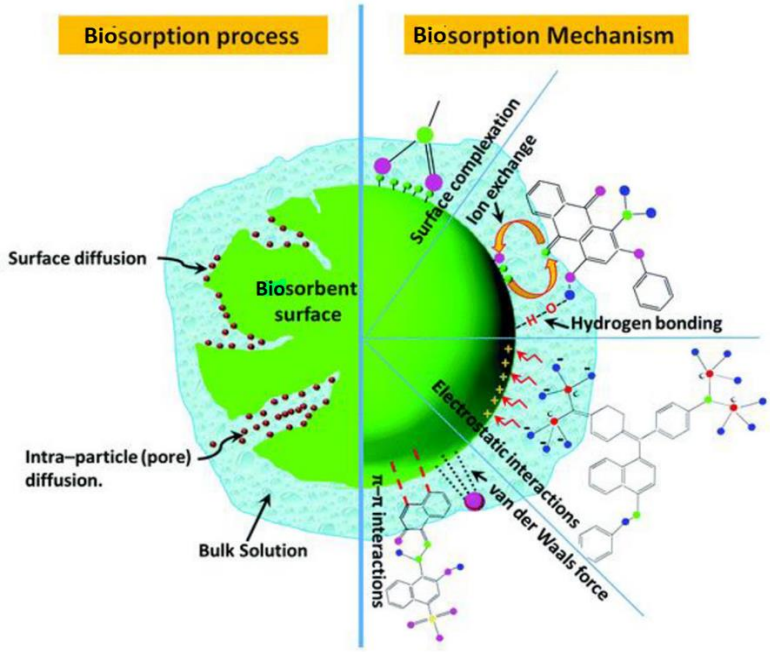


Figure 2. Biosorption process mechanism (Kausar et al., 2023).

Biosorpsion Varieties

There are various biosorbents used in biosorption processes. The biosorbents used are shown in Figure 3.

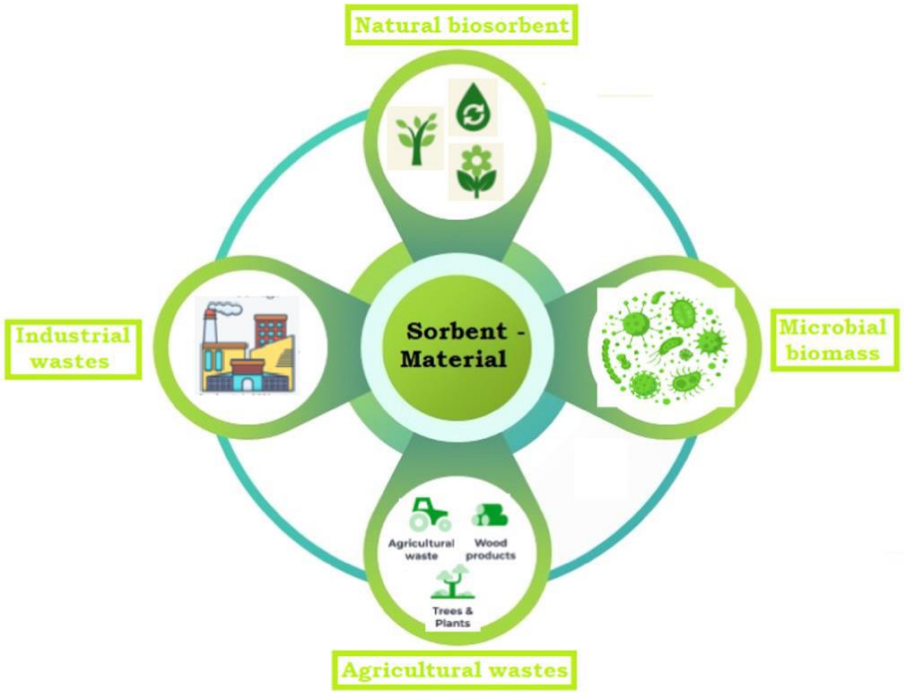


Figure 3. Biosorbents used in biosorption processes (Yaashikaa et al., 2021).

Natural Biosorbents: Clay, chitosan, and zeolites are natural materials used in the removal of synthetic dyes (Azimi et al., 2017).

Microbial Biomass: Biological biosorbents are derived from microbes such as bacteria, fungi, algae, yeast, etc. These biological biomasses play an active role in the removal of synthetic dyes (Dai et al., 2019).

Agricultural Wastes: Agricultural wastes such as rice husk, wheat bran, shell, dry leaves, sugar peat pulp, vegetable and fruit peels such as banana, orange, pomegranate, corn cob, and peanut shells can be given as examples of biosorbents used in synthetic dye removal (Saravanan et al., 2021).

Industrial Wastes: Waste produced from industries is used to remove synthetic dyes. The surface of these biosorbents used is chemically modified to increase the removal efficiency of synthetic dyes (Ahmaruzzaman, 2011). Synthetic dyes are removed using industrial waste biosorbents such as fly ash, furnace slag, sludge, sugar cane pulp (Regkouzas & Diamadopoulos, 2019).

Table 4 shows the comparison of different synthetic dye removal of different biosorbents. As a result, the biosorption process, which is a biotechnological treatment method for the removal of synthetic dyes, is considered to be a promising process for the future due to its effective treatment, low cost, sustainability, and ecofriendly.

Table 4. Applications and toxic effects of synthetic dyes.

Biosorbent	**Dye Removed**	**Dye Removal Percentage (%)**	**References**
Durian (Durio zibethinus murray)	Basic Brown	74.26	Gopalakrishnan et al., 2020
Watermelon shell (Citrullus vulgaris)	Orange G	100	Chigbundu & Adebowale, 2017
***Carica papaya* wood**	Methylene Blue	94.5	Rangabhashiyam et al., 2018
Streptomyces bacillaris	Methyl Violet	91.8	Adenan et al., 2024
Pomegranate peel	Basic Red 46	86	Akkari et al., 2023
Pterocladia capillacea	Crystal Violet	98	Al-Saadi et al., 2023
Durian (*Durio zibethinus*)	Evans Blue	96	Basirun et al., 2023
Casuarina equisetifolia	Methylene Blue	95.7	Chandarana et al., 2021
Ocimum gratissimum	Indigo Carmine	92.6	Dada et al., 2020
Teak leaf powder (*Tectona grandis*)	Congo red	90	Gedam et al., 2019
***Chlorella* sp.**	Congo red	97.1	Yu et al., 2021
Burned root of *Eichhornia crassipes*	Congo Red	95	Roy & Mondal, 2017
***Fraxinus Excelsior* L. (Oleaceae)**	Methylene Blue	89	Güllüce et al., 2022
Sunflower Straw (*Helianthus annuus* L.)	Malachite Green	97	Güllüce et al., 2020
***Syringa vulgaris* L. hull**	Methylene Blue	97.5	Gulluce et al., 2020

REFERENCES

Adeleke, A.O., Omar, R.C., Katibi, K.K., Dele-Afolabi, T.T., Ahmad, A., Quazim, J.Q., Amusa, A.A., & Alshammari, M.B. (2024). Process optimization of superior biosorption capacity of biogenic oyster shells nanoparticles for Congo red and Bromothymol blue dyes removal from aqueous solution: Response surface methodology, equilibrium isotherm, kinetic, and reusability studies. *Alexandria Engineering Journal, 92,* 11–23.

Adenan, N.H., Lim, Y.Y., & Ting, A.D.S.Y. (2022). Removal of triphenylmethane dyes by *Streptomyces bacillaris*: A study on decolorization, enzymatic reactions and toxicity of treated dye solutions. *Journal of Environmental Management, 318,* 115520.

Ahmaruzzaman, M. (2011). Industrial wastes as low-cost potential adsorbents for the treatment of wastewater laden with heavy metals. *Adv. Colloid Interface, 166,* 36–59.

Akkari, I., Graba, Z., Bezzi, N., Merzeg, F.A., Bait, N., & Ferhati, A. (2023). Raw pomegranate peel as promise efficient biosorbent for the removal of Basic Red 46 dye: equilibrium, kinetic, and thermodynamic studies. *Biomass Conversion and Biorefinery, 13,* 8047–8060.

Al-Saeedi, S.I., Ashour, M., & Alprol, A.E. (2023). Adsorption of toxic dye using red seaweeds from synthetic aqueous solution and its application to industrial wastewater effluents. *Frontiers in Marine Science, 10,* 1202362.

Amalraj, R., Ramsenthil, R., Durai, G., Jayakumar, R., & Palaniraj, R. (2021). Dyes Removal Using Novel Sorbents – A Review. *Journal of Pharmaceutical Research International, 233(45a),* 355–382.

Aragaw, T.A., & Bogale, F.M. (2021). Biomass-based adsorbents for removal of dyes from wastewater: A Review. *Frontiers in Environmental Science, 9,* 764958.

Aruna, Bagotia, N., Sharma, A.K., & Kumar, S. (2021). A review on modified sugarcane bagasse biosorbent for removal of dyes. *Chemosphere. 268,* 129309.

Azimi, A., Azari, A., Rezakazemi, M., & Ansarpour, M. (2017). Removal of heavy metals from industrial wastewaters: a review. *Chem Bio Eng Rev., 4,* 37–59.

Basirun, A.A., Othman, A.R., Yasid, N.A., Halmi, M.I.E., Gunasekaran, B., & Shukor, M.Y.A. (2023). Adsorption kinetics, equilibrium, and thermodynamic studies to understand adsorption behavior of Evans blue dye by durian husk. *Korean J. Chem. Eng., 40(6),* 1440–1456.

Chandarana, H., Kuma, P.S., Seenuvasan, M., & Kumar, M.A. (2021). Kinetics, equilibrium and thermodynamic investigations of methylene blue dye removal using *Casuarina equisetifolia* pines. *Chemosphere, 285,* 131480.

Chigbundu, E.C., & Adebowale, K.O. (2017). Equilibrium and fractal-like kinetic studies of the sorption of acid and basic dyes onto watermelon shell (*Citrullus vulgaris*). *Cellulose 24(11),* 4701–4714.

Dada, A.O., Adekola, F.A., Odebunmi, E.O., Dada, F.E., Bello, O.M., Akinyemi, B.A., Bello, O.S., & Umukoro, O.G. (2020). Sustainable and low-cost *Ocimum gratissimum* for biosorption of indigo carmine dye: kinetics, isotherm, and thermodynamic studies *International Journal of Phytoremediation, 22(14),*1524–1537.

Dai, Q.H., Bian, X.Y., Li, R., Jiang, C.B., Ge, J.M., Li, B.L., & Ou, J. (2019). Biosorption of lead (II) from aqueous solution by lactic acid bacteria. *Water Science and Technology, 79,* 627–634.

Das, S., Samal, P.P., Qaiyum, M.A., Dutta, S., Dey, B., & Dey, S. (2024). *Neolamarckia cadamba* (cadamba) waste pulp as a natural and techno-economic scavenger for methylene blue from aqueous solutions. *International Journal of Phytoremediation. 26(2),* 208–218.

Gedam, V.V., Raut, P., Chahande, A., & Pathak, P. (2019). Kinetic, thermodynamics and equilibrium studies on the removal of Congo red dye using activated teak leaf powder. *Applied Water Science, 9,*55.

Gopalakrishnan, Y., Al-Gheethi, A., Abdul Malek, M., Marisa Azlan, M., Al-Sahari, M., Radin Mohamed, R.M., Alkhadher, S., & Noman, E. (2020). Removal of basic Brown 16 from aqueous solution using durian shell adsorbent, optimisation and technoeconomic analysis. *Sustainability, 12(21),* 8928.

Güllüce, E., Aslan, E., Sulanç, Y.N., & Başak, A. (2020). biosorption process using by sunflower straw (*helianthus annuus* l.) biosorbent for removal malachite green dye from aqueous solutions. *Anatolian Journal of Biology, 1(2),* 84–92.

Gulluce, E., Karadayi, M., Gulluce, M., Karadayi, G., Yildirim, V., Egamberdieva, D., & Alaylar, B. (2020). Bioremoval of methylene blue from aqueous solutions by *Syringa vulgaris* L. hull biomass. *Environmental Sustainability 3,* 303–312.

Güllüce, E., Koç, T.Y., Güllüce, M., & Karadayı, M. (2022). Isolation of Methylene Blue from Aqueous Solution Using a *Fraxinus Excelsior* L. (Oleaceae) Based Biosorbent: Isotherm, Kinetics, and Thermodynamics. *Analytical Letters, 56(3),* 422–432.

Kainth, S., Sharma P., & Pandey, O.P. (2024). Green sorbents from agricultural wastes: A review of sustainable adsorption materials. *Applied Surface Science Advances, 19,* 100562.

Kausar, A., Zohra, S.T., Ijaz, S., Iqbal, M., Bibi, I., Nouren, S., El Messaoudi, N., & Nazir, A. (2023). Cellulose-based materials and their adsorptive removal efficiency for dyes: A review. *International Journal of Biological Macromolecules, 224,* 1337–1355.

Lima, H.D.P., & Asencios, Y.J.O. (2021). *Eichhornia crassipes* (Mart.) Solms (natural or carbonized) as biosorbent to remove pollutants in water. *SN Appl. Sci. 3,* 750.

Martini, S., Afroze, S., Roni, K.A., Setiawati, M., & Kharismadewi, D. (2021). A review of fruit waste-derived sorbents for dyes and metals removal from contaminated water and wastewater. *Desalination and Water Treatment, 235,* 300–323.

Mehrez, M., Chaari, I., & Medhioub, M. (2024). Biosorption of a leather dye (Dycem TTO) onto banana peel: Characterization, isotherm, kinetic, thermodynamic, and mechanism studies. *Water Air Soil Pollut., 235,* 119.

Rangabhashiyam, S., Lata, S., & Balasubramanian, P. (2018). Biosorption characteristics of methylene blue and malachite green from simulated wastewater onto *Carica papaya* wood biosorbent. *Surfaces and Interfaces, 10,* 197–215.

Rashid, T.U., Kabir, S.M.F., Biswas, M.C. & Bhuiyan, M.A.R. (2020). Sustainable wastewater treatment via dye−surfactant interaction: A Critical Review. *Industrial & Engineering Chemistry Research 59,* 9719−9745.

Regkouzas, P., & Diamadopoulos, E. (2019). Adsorption of selected organic micro-pollutants on sewage sludge biochar. *Chemosphere, 224*, 840–851.

Roy, T.K., & Mondal, N.K. (2019). Potentiality of *Eichhornia* shoots ash towards removal of Congo red from aqueous solution: Isotherms, kinetics, thermodynamics and optimization studies. *Groundwater for Sustainable Development, 9*, 100269.

Saranya, N., Karishma S.K., Hemapriya, M., Abishek, V.S., Vasantharaj, K., & Sivasubramanian, V. (2023). Fabricating a biochar from *Artocarpus heterophyllus* peels for methylene blue sequestration from synthetic wastewater: Isotherm, kinetic, and thermodynamic analysis. *Industrial & Engineering Chemistry Research 59*, 9719–9745.

Saravanan, A., Kumar, P.S., Yaashikaa, P.R., Karishma, S., Jeevanantham, S., & Swetha, S. (2021). Mixed biosorbent of agro waste and bacterial biomass for the separation of Pb (II) ions from water system. *Chemosphere, 277*, 130236.

Ungureanu, E.L., Mocanu, A.L., Stroe, C.A., Panciu, C.M., Berca, L., Sionel, R.M., & Mustatea, G. (2023). Agricultural byproducts used as low-cost adsorbents for removal of potentially toxic elements from wastewater: A Comprehensive Review. *Sustainability.15*, 5999.

Upadhyay, R., Przystaś, W., & Dave, B. (2024). Myco-remediation of synthetic dyes: a comprehensive review on contaminant alleviation mechanism, kinetic study and toxicity analysis. *International Journal of Environmental Science and Technology*.

Yaashikaa, P.R., Senthil Kumar, P., Saravanan, A., & Dai-Viet, N. (2021). Advances in biosorbents for removal of environmental pollutants: A review on pretreatment, removal mechanism and future Outlook. *Journal of Hazardous Materials.* 126596.

Yu, K.Y., Lee, X.J., Ong, H.C., Chen, W.H., Chang, J.S., Lin, C.S., Show, P.L., & Ling, T.C. (2021). Adsorptive removal of cationic methylene blue and anionic Congo red dyes using wet-torrefied microalgal biochar: Equilibrium, kinetic and mechanism modeling. *Environmental Pollution, 272*, 115986.

BACTERIOPHAGES: FROM HISTORICAL THERAPEUTIC AGENTS TO CONTEMPORARY REDISCOVERY AND BIOTECHNOLOGICAL APPLICATIONS

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Introduction

Phages, also known as bacteriophages, are viruses that exclusively target bacteria. Their discovery can be traced to the early 1900s. The first identification of bacteriophages occurred independently by Frederick Twort in 1915 and Félix d'Hérelle in 1917, marking a significant milestone in microbiology. In the beginning, these organisms were regarded as promising treatments for bacterial infections, especially prior to the introduction of antibiotics in the 1940s. However, the lack of understanding regarding their biological nature and mechanisms of action led to a decline in their use as antibiotics gained prominence (Aswani & Shukla, 2021; Nóbrega et al., 2015; Summers, 2001).

The historical context of bacteriophage therapy reveals a complex narrative. Early applications of phage therapy were promising, especially in Eastern Europe, where it was utilized extensively for treating bacterial infections. The emergence of antibiotics diminished the focus on phage therapy, causing a reduction in both research efforts and clinical use (Squires, 2018; Verbeken et al., 2014). The renewed focus on bacteriophages in recent times is largely due to the escalating issue of antibiotic resistance, pushing scientists and healthcare professionals to reconsider phage therapy as either a supplement or alternative to conventional antibiotics (Farmehr, 2021; Altamirano & Barr, 2019; Doub, 2020).

In the biotechnological realm, bacteriophages have been instrumental in advancing molecular biology and genetic engineering. They have served as tools for understanding fundamental biological processes, such as gene expression and regulation, and have facilitated the development of recombinant DNA technology (Squires, 2018; Abedon, 2017; Nóbrega et al., 2015). Moreover, the unique properties of bacteriophages, including their specificity for bacterial hosts and their ability to evolve alongside bacterial pathogens, make them promising candidates for targeted therapies against antibiotic-resistant infections (Feng, 2024; Pires et al., 2017; Baker et al., 2016). Recent research has investigated the use of modified phages to improve treatment effectiveness and minimize the chances of bacteria developing resistance to phages (Doub et al., 2020; Farmehr, 2021; Kwiatek et al., 2019).

In summary, the history of bacteriophages is marked by initial excitement, subsequent neglect, and a contemporary resurgence driven by the urgent need for new antimicrobial strategies. Their biotechnological

applications continue to evolve, offering hope for innovative treatments in the face of rising antibiotic resistance.

History of Bacteriophages

Bacteriophages were first discovered in 1896 by Ernest Hankin. Observing that people in India were recovering from cholera by bathing in the Ganges River, the bacteriologist speculated that this was due to the action of a living entity. He found that the antibacterial effects in the river persisted even when the Ganges water was filtered through fine porcelain (Hankin, 1896). This initiated phage research, which was later expanded upon by Frederick Twort in 1913, who described "an agent infecting and killing bacteria." The term "bacteriophage" was coined by Felix d'Herelle in 1917 after discovering an "invisible microbe" responsible for dysentery and documenting the first literature on their antimicrobial activities. Australian microbiologist Macfarlane Burnet reported in 1937 that bacteriophages exhibited varying resistance to physicochemical agents. By 1940, Helmut Ruska had used electron microscopy to visualize phages (Ustaçelebi et al., 1968). Phages were extensively used in treatments until the discovery of antibiotics, after which their use waned, though research continued steadily in countries like Poland and Georgia. Institutes such as Georgia's EIBMV (Eliava Institute of Bacteriophage Microbiology and Virology) and Poland's HIIET (Hirszfeld Institute of Immunology and Experimental Therapy) have played significant roles in phage isolation and therapeutic research (Sulakvelidze et al., 2001).

The history of bacteriophages began with the co-discovery by Frederick Twort in 1915 and Felix d'Hérelle in 1917. These early researchers identified bacteriophages as agents capable of lysing bacteria, which opened new avenues for understanding microbial interactions and disease treatment (Pelzek et al., 2008). The term "bacteriophage," derived from the Greek words "bacterion" (bacteria) and "phagein" (to eat), aptly describes their predatory nature. Over the decades, bacteriophages have been pivotal in advancing molecular biology, particularly in elucidating the mechanisms of genetic material and the principles of gene therapy (Pelzek et al., 2008; Jones et al., 2020).

The initial fascination with bacteriophages stemmed from their potential as therapeutic agents against bacterial infections. In the early 20th century, phage therapy was widely practiced, especially in Eastern Europe,

where it was used to treat various bacterial diseases (Jaglan et al., 2022; Wittebole et al., 2013). However, the advent of antibiotics in the 1940s led to a decline in phage therapy's popularity in Western medicine, overshadowing the significant role bacteriophages played in microbial ecology and evolution (Wittebole et al., 2013).

Despite this decline, bacteriophages continued to be utilized as model organisms in molecular biology. They were instrumental in the discovery of DNA as the genetic material and in the development of recombinant DNA technology (Squires, 2018). The study of bacteriophage lambda, for instance, provided insights into gene regulation and the mechanisms of lysogeny and lysis (Catalano, 2018).

In recent years, there has been a resurgence of interest in bacteriophage therapy, driven by the increasing prevalence of antibiotic-resistant bacterial infections (Jaglan et al., 2022; Wittebole et al., 2013). Modern research has revitalized phage therapy, demonstrating its efficacy in treating infections that are resistant to conventional antibiotics (Squires, 2018). This revival is supported by a growing body of evidence highlighting the safety and effectiveness of phages in clinical settings (Jaglan et al., 2022; Wittebole et al., 2013).

Phage therapy's potential extends beyond human medicine; it has also been explored in veterinary medicine and agriculture, where bacteriophages are used to control bacterial pathogens in livestock and crops (Smirnov et al., 2020). The versatility of bacteriophages as biocontrol agents underscores their importance in contemporary microbiological research and applications.

Bacteriophages have significantly contributed to the field of molecular biology. Their simple structure and rapid replication cycles make them ideal subjects for genetic studies. For example, bacteriophage T4 has been extensively studied to understand DNA replication and repair mechanisms (Letarov, 2020). Moreover, the use of bacteriophages as vectors in genetic engineering has facilitated advancements in biotechnology, including the development of phage display technology, which is employed in drug discovery and vaccine development (Pelzek et al., 2008; Letarov, 2020).

The study of bacteriophage proteins has also provided insights into viral assembly and host interactions. Research on the capsid proteins of bacteriophage P22, for instance, has elucidated the principles of virus

morphogenesis and the interactions between viral and host proteins (Essus et al., 2023). Such studies have paved the way for the application of bacteriophages in nanotechnology and theranostics, where they are being explored as delivery systems for therapeutic agents (Catalano, 2018).

Bacteriophages play a crucial role in shaping bacterial populations and ecosystems. They exert top-down control on bacterial communities, influencing diversity and dynamics through predation (Meijer et al., 2023; Wommack & Colwell, 2000). The co-evolution of bacteriophages and their bacterial hosts has led to complex interactions that drive genetic diversity and adaptation in both groups (Brüssow et al., 2004). This interplay is evident in the emergence of bacteriophage-resistant bacterial strains, which can exhibit altered virulence and pathogenicity (León & Bastías, 2015; Brüssow et al., 2004).

Recent studies have highlighted the ecological significance of bacteriophages in aquatic environments, where they contribute to nutrient cycling and the regulation of microbial communities (Wommack & Colwell, 2000). The concept of virioplankton, which refers to the viral component of planktonic communities, emphasizes the importance of bacteriophages in aquatic ecosystems (Wommack & Colwell, 2000).

The history of bacteriophages is a testament to their multifaceted roles in science and medicine. From their initial discovery as bacterial predators to their current applications in therapy and biotechnology, bacteriophages have profoundly influenced our understanding of microbial life and disease. As antibiotic resistance continues to pose a significant challenge in healthcare, the exploration of bacteriophages as therapeutic agents is more relevant than ever. Future research will undoubtedly uncover new dimensions of bacteriophage biology and their potential applications in various fields.

Morphology of Bacteriophages

Bacteriophages, or phages, are the most abundant biological entities on Earth, playing a pivotal role in regulating bacterial populations in various ecosystems. Their morphology is not only fascinating but also essential for their classification and understanding of their life cycles. The International Committee on Taxonomy of Viruses (ICTV) has established a classification system based on morphological characteristics, which includes the presence of tails, head shapes, and sizes (Amarillas et al., 2017). This article aims to

explore the morphological diversity of bacteriophages, focusing on the major families and their structural features.

Bacteriophages are primarily classified into three families based on their tail morphology: Myoviridae, Siphoviridae, and Podoviridae.

Myoviridae: These phages are characterized by their long, contractile tails and icosahedral heads. The contractile tail allows for the injection of viral DNA into the host cell, making them effective in lytic infections. For instance, the bacteriophage DW-EC, isolated from food sources, has been identified as belonging to the Myoviridae family due to its larger head size and long, rigid tail (Dewanggana et al., 2022).

Siphoviridae: Members of this family possess long, non-contractile tails and an icosahedral head. They are known for their flexibility and ability to form stable complexes with host bacteria. For example, bacteriophages vB_VpS_BA3 and vB_VpS_CA8, isolated from sewage, exhibit characteristics typical of the Siphoviridae family, including an icosahedral head and a long tail (Tan et al., 2021).

Podoviridae: These phages are characterized by short, stubby tails and are often associated with rapid infection cycles. Their morphology allows for quick attachment and penetration of host bacterial cells. The bacteriophage phiLLS, which targets multidrug-resistant Escherichia coli, has been classified under this family due to its distinct morphological features (Amarillas et al., 2017).

The morphology of bacteriophages is closely linked to their function and mode of infection. The structural components of phages include:

The head of a bacteriophage typically contains the viral genome and is composed of proteins that form a protective capsid. The shape and size of the head can vary significantly among different phage families. For instance, the head of the bacteriophage PAP 1-1 is similar to that of siphoviruses, indicating a common structural theme among phages that infect similar hosts.

The tail structure is critical for the attachment and penetration of the phage into the bacterial cell. Contractile tails, as seen in Myoviridae, facilitate the injection of the viral genome into the host, while non-contractile tails, characteristic of Siphoviridae, allow for stable binding to the bacterial surface

(Lukman et al., 2020). The tail morphology can also influence the specificity of the phage for its bacterial host.

These structures play a crucial role in host recognition and attachment. The tail fibers can vary in length and shape, contributing to the phage's ability to infect specific bacterial strains. For example, the tailspikes of bacteriophage Epsilon15 interact with lipopolysaccharides on the surface of Salmonella, facilitating infection (Chang et al., 2010).

The morphological characterization of bacteriophages is primarily conducted using transmission electron microscopy (TEM), which provides high-resolution images of phage structures. Techniques such as negative staining are commonly employed to enhance visibility. For instance, the morphology of bacteriophages isolated from poultry was examined using TEM, revealing distinct icosahedral heads and tails (Alomari et al., 2016).

Additionally, molecular techniques, including genomic analysis and restriction fragment length polymorphism (RFLP), complement morphological studies by providing insights into the genetic diversity and evolutionary relationships among phages (Alič et al., 2017).

Understanding the morphology of bacteriophages has significant implications for their ecological roles and applications in biotechnology. Phages are integral to microbial ecology, influencing bacterial population dynamics and genetic exchange through horizontal gene transfer (Wommack & Colwell, 2000). Their ability to specifically target bacterial pathogens makes them valuable tools in phage therapy, a promising alternative to antibiotics in combating multidrug-resistant infections (Doub et al., 2020).

Moreover, the morphological characteristics of phages can inform their use in biocontrol strategies in agriculture and food safety. For example, phages with specific morphologies have been isolated for their effectiveness against foodborne pathogens, highlighting the importance of morphology in selecting appropriate phages for therapeutic applications (Waturangi et al., 2021; Lopez et al., 2015).

The morphology of bacteriophages is a critical aspect of their biology, influencing their classification, host interactions, and applications in medicine and biotechnology. The diverse structural features of phages, including their heads and tails, reflect their evolutionary adaptations to infect specific

bacterial hosts. As research continues to uncover the complexities of bacteriophage morphology, it will enhance our understanding of their ecological roles and therapeutic potential.

Bacteriophages, or simply phages, are viruses that infect bacteria. These microorganisms, harmless to all organisms except their target bacterial hosts, are a major driving force in bacterial adaptive evolution (Pirnay et al., 2015). Measurements of bacteriophages using electron microscopy reveal that they generally range in size from 20 to 200 nm. Despite exhibiting different morphological characteristics, the majority of phages have cubic symmetry with dsDNA (double-stranded) genomes in their head structures. Although 90% of their main structures consist of DNA, they can have genetic material composed of either DNA or RNA (Haq et al., 2012).

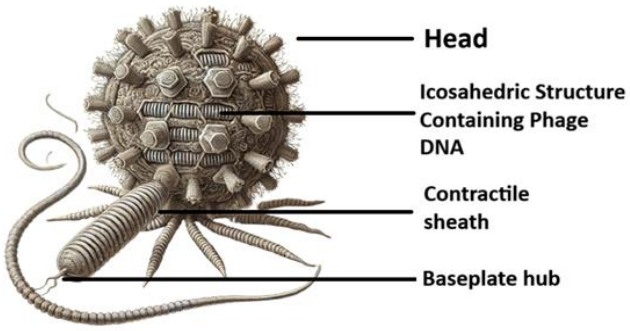

Figure 1. Bacteriophage and parts (Image was designed for book using Adobe Illustrator)

Phages have contributed significantly to the development of many molecular applications over time. Alfred Hershey and Martha Chase used phages in their "Waring Blender Experiment" or Hershey-Chase experiment to prove that genes are made of DNA (Elois et al., 2023). Phages, being suitable for gene and enzyme studies, play a crucial role in the genetic control of enzymes. They also show great potential in studies addressing bacterial gene mutagenesis (Elois et al., 2023). The abundance of uncharacterized genes in bacteriophages increases their biotechnological importance, making them especially valuable for biotechnologists in recent years. Phage genomes encode proteins that are beneficial in various fields of biotechnology, including food preservation, control of multi-drug resistant infections, DNA transfer applied technologies, and more. The rapid circulation of phage infections in bacterial cells, their replication, and the release of new virions

that infect other bacteria are of great significance for research. These studies are essential for understanding the evolution of genomes, bacterial adaptive evolution, and the expression and replication of DNA, which could lead to the development of potentially new biotechnological products.

Bacteriophages' Role in Molecular Biology

Bacteriophages have played a crucial role in developing many molecular applications since their identification. Alfred Hershey and Martha Chase, through the "Waring Blender Experiment" or Hershey-Chase experiment, demonstrated that genes are composed of DNA using phages (Elois et al., 2023). Phages, which are suitable for use in gene and enzyme studies, hold great importance in the genetic control of enzymes. They also show significant potential in studies addressing bacterial gene mutagenesis (Elois et al., 2023).

The abundance of uncharacterized genes in bacteriophages increases their biotechnological importance, making them particularly valuable for biotechnologists in recent years. Bacteriophage genomes encode proteins that are useful in various fields of biotechnology, such as food preservation and control, the antibacterial therapy of multi-drug-resistant infections, and DNA transfer technologies. The continued circulation and infection rate of phages as they infect bacterial cells, replicate, and release new virions to infect other bacterial cells are of great importance for research. These studies are crucial for understanding genome evolution, bacterial adaptive evolution, and the expression and replication of DNA, which may lead to the development of potentially new biotechnological products.

Bacteriophages do not possess metabolic mechanisms and do not show affinity for eukaryotic cells. This increases their potential for use as drugs and in various biotechnological products (Chan & Abedon, 2012). Specifically, bacteriophage particles show potential in various preventive strategies, including phage-based vaccines, gene transfer in animals and plants, the creation of bacterial biosensors, gene delivery systems, food preservation and safety, plant pathogen biocontrol, biofilm management, surface sanitization, and corrosion prevention. There are numerous biotechnological applications for bacteriophages in fields ranging from human antibacterial therapy to environmental disinfection and food preservation, with high potential for further development and application in various other biotechnological fields.

In the past five decades, researchers have studied and identified over 5,100 bacteriophages. It has been noted that more than 90% of these phages are classified within the Myoviridae, Siphoviridae, and Podoviridae families. (Harada et al., 2018; Wittebole et al., 2013).

Applications of Phages

Phages, or bacteriophages, are viruses that specifically infect bacteria, offering a range of applications in medicine, biotechnology, and environmental sciences. In medicine, phage therapy is being explored as an alternative to antibiotics, particularly for treating antibiotic-resistant infections. Phages are also used in food safety to prevent bacterial contamination and spoilage. In biotechnology, they serve as tools for genetic engineering, acting as vectors for gene transfer. Additionally, phages play a role in environmental applications, such as controlling harmful bacteria in water systems and agriculture. Their specificity and adaptability make them a valuable tool across various fields (Table 1).

Table 1. Phage applications in various fields.

Host	Area	Notes	Reference
Salmonella sp.	Food	The application of a bacteriophage preparation specifically targeting *Salmonella* through dipping or surface treatment of chicken breast fillets, followed by storage at 4°C, resulted in a significant reduction in *Salmonella* contamination.	(Sukumaran et al., 2016)
Staphylococcus aureus	Food	The use of phage-treated milk in cheese production led to a significant reduction in *Staphylococcus aureus* counts compared to cheese produced from untreated milk. Importantly, the inclusion of phages did not alter the overall microbiota of the cheese.	(Bueno et al., 2012)
Xanthomonas axonopodis pv. citrumelo	Agriculture	A suspension containing phages at a concentration of 10^8 PFU/mL in 75% skim milk powder was applied via spraying at dawn to manage a naturally occurring population of bacterial pathogens	(Balogh et al., 2006)
E. coli *Proteus* *Pseudomonas* *Staphylococcus*	Medical	In 22 patients with ulcers, healing was observed in association with the reduction or eradication of the target organisms.	(Markoishvili et. al., 2002)
Proteus *Staphylococcus* *Streptococcus*	Medical	The use of Pyophage resulted in a reduction of septic complications, improved temperature normalization, a two-fold decrease in Staphylococcus and *Streptococcus* populations, and a 1.5-fold reduction in Proteus	(Lazareva et al., 2001)
E. coli *Proteus* *Staphylococcus*	Medical	Marked clinical improvement was observed in 92% of cases, with bacteriological clearance achieved in 84% of instances	(Perepanova et al., 1995)

Beyond their therapeutic use in humans and animals, phages have various other applications:

Biological control in food and agriculture: Bacteriophages can be used as natural preservatives to reduce contamination on carcasses, processed products, and fresh fruits and vegetables, as well as to disinfect equipment and contact surfaces, thereby extending the shelf life of perishable food products. They are also frequently used in food control to detect contaminants in foods. Phages play a crucial role in controlling major food pathogens such as *Campylobacter, Listeria, Salmonella,* and *E. coli*. Additionally, phages have been effectively used in agriculture to combat *Erwinia amylovora,* the causative agent of apple blossom infections.

Biotechnology: Studies have shown that phages can be used in modern biotechnology. Phages can be used as vectors in gene therapy, as tools for screening protein, peptide, or antibody libraries, and as carriers for vaccines and proteins, which significantly increases their biotechnological importance.

Molecular biology: In molecular biology, phages are used as model systems. They are used as vectors in recombinant DNA technology to express desired genes in bacteria. Today, phage display technology, which involves using immune techniques to evaluate gene technology, is used to develop next-generation recombinant antibodies.

Bacterial identification: Phages can be used as tools for the direct detection and identification of foodborne and clinical bacterial pathogens.

Aquaculture: Phages have the potential to be used as alternative treatments or prophylactics against diseases in aquaculture, although few studies have been conducted in this area.

Biological control of dental caries: The oral cavity, colonized by approximately 6 billion bacteria and at least 35 times more viruses, is one of the largest ecosystems in the human body. Accordingly, bacteriophage therapies have become an alternative option for combating orodental infections (Parasion et al., 2014).

Application of phages in nanotechnology: The intersection of bacteriophages and nanotechnology has emerged as a promising frontier in biomedical research, offering innovative solutions for drug delivery, biosensing, and therapeutic applications. Bacteriophages, or phages, are

viruses that specifically infect bacteria, and their unique properties make them suitable candidates for various nanotechnological applications. This article explores the diverse applications of phages in nanotechnology, focusing on their roles in drug delivery systems, biosensors, and their potential in cancer therapy.

One of the most significant applications of phages in nanotechnology is their use as targeted drug delivery systems. Phages can be genetically engineered to carry therapeutic agents, such as drugs or genes, directly to specific cells or tissues. This targeted approach enhances the efficacy of treatments while minimizing side effects. For instance, phages can be modified to display peptides or proteins on their surfaces, allowing them to bind selectively to cancer cells or infected tissues (Li, 2023). This specificity is crucial in improving the therapeutic index of anticancer drugs and antibiotics, as it reduces off-target effects and enhances drug accumulation at the desired site (Li, 2023).

Recent advancements have demonstrated the potential of phage-based nanoplatforms for tumor therapy. By encapsulating anticancer drugs within phage particles, researchers have achieved improved drug stability and controlled release profiles (Li, 2023). Additionally, the use of phages in combination with nanoparticles, such as gold or silver nanoparticles, has shown promise in enhancing the therapeutic effects of these agents (El-Sayed et al., 2023). The synergistic effects of phages and nanoparticles can lead to improved drug delivery efficiency and enhanced therapeutic outcomes, although some studies indicate potential stability issues when combining phages with metal nanoparticles (Abdelsattar et al., 2021).

Phages are also being explored for their potential in biosensing applications. The ability of phages to specifically bind to their bacterial hosts can be harnessed to develop sensitive biosensors for detecting bacterial pathogens. For example, phage display technology has been utilized to engineer phages that can selectively bind to specific bacterial strains, allowing for the detection of pathogens in food and clinical samples (Stachurska et al., 2022).

In a notable study, engineered phages were combined with gold nanoparticles to create a colorimetric biosensor. The phages displayed receptor-binding proteins that targeted specific bacteria, and upon binding, the aggregation of gold nanoparticles resulted in a visible color change, indicating

the presence of the target bacteria (Stachurska et al., 2022). This approach not only enhances the sensitivity of bacterial detection but also provides a rapid and cost-effective method for monitoring bacterial contamination in various settings.

The unique structural properties of phages have inspired their use in the fabrication of nanostructures. Phages can serve as templates for the assembly of nanoparticles, leading to the creation of novel nanomaterials with tailored properties. For instance, studies have demonstrated the successful templating of magnetic nanoparticles using bacteriophages, resulting in materials with potential applications in nanomedicine and targeted drug delivery (Olszewska-Widdrat et al., 2021). The ability to control the size and distribution of nanoparticles on phage templates allows for the development of multifunctional nanomaterials that can be used in diagnostics and therapeutics.

Moreover, phages can be utilized to create virus-like particles (VLPs) that mimic the structure of viruses but lack the viral genome. These VLPs can be engineered to display specific antigens, making them suitable candidates for vaccine development and targeted therapy (Aljabali et al., 2021). The versatility of phage-based nanostructures opens new avenues for the design of advanced materials with applications in biomedicine, including drug delivery and immunotherapy.

Despite the promising applications of phages in nanotechnology, several challenges remain. The stability of phage-nanoparticle conjugates in biological environments is a critical concern, as factors such as pH, temperature, and enzymatic activity can affect their performance (El-Sayed et al., 2023). Additionally, the potential immunogenicity of phage-based systems must be carefully evaluated to ensure their safety in clinical applications.

Future research should focus on optimizing the engineering of phages for specific applications, enhancing their stability, and exploring their interactions with biological systems. The integration of phages with other nanomaterials, such as liposomes and hydrogels, could further improve their functionality and expand their applications in targeted therapy and diagnostics (Portilla et al., 2020).

Phages represent a versatile tool in the field of nanotechnology, with applications ranging from targeted drug delivery to biosensing and the

development of novel nanomaterials. Their unique properties and ability to be engineered for specific functions make them valuable assets in the quest for innovative solutions to pressing biomedical challenges. As research in this area continues to advance, phage-based nanotechnology holds great promise for improving therapeutic outcomes and enhancing our understanding of complex biological systems.

Techniques for Using Phages in Biotechnology

Phage Display

Phage display technology, first introduced by George P. Smith in 1985, has emerged as a revolutionary tool in molecular biology, enabling the identification and selection of peptides, proteins, and antibodies with high specificity and affinity for various targets. This technique involves the fusion of a peptide or protein of interest to a bacteriophage coat protein, allowing the displayed peptide to be presented on the surface of the phage. This unique approach links phenotype (the displayed peptide) to genotype (the encoding DNA), facilitating the selection of desired ligands through a process known as biopanning (Wu et al., 2016; Anand et al., 2021).

The versatility of phage display is evident in its broad range of applications across various fields, including immunology, drug discovery, and vaccine development. For instance, phage display has been utilized to identify specific peptides that bind to viral proteins, thereby distinguishing pathogenic viruses from non-pathogenic strains. demonstrated the use of phage-displayed peptides to recognize the N protein of the porcine reproductive and respiratory syndrome virus, showcasing the potential of this technology in virology (Ren et al., 2010). Similarly, phage display has been employed to develop peptide ligands targeting the transmissible gastroenteritis coronavirus, highlighting its utility in antiviral research (Ren et al., 2011).

In the realm of immunotherapy, phage display has significantly advanced the development of antibody-based therapeutics. By constructing diverse antibody libraries displayed on phages, researchers can efficiently screen for antibodies with high affinity and specificity for their targets. emphasized the impact of phage display on antibody engineering, noting its role in generating fully human antibodies that are now in clinical use (Almagro et al., 2019). Furthermore, phage display has been instrumental in identifying

autoantigens relevant to autoimmune diseases, as demonstrated by in their study on acute anterior uveitis (Kim et al., 2011).

Phage display technology also plays a crucial role in vaccine development. By displaying immunogenic peptides on the surface of phages, researchers can elicit robust immune responses. reviewed various phage display systems and their applications in vaccine design, underscoring the potential of phage-based vaccines to stimulate effective immunity (Gao et al., 2010). Moreover, the safety profile of phage vaccines is enhanced due to their long-standing evolutionary relationship with mammalian hosts, making them promising candidates for future vaccine strategies (Aghebati-Maleki et al., 2016).

The application of phage display extends beyond immunology and vaccine development; it has also made significant strides in biomaterials engineering and nanotechnology. For instance, phage display has been utilized to select peptides that bind to specific materials, facilitating the development of biocompatible materials for various applications (Şeker & Demir, 2011; Martins et al., 2016). This capability is particularly valuable in regenerative medicine, where tailored biomaterials can enhance tissue engineering and repair processes.

Despite its numerous advantages, phage display technology is not without challenges. One notable limitation is the absence of post-translational modifications (PTMs) in phage-displayed peptides, which can affect the biological activity and functionality of the selected ligands (Somers et al., 2011). Addressing this limitation through the incorporation of PTMs into phage display systems could significantly broaden the applicability of the technology.

In conclusion, phage display technology has established itself as a powerful and versatile tool in modern biomedical research. Its ability to link genotype and phenotype has facilitated the discovery of novel peptides, proteins, and antibodies with applications in diagnostics, therapeutics, and vaccine development. As advancements continue to be made in phage display methodologies, its potential to address complex biological challenges will undoubtedly expand, paving the way for innovative solutions in medicine and biotechnology.

Bacteriophage Recombineering of Electroporated DNA (BRED) Method

The BRED method is a technique for genetically modifying phages. It leverages the recombination systems of natural phages to enhance the frequency of homologous recombination. To facilitate this process, the phage DNA template and donor DNA are electroporated into cells that express phage-encoded recombination system proteins. This approach is particularly useful for studies involving deletions, replacements, and the insertion of foreign genes, offering significant benefits and promising potential for phage engineering. The method may also rely on the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas system. CRISPR is an immune process of prokaryotic organisms. It grants bacteria immunity against exogenous elements and has recently been applied in bioengineering. These applications of bacteriophages have rapidly increased their use in the CRISPR-Cas system in recent years. Phage genome engineering can be applied both in vivo and in vitro. In these applications, the genome is assembled and placed into the host cell, and the phage's replication cycle within the bacterium promotes the assembly of newly modified viruses (Chen, 2019).

In the treatment of a 15-year-old patient with a widespread infection caused by Mycobacterium abscessus, a cocktail of three phages produced using the BRED method was administered, resulting in significant improvement after treatment. This study is one of the first examples of using modified phages in human mycobacterial infections and is a pioneering study combining therapeutic use with bioengineering (Dedrick, 2019).

Vaccine Development Using Phages

The emergence of bacteriophages (phages) as innovative platforms for vaccine development has garnered significant attention in recent years. Phages, which are viruses that specifically infect bacteria, have been recognized for their unique properties that make them suitable for immunization strategies. This article explores the various aspects of phage-based vaccines, including their mechanisms of action, types, advantages, and potential applications in combating infectious diseases and cancer.

Phage-based vaccines utilize phages as carriers to present antigens to the immune system, thereby eliciting a robust immune response. The primary

mechanisms involve the display of antigens on the phage surface, which can stimulate both humoral and cellular immunity. Phages can be engineered to express multiple copies of an antigen, enhancing the immunogenicity of the vaccine (Palma, 2023; Aghebati-Maleki et al., 2016). This is particularly beneficial as phages can engage pattern recognition receptors on antigen-presenting cells, leading to improved antigen presentation to B-cells and T-cells (Islam, 2023).

Moreover, phages possess inherent adjuvant properties, which can enhance the immune response without the need for additional adjuvants (Staquicini et al., 2021). The ability of phages to induce antigen-specific antibodies by recruiting helper T cells further underscores their potential as effective vaccine platforms (Balcıoğlu et al., 2014).

There are several types of phage-based vaccines currently under investigation. These include display vaccines, DNA vaccines, and hybrid vaccines (Palma, 2023). Display vaccines utilize phage display technology to present antigens or epitopes on the phage surface, which can be tailored to target specific pathogens (Aghebati-Maleki et al., 2016). DNA vaccines involve the introduction of phage DNA encoding antigens, which can be expressed in host cells to elicit an immune response (Palma, 2023). Hybrid vaccines combine elements from both phage and traditional vaccine platforms, potentially enhancing efficacy and safety (Palma, 2023).

Recent studies have demonstrated the efficacy of phage-based vaccines in various contexts. For instance, a phage-based vaccine targeting the hepatitis B virus surface antigen was shown to be cost-effective and capable of inducing a strong immune response in animal models (Balcıoğlu et al., 2014). Similarly, phage-displayed peptides have been explored as candidates for vaccines against Leishmania infantum, demonstrating protective efficacy in preclinical models (Costa et al., 2014; Ramos et al., 2017).

Phage-based vaccines offer several advantages over traditional vaccine platforms. Firstly, they are highly stable and can withstand harsh environmental conditions, making them suitable for storage and transport (Haq et al., 2023). Additionally, phages can be produced in large quantities at a relatively low cost, facilitating widespread vaccination efforts (Haq et al., 2023).

The safety profile of phages is another significant advantage. Phages have co-evolved with mammals, leading to a long-standing relationship that minimizes

adverse immune reactions (Aghebati-Maleki et al., 2016; Zalewska-Piątek & Piątek, 2021). This safety aspect is particularly crucial for vaccines intended for human use, as demonstrated in clinical trials involving phage-based cancer vaccines (Roehnisch et al., 2014).

The versatility of phage-based vaccines extends to various infectious diseases and cancer immunotherapy. For instance, engineered filamentous phages have been explored as potential vaccines against COVID-19, with studies indicating their ability to induce strong humoral responses against SARS-CoV-2 (Haq et al., 2023). Furthermore, phage-based platforms have been utilized to develop vaccines targeting antibiotic-resistant bacterial strains, addressing a critical public health challenge (Sartorius et al., 2019).

In the realm of cancer immunotherapy, phages can be engineered to display tumor-associated antigens, thereby stimulating an immune response against cancer cells (Ragothaman & Yoo, 2023). Clinical trials have shown promising results for phage-based vaccines in treating multiple myeloma, highlighting their potential in oncology (Roehnisch et al., 2014).

Despite the promising potential of phage-based vaccines, several challenges remain. The complexity of phage engineering and the need for rigorous safety evaluations are critical considerations in the development process (González-Mora et al., 2020). Furthermore, regulatory pathways for phage-based vaccines are still evolving, necessitating collaboration between researchers, clinicians, and regulatory bodies to ensure safe and effective products reach the market (Zalewska-Piątek & Piątek, 2021).

Future research should focus on optimizing phage engineering techniques, exploring novel phage-antigen combinations, and conducting comprehensive clinical trials to establish the efficacy and safety of phage-based vaccines across diverse populations and disease contexts (Sartorius et al., 2019; Ragothaman & Yoo, 2023).

Phage-based vaccines represent a revolutionary approach to immunization, leveraging the unique properties of phages to enhance vaccine efficacy and safety. With ongoing research and development, phages hold the potential to address pressing public health challenges, including infectious diseases and cancer. As the field advances, it is crucial to continue exploring the vast possibilities that phage technology offers in the realm of vaccine development.

REFERENCES

Abdelsattar, A. S., Dawooud, A., Rezk, N., Makky, S., Safwat, A., Richards, P. J., & El-Shibiny, A. (2021). How to train your phage: The recent efforts in phage training. *Biologics, 1*(2), 70-88.

Abedon, S. T. (2017). Information phage therapy research should report. *Pharmaceuticals, 10*(2), 43.

Aghebati-Maleki, L., Bakhshinejad, B., Baradaran, B., Motallebnezhad, M., Aghebati-Maleki, A., Nickho, H., Yousefi, M., Majidi, J. (2016). Phage display as a promising approach for vaccine development. *Journal of Biomedical Science, 23*, 1-18.

Alič, Š., Naglič, T., Tušek-Žnidarič, M., Ravnikar, M., Rački, N., Peterka, M., & Dreo, T. (2017). Newly isolated bacteriophages from the Podoviridae, Siphoviridae, and Myoviridae families have variable effects on putative novel Dickeya spp. *Frontiers in Microbiology, 8*, 1870.

Aljabali, A. A., Al Zoubi, M. S., Al-Batayneh, K. M., Pardhi, D. M., Dua, K., Pal, K., & Tambuwala, M. M. (2021). Innovative applications of plant viruses in drug targeting and molecular Imaging-A review. *Current Medical Imaging, 17*(4), 491-506.

Almagro, J. C., Pedraza-Escalona, M., Arrieta, H. I., & Pérez-Tapia, S. M. (2019). Phage display libraries for antibody therapeutic discovery and development. *Antibodies, 8*(3), 44.

Alomari, M. M. M., Dec, M., & Urban-Chmiel, R. (2021). Bacteriophages as an alternative method for control of zoonotic and foodborne pathogens. *Viruses, 13*(12), 2348.

Altamirano, F. G., & Barr, J. J. (2019). Phage therapy in the postantibiotic era. *Clinical Microbiology Reviews, 32*(2).

Amarillas, L., Lightbourn-Rojas, L., Angulo-Gaxiola, A. K., Basilio Heredia, J., González-Robles, A., & León-Félix, J. (2018). The antibacterial effect of chitosan-based edible coating incorporated with a lytic bacteriophage against *Escherichia coli* O157: H7 on the surface of tomatoes. *Journal of Food Safety, 38*(6), e12571.

Anand, U., Bianco, F., Suresh, S., Tripathi, V., Nunez-Delgado, A., & Race, M. (2021). SARS-CoV-2 and other viruses in soil: an environmental outlook. *Environmental Research, 198*, 111297.

Aswani, V., & Shukla, S. K. (2021). An early history of phage therapy in the united states: is it time to reconsider?. *Clinical Medicine & Research, 19*(2), 82-89.

Baker, C. W., Miller, C. R., Thaweethai, T., Yuan, J., Baker, M. H., Joyce, P., … & Weinreich, D. (2016). Genetically determined variation in lysis time variance in the bacteriophage φx174. *G3 Genes Genomes Genetics, 6*(4), 939-955.

Balogh, B. (2006). Characterization and use of bacteriophages associated with citrus bacterial pathogens for disease control. University of Florida PhD thesis.

Brüssow, H., Canchaya, C., & Hardt, W. D. (2004). Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. *Microbiology and Molecular Biology Reviews, 68*(3), 560-602.

Bueno, E., García, P., Martínez, B., & Rodríguez, A. (2012). Phage inactivation of *Staphylococcus aureus* in fresh and hard-type cheeses. *International Journal of Food Microbiology, 158*(1), 23-27.

Catalano, C. E. (2018). Bacteriophage lambda: The path from biology to theranostic agent. Wiley Interdisciplinary Reviews: *Nanomedicine and Nanobiotechnology, 10*(5), e1517.

Chan, B.K., Abedon, S.T., (2012). Phage therapy pharmacology phage cocktails. In: In: Laskin, A.I., Sariaslani, S., Gadd, G.M. (Eds.), Advances in Applied Microbiology Vol. 78. Elsevier Academic Press Inc., San Diego, pp. 1–23.

Chang, R. Y. K., Wallin, M., Lin, Y., Leung, S. S. Y., Wang, H., Morales, S., & Chan, H. K. (2018). Phage therapy for respiratory infections. *Advanced Drug Delivery Reviews, 133*, 76-86.

Chen, Y., Batra, H., Dong, J., Chen, C., Rao, V.B., Tao, P. (2019). Genetic Engineering of Bacteriophages Against Infectious Diseases. *Frontiers in Microbiology, 10*, 954.

Costa, L. E., Lima, M. I., Chávez-Fumagalli, M. A., Menezes-Souza, D., Martins, V. T., Duarte, M. C., Lage, P. S., Lopes, E. G. P., Lage, D. P., Ribeiro, T. G., Andrade, P. H. R., de Magalhães-Soares, D. F., Manuel Soto, Carlos A. P. Tavares, Luiz R. Goulart, Coelho, E. A. (2014). Subtractive phage display selection from canine visceral leishmaniasis identifies novel epitopes that mimic *Leishmania infantum* antigens with potential serodiagnosis applications. *Clinical and Vaccine Immunology, 21*(1), 96-106.

Dedrick, R.M., Guerrero-Bustamante, C.A., Garlena, R.A., Russell, D.A., Ford, K., Harris, K., Gilmour, K.C., Soothill, J., Jacobs-Sera, D., Schooley, R.T., Hatfull, G.F., Spencer, H. (2019). Engineered Bacteriophages for Treatment of a Patient with a Disseminated Drug-Resistant *Mycobacterium abscessus*. *Nature Medicine, 25*, 730–733.

Dewanggana, M. N., Evangeline, C., Ketty, M. D., Waturangi, D. E., Yogiara, & Magdalena, S. (2022). Isolation, characterization, molecular analysis and application of bacteriophage DW-EC to control Enterotoxigenic *Escherichia coli* on various foods. *Scientific Reports, 12*(1), 495.

Doub, J. B. (2020). Bacteriophage therapy for clinical biofilm infections: parameters that influence treatment protocols and current treatment approaches. *Antibiotics, 9*(11), 799.

Elois, M. A., Silva, R. D., Pilati, G. V. T., Rodríguez-Lázaro, D., & Fongaro, G. (2023). Bacteriophages as biotechnological tools. *Viruses, 15*(2), 349.

El-Sayed, A. I., El-Sayed, I. M., & Awad, S. (2023). Bacteriocins: Nisin as an Alternative Source to Chemical Preservatives. In Natural Food Preservatives (pp. 51-85). Jenny Stanford Publishing.

Essus, V. A., Souza Júnior, G. S. E., Nunes, G. H. P., Oliveira, J. D. S., de Faria, B. M., Romão, L. F., & Cortines, J. R. (2023). Bacteriophage P22 capsid as a pluripotent nanotechnology tool. *Viruses, 15*(2), 516.

Farmehr, P. (2021). Phage therapy is an important replacement for the antibiotic resistance. *Pakistan Journal of Medical and Health Sciences, 15*(5), 1236-1240.

Feng, L., Chen, H., Qian, C., Zhao, Y., Wang, W., Liu, Y., … & Wu, Q. (2024). Resistance, mechanism, and fitness cost of specific bacteriophages for *Pseudomonas aeruginosa*. *mSphere, 9*(2).

Gao, J., Wang, Y., Liu, Z., & Wang, Z. (2010). Phage display and its application in vaccine design. *Annals of Microbiology, 60*, 13-19.

González-Mora, A., Hernández-Pérez, J., Iqbal, H. M., Rito-Palomares, M., & Benavides, J. (2020). Bacteriophage-based vaccines: A potent approach for antigen delivery. *Vaccines*, *8*(3), 504.

Hankin, E. H., (1896). L‟action bactericide des eaux de la Jumna et du Gange sur le vibrion du cholera, *Ann Inst Pasteur, 10*(5), ll.

Haq, I. U., Khan, Z. I., Aziz, I., Basit, A., Hussain, F., Bibi, A., Aqib, A. I., Siddique, F., Younas, U., Rahim, K. (2023). Phages and SARS-CoV-2. In Application of Natural Products in SARS-CoV-2 (pp. 273-292). Academic Press.

Haq, I. U., Chaudhry, W. N., Akhtar, M. N., Andleeb, S., & Qadri, I. (2012). Bacteriophages and their implications on future biotechnology: a review. *Virology Journal*, *9*, 1-8.

Harada, L. K., Silva, E. C., Campos, W. F., Del Fiol, F. S., Vila, M., Dąbrowska, K., ... & Balcão, V. M. (2018). Biotechnological applications of bacteriophages: State of the art. *Microbiological Research, 212*, 38-58.

Islam, M. S., Nime, I., Pan, F., & Wang, X. (2023). Isolation and characterization of phage ISTP3 for bio-control application against drug-resistant Salmonella. *Frontiers in Microbiology, 14*, 1260181.

Jaglan, A. B., Anand, T., Verma, R., Vashisth, M., Virmani, N., Bera, B. C., Valid, R. K., Tripathi, B. N. (2022). Tracking the phage trends: A comprehensive review of applications in therapy and food production. *Frontiers in Microbiology, 13*, 993990.

Jones, E. H., Letarov, A. V., & Clokie, M. (2020). Neat science in a messy world: the global impact of human behavior on phage therapy, past and present. *Phage, 1*(1), 16-22.

Kim, J., Kim, M., Kim, S., & Ryu, S. (2017). Sensitive detection of viable *Escherichia coli* O157: H7 from foods using a luciferase-reporter phage phiV10lux. *International Journal of Food Microbiology, 254*, 11-17.

Klaartje, S., Piet, G., Dirk, E., Filip, D. K., Jean-Luc, R., Marieke, C., Marlies, B., Piet, S., Veerle, S. (2011). Novel autoantibody markers for early and seronegative rheumatoid arthritis. *Journal of Autoimmunity, 36*(1), 33-46.

Kwiatek, M., Parasion, S., & Nakonieczna, A. (2019). Therapeutic bacteriophages as a rescue treatment for drug-resistant infections – anin vivostudies overview. *Journal of Applied Microbiology, 128*(4), 985-1002.

Lazareva, E.B., Smirnov, S.V., Khvatov, V.B., Spiridonova, T.G., Bitkova, E.E., Darbeeva, O.S. (2001). Efficacy of bacteriophage use in complex treatment of the patients with burn wounds. *Antibiotics and Chemoterapy, 46*(1), 10-14.

León, M., & Bastías, R. (2015). Virulence reduction in bacteriophage resistant bacteria. *Frontiers in Microbiology, 6*, 343.

Letarov, A. V. (2020). History of early bacteriophage research and emergence of key concepts in virology. *Biochemistry (Moscow), 85*, 1093-1112.

Li, J., Zheng, H., & Leung, S. S. Y. (2023). Potential of bacteriophage therapy in managing *Staphylococcus aureus* infections during chemotherapy for lung cancer patients. *Scientific Reports, 13*(1), 9534.

Lopez-Perez, M., Haro-Moreno, J. M., Gonzalez-Serrano, R., Parras-Molto, M., & Rodriguez-Valera, F. (2017). Genome diversity of marine phages recovered from Mediterranean metagenomes: size matters. *PLoS Genetics, 13*(9), e1007018.

Lukman, C., Yonathan, C., Magdalena, S., & Waturangi, D. E. (2020). Isolation and characterization of pathogenic *Escherichia coli* bacteriophages from chicken and beef offal. *BMC Research Notes, 13*, 1-7.

Markoishvili, K., Tsitlanadze, G., Katsarava, R., Glenn, J., Morris Jr, M. D., & Sulakvelidze, A. (2002). A novel sustained-release matrix based on biodegradable poly (ester amide) s and impregnated with bacteriophages and an antibiotic shows promise in management of infected venous stasis ulcers and other poorly healing wounds. *International Journal of Dermatology*, *41*(7), 453-458.

Martins, I. M., Reis, R. L., & Azevedo, H. S. (2016). Phage display technology in biomaterials engineering: progress and opportunities for applications in regenerative medicine. *ACS Chemical Biology*, *11*(11), 2962-2980.

Meijer, J., Skiadas, P., Rainey, P. B., Hogeweg, P., & Dutilh, B. E. (2023). Eco-evolutionary dynamics of massive, parallel bacteriophage outbreaks in compost communities. *Biorxiv*, 2023-07.

Nóbrega, F. L., Costa, A. R., Kluskens, L., & Azeredo, J. (2015). Revisiting phage therapy: new applications for old resources. *Trends in Microbiology*, *23*(4), 185-191.

Olszewska-Widdrat, A., Bennet, M., Mickoleit, F., Widdrat, M., Tarabout, C., Reichel, V., Arndt, K., Schüler, D., Faivre, D. (2021). Bacteriophage-templated assembly of magnetic nanoparticles and their actuation potential. *ChemNanoMat, 7*(8), 942-949.

Palma, M. (2023). Aspects of phage-based vaccines for protein and epitope immunization. Vaccines, 11(2), 436.

Parasion, S., Kwiatek, M., Gryko, R., Mizak, L., Malm, A., (2014). Bacteriophages as an alternative strategy for fighting biofilm development. *Polish Journal of Microbiolgy*, *63*(2), 137–145.

Pelzek, A. J., Schuch, R., Schmitz, J. E., & Fischetti, V. A. (2013). Isolation of bacteriophages from environmental sources, and creation and functional screening of phage DNA libraries. *Current Protocols Essential Laboratory Techniques*, *7*(1), 13-3.

Perepanova, T. S., Darbeeva, O. S., Kotliarova, G. A., Kondrat'eva, E. M., Maĭskaia, L. M., Malysheva, V. F., Baĭguzina, F. A., Grishkova, N. V. (1995). The efficacy of bacteriophage preparations in treating inflammatory urologic diseases. *Urologiia i Nefrologiia*, *5*, 14-17.

Pires, D. P. P., Melo, L. D. R., Boas, D. P. A. V., Sillankorva, S., & Azeredo, J. (2017). Phage therapy as an alternative or complementary strategy to prevent and control biofilm-related infections. *Current Opinion in Microbiology*, *39*, 48-56.

Pirnay, J.P., Blasdel, B.G., Bretaudeau, L., Buckling, A., Chanishvili, N., Clark, J.R., Corte-Real, S., Debarbieux, L., Dublanchet, A., De, V.D., Gabard, J., Garcia, M.,Goderdzishvili, M., Górski, A., Hardcastle, J., Huys, I., Kutter, E., Lavigne, R., Merabishvili, M., Olchawa, E., Parikka, K.J., Patey, O., Pouilot, F., Resch, G., Rohde, C., Scheres, J., Skurnik, M., Vaneechoutte, M., Van, P.L., Verbeken, G., Zizi, M., Vanden Eede, G., (2015). Quality and safety requirements for sustainable phage therapy products. *Pharmaceutical Research*, *32*, 2173–2179.

Portilla, S., Fernández, L., Gutiérrez, D., Rodríguez, A., & García, P. (2020). Encapsulation of the antistaphylococcal endolysin LysRODI in pH-sensitive liposomes. *Antibiotics, 9*(5), 242.

Ragothaman, M., & Yoo, S. Y. (2023). Engineered phage-based cancer vaccines: current advances and future directions. *Vaccines, 11*(5), 919.

Ramos, F., Costa, L., Dias, D. S., Santos, T. T. O., Rodrigues, M. R., Lage, D. P., Salles, B. C. S., Martins, V. T., Ribeiro, P. A. F., Chávez-Fumagalli, M. A., Dias, A. C. S., Alves, P. T., Vieira, É. L. M., Roatt, B. M., Menezes-Souza, D., Duarte, M. C., Teixeira, A. L. G (2017). Selection strategy of phage-displayed immunogens based on an in vitro evaluation of the Th1 response of PBMCs and their potential use as a vaccine against *Leishmania infantum* infection. *Parasites & Vectors, 10*, 1-14.

Ren, H. J., Liu, R. D., Wang, Z. Q., & Cui, J. (2013). Construction and use of a Trichinella spiralis phage display library to identify the interactions between parasite and host enterocytes. *Parasitology Research, 112*, 1857-1863.

Roehnisch, T., Then, C., Nagel, W., Blumenthal, C., Braciak, T., Donzeau, M. Böhm, T., Bourquin, C., Oduncu, F. (2013). Chemically linked phage idiotype vaccination in the murine B cell lymphoma 1 model. *Journal of Translational Medicine, 11*, 1-7.

Sartorius, R., D'Apice, L., Prisco, A., & De Berardinis, P. (2019). Arming filamentous bacteriophage, a nature-made nanoparticle, for new vaccine and immunotherapeutic strategies. *Pharmaceutics, 11*(9), 437.

Seker, U. O. S., & Demir, H. V. (2011). Material binding peptides for nanotechnology. *Molecules, 16*(2), 1426-1451.

Smirnov, D. D., Kapustin, A. V., Yakimova, E. A., Savinov, V. A., & Laishevtsev, A. I. (2020). Perspectives of the use of bacteriophages in agriculture, food and processing industries. In IOP conference series: *Earth and Environmental Science, 548*, 072058.

Squires, R. A. (2018). Bacteriophage therapy for management of bacterial infections in veterinary practice: what was once old is new again. *New Zealand Veterinary Journal, 66*(5), 229-235.

Stachurska, X., Cendrowski, K., Pachnowska, K., Piegat, A., Mijowska, E., & Nawrotek, P. (2022). Nanoparticles influence lytic phage T4-like performance *in vitro*. *International Journal of Molecular Sciences, 23*(13), 7179.

Staquicini, D. T., Barbu, E. M., Zemans, R. L., Dray, B. K. Staquicini, E. I., Dogra, P., Cardó-Vila, M., Miranti, C. K., Baze, W. B., Villa L. L., Kalil, J., Sharma, G., Prossnitz, E. R., Wang, Z., Cristini, V., Sidman, R. L., Berman, A. R., Panettieri, R., A., Tuder, R. M., Pasqualini, R., Arap, W. (2021). Targeted phage display-based pulmonary vaccination in mice and non-human primates. *Med, 2*(3), 321-342.

Sukumaran, A.T., Nannapaneni, R., Kiess, A., Sharma, C.S. (2016). Reduction of Salmonella on chicken breast fillets stored under aerobic or modified atmosphere packaging by the application of lytic bacteriophage preparation SalmoFreshTM. *Poultry Science, 95*, 668–675

Sulakvelidze, A., Alavidze, Z., & Morris Jr, J. G. (2001). Bacteriophage therapy. Antimicrobial Agents and Chemotherapy, 45(3), 649-659.

Summers, W. C. (2001). Bacteriophage therapy. *Annual Review of Microbiology, 55*(1), 437-451.

Tan, X., Chen, H., Zhang, M., Zhao, Y., Jiang, Y., Liu, X., Huang, W., Ma, Y. (2021). Clinical experience of personalized phage therapy against carbapenem-resistant *Acinetobacter baumannii* lung infection in a patient with chronic obstructive pulmonary disease. *Frontiers in Cellular and Infection Microbiology*, *11*, 631585.

Ustaçelebi Ş., Okuyan M., Odabaşıoğlu N. (1968). T4 R mutant bakteriyofajları ile serolojik deneyler ve normal insan serumlarının faj enfeksiyonunda stimülan etkisi. *Mikrobiyoloji Bülteni, 4*, 131-141.

Verbeken, G., Pirnay, J., Lavigne, R., Jennes, S., Vos, D. D., Casteels, M., … & Huys, I. (2014). Call for a dedicated european legal framework for bacteriophage therapy. *Archivum Immunologiae Et Therapiae Experimentalis, 62*(2), 117-129.

Waturangi, D. E., Kasriady, C. P., Guntama, G., Sahulata, A. M., Lestari, D., & Magdalena, S. (2021). Application of bacteriophage as food preservative to control enteropathogenic *Escherichia coli* (EPEC). *BMC Research Notes, 14*(1), 336.

Wittebole, X., de Roock, S., Opal, S.M., (2013). A historical overview of bacteriophage therapy as an alternative to antibiotics for the treatment of bacterial pathogens. *Virulence 4*(8), 1–10

Wommack, K. E., & Colwell, R. R. (2000). Virioplankton: viruses in aquatic ecosystems. *Microbiology and Molecular Biology Reviews*, *64*(1), 69-114.

Wu, C. H., Liu, I. J., Lu, R. M., & Wu, H. C. (2016). Advancement and applications of peptide phage display technology in biomedical science. *Journal of Biomedical Science, 23*, 1-14.

Zalewska-Piątek, B. (2023). Phage therapy—challenges, opportunities and future prospects. *Pharmaceuticals, 16*(12), 1638.

APLICATION OF BIOTECHNOLOGICAL APPROACHES IN ELECTRONIC DEVICE APPLICATIONS

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Introduction

The convergence of biology and electronics has facilitated substantial progress in the creation of innovative electronic devices, resulting in the emergence of bioelectronics. This interdisciplinary domain utilises biological molecules, cells, and tissues to interface with electronic components, facilitating novel applications from medical diagnostics to energy harvesting. Biotechnological approaches have transformed the design and performance of electronic devices by using the intrinsic capabilities of biological systems, including self-assembly, sensing, and adaptation.

A key area where biotechnology plays a critical role is in the development of biosensors. Biosensors combine biological recognition elements, such as enzymes or antibodies, with transducers that convert biological interactions into measurable electronic signals. These devices have found widespread use in healthcare for monitoring glucose levels in diabetic patients and detecting pathogens in clinical diagnostics (Turner, 2013).

Furthermore, bio-inspired materials, like nanocellulose and protein-based conductors, have been investigated as substitutes for conventional semiconductor materials. These materials provide biocompatibility, flexibility, and environmental sustainability, rendering them appropriate for use in wearable electronics and implantable medical devices (Liu et al., 2017). Conductive polymers originating from biological sources are being investigated for their possible application in organic electronic devices, providing enhanced flexibility and biodegradability (Moya et al., 2021).

Molecular electronics has also brought new paradigms to data processing and storage with the introduction of biological molecules like DNA. According to Guo and Tao (2004), DNA-based circuits and nanowires are made possible by the highly ordered and predictable structure of DNA, which makes it possible to precisely assemble electronic components on a nanoscale. The aforementioned developments indicate that biotechnology has promise in offering scalable solutions for the upcoming generation of electrical devices with biointegration.

As research progresses in exploring the molecular mechanisms of biological systems, the collaboration between biotechnology and electronics is anticipated to propel advancements in areas such as neuroprosthetics, bioelectronic medicine, and renewable energy devices (Cui & Lieber, 2001).

The capacity to leverage the distinctive characteristics of biological systems is expected to result in innovative hybrid devices that function at the intersection of biological and digital realms.

Moreover, bio-inspired materials, like nanocellulose and protein-based conductors, have been investigated as substitutes for conventional semiconductor materials. These materials provide biocompatibility, flexibility, and environmental sustainability, rendering them appropriate for use in wearable electronics and implantable medical devices (Liu et al., 2017). Conductive polymers originating from biological sources are being investigated for their possible application in organic electronic devices, providing enhanced flexibility and biodegradability (Moya et al., 2021).

The integration of living molecules like DNA in molecular electronics has established novel paradigms in data storage and processing. The highly organised and predictable characteristics of DNA facilitate the meticulous organisation of electronic components at the nanoscale, resulting in the development of DNA-based circuits and nanowires (Guo & Tao, 2004). These breakthroughs illustrate biotechnology's capacity to deliver scalable solutions for the forthcoming generation of bio-integrated electronic gadgets.

Another promising avenue is the use of nanoparticle-doped thin films in the design of hybrid diodes. Polymeric or metal oxide thin films frequently include nanoparticles like gold (Au) or silver (Ag) to enhance the electrical and optical characteristics of these devices. Organic light-emitting diodes (OLEDs), photodetectors, and solar cells have effectively utilised nanoparticle-doped thin films. The incorporation of metal nanoparticles in polymer-based diodes augments charge transport efficiency and enhances overall device performance (Cui & Lieber, 2001). Moreover, interface engineering is essential for optimising the electrical characteristics of these films, hence enhancing device performance and longevity (Guo & Tao, 2004).

Recent studies indicate that nanoparticle-assisted Schottky diodes exhibit enhanced rectifying behavior and increased sensitivity in photodetector applications. Hybrid structures leverage plasmonic effects from metallic nanoparticles, enhancing light absorption and electron mobility at the interface of the thin film and substrate (Moya et al., 2021). Additionally, these devices can be engineered to function at low voltages, enhancing their efficiency for portable and wearable electronics (Liu et al., 2017). An emerging area of interest is the use of bacterially synthesized nanoparticles in

electronic devices. Bacterial systems have been utilised for the eco-friendly manufacture of metallic nanoparticles, including silver (Ag) and gold (Au), via bioreduction techniques. Biologically synthesised nanoparticles are being extensively investigated for their use in thin film transistors (TFTs), diodes, and biosensors owing to their regulated size distribution and biocompatibility. The bacterial reduction of metal ions provides a cost-effective and environmentally sustainable method for synthesising nanoparticles with customised characteristics for electrical applications (Narayanan & Sakthivel, 2010). Silver nanoparticles produced via bacterial reduction have demonstrated potential in improving the conductivity and sensitivity of Schottky barrier diodes and organic solar cells (Ramanathan et al., 2019). This eco-friendly approach to nanoparticle creation minimises the chemical waste commonly linked to nanoparticle synthesis and facilitates the incorporation of biologically sourced components in biocompatible electronics.

Current investigations into the molecular mechanisms of biological systems is anticipated to enhance the collaboration between biotechnology and electronics, leading to significant advancements in neuroprosthetics, bioelectronic medicine, and renewable energy devices (Cui & Lieber, 2001). The capacity to exploit the distinctive characteristics of biological systems is expected to result in novel hybrid technologies functioning at the intersection of biological and digital realms.

The advancements in biotechnological methods, especially the microbial green synthesis of nanoparticles, have attracted considerable interest due to their potential to transform electronic devices. In 2019, Çakıcı et al. investigated the synthesis of ZnSe and CuSe nanoparticles using microbial methods, analysing their electrical properties in Au/ZnSe/p-Si/Al and Au/CuSe/p-Si/Al configurations (Çakıcı et al., 2019). In the same year, they investigated the production and characterisation of GO nanoparticles, demonstrating their application in Ag/GO/p-Si devices (Çakıcı, 2019). In 2020, Çakıcı conducted a study on GO nanoparticles and their integration into Au/GO/p-Si/Al diodes, highlighting the efficacy of green synthesis methods (Çakıcı, 2020). In 2023, significant advancements were made in the field as Çakıcı et al. synthesised bacterial MgSe complex nanoparticles and characterised Ag/MgSe/p-Si heterostructures under various conditions (Çakıcı et al., 2023). The surface activation of Si-based Schottky diodes was enhanced through the use of bacterial biosynthesised AgInSe2 trimetallic alloy nanoparticles, resulting in significant negative capacitance and improved

electro-dielectric performance (Çakıcı et al., 2023). These studies highlight the significant potential of biotechnologically synthesised materials in semiconductor technologies, consistent with the principles of green chemistry and sustainable development.

Bacterial Synthesis of Compound and Doped Semiconductor Nanoparticles

Nanotechnology has achieved significant momentum in recent years, especially via the eco-friendly creation of nanoparticles by biological techniques. This method utilises the distinctive characteristics of biological organisms, rendering it a promising pathway for the synthesis of metallic nanoparticles.

The eco-friendly synthesis of metallic nanoparticles employs many biological organisms including plants, bacteria, fungi, and algae. These biological approaches are beneficial because to their environmental sustainability, financial efficiency, and ease of implementation. Conversely, conventional physical and chemical techniques for nanoparticle synthesis frequently utilise hazardous substances, leading to environmentally detrimental consequences and requiring costly, high-vacuum apparatus (Hulkoti & Taranath, 2014; Shah et al., 2015). Moreover, researchers utilize several synthesis approaches, encompassing chemical, physical, and biological procedures, to generate nanoparticles with precise compositions, morphologies, and dimensions. These attributes profoundly affect the material qualities and their utilizations.

A wide range of metallic nanomaterials, including magnesium, gold, graphene, selenium, copper, zinc, silver, iron, titanium, and cadmium, is being manufactured for varied uses in industries such as electronics, textiles, energy, computing, medicine, food, optics, and aerospace. Certain microorganisms exhibit tolerance to elevated chemical concentrations, rendering them especially suitable for bacterial synthesis techniques. The utilisation of microbes for nanoparticle synthesis can be efficiently scaled for extensive applications (Khanna et al., 2007; Rasool & Hemalatha, 2017). Copper nanoparticles demonstrate a diverse array of properties, encompassing optical, catalytic, antibacterial, antifungal, and anticancer actions (Malik et al., 1999; Oremland et al., 2004).

Green Synthesis of Selenide-Based Nanoparticles

The green synthesis of selenide-based nanoparticles has attracted considerable interest in recent years because of its environmentally benign, economical, and non-toxic benefits compared to traditional chemical and physical processes. Selenium, a semiconductor with adjustable electrical and optical characteristics, has significant potential in diverse applications including photovoltaics, optoelectronics, and biomedical devices. Green synthesis approaches, employing biological entities including plants, bacteria, fungi, and algae, provide an environmentally friendly strategy for nanoparticle creation.

Recent studies have shown that bacteria such as Bacillus cereus and Pseudomonas aeruginosa are effective in reducing selenium salts into selenium nanoparticles, a process that takes place under ambient conditions, avoiding toxic byproducts and the need for complex equipment (Shah et al., 2015; Rasool & Hemalatha, 2017). In addition, fungi such as Aspergillus terreus have been utilized for the biosynthesis of selenium nanoparticles, which has been found to yield nanoparticles with excellent uniformity in size and shape (Hulkoti & Taranath, 2014).

The microbial synthesis of selenium nanoparticles is not only limited to elemental selenium. Various studies have reported the production of selenide-based compounds such as zinc selenide (ZnSe) and cadmium selenide (CdSe) nanoparticles using microorganisms. For example, Khanna et al. (2007) synthesized copper selenide (CuSe) nanoparticles through microbial methods, showcasing the versatility of biological systems in producing complex selenide structures. Similarly, Malik et al. (1999) demonstrated the biosynthesis of copper indium diselenide ($CuInSe_2$) nanoparticles, which are known for their applications in thin-film photovoltaics and electronic devices.

Biogenic synthesis techniques have several benefits, such as meticulous regulation of particle dimensions, crystallinity, and shape. Oremland et al. (2004) demonstrated that selenium-respiring bacteria can generate selenium nanospheres exhibiting unique structural and spectrum characteristics. The morphology and dimensions of these nanoparticles are significantly influenced by the organism employed and the ambient parameters during synthesis, including pH and temperature. These parameters can be optimized to provide nanoparticles with specific attributes, which is crucial for applications in nanoelectronics and catalysis.

Besides microbiological techniques, plant extracts have been employed for the eco-friendly synthesis of selenide nanoparticles. The extract of *Cymbopogon citratus* (lemongrass) has been effectively utilized to synthesize selenium nanoparticles characterized by excellent purity and uniform shape. The plant-mediated synthesis presents a straightforward and economical alternative, circumventing the necessity for harsh chemical reducing agents (Li et al., 2013).

Selenide nanoparticles, particularly as quantum dots such as CdSe, exhibit significant potential in biological imaging and photothermal therapy owing to their size-tunable optical characteristics (Wang et al., 2017). These nanoparticles can be synthesized under mild circumstances, rendering green synthesis approaches a compelling choice for biomedical applications. Selenium nanoparticles demonstrate antimicrobial characteristics, advantageous for applications such as antibacterial coatings and medication delivery systems (Shah et al., 2015).

The scalability of green synthesis processes is a further factor that has attracted interest. Researchers have shown the viability of enhancing microbial production of nanoparticles for industrial use, minimising environmental impact while preserving the quality of the produced nanoparticles (Hulkoti & Taranath, 2014). The scalability, along with the reproducibility and environmentally sustainable characteristics of the process, renders green synthesis a promising method for the large-scale manufacture of selenide nanoparticles.

The green synthesis of selenide-based nanoparticles, employing both microbial and plant-mediated processes, provides a sustainable alternative to conventional techniques. These biosynthesised nanoparticles possess distinctive features that are highly sought after across various applications, including electronics and medicine. Ongoing research suggests that green synthesis methods will significantly influence the future of nanotechnology.

The synthesis of binary and ternary semiconductor nanoparticles based on compound selenide using the bacterial synthesis method and their use as circuit elements have been introduced to the literature for the first time by us (Çakıcı 2019; Çakıcı et al 2019; Çakıcı et al 2020; Çakıcı et al 2021; Çakıcı et al 2022; Çakıcı et al 2023). Figure 1 shows TEM micrograf of ZnSe, CuSe and CuZnSe nanoparticles at 100 nm scale. The sizes of nanoparticles vary

approximately in the range of 20-50 nm.

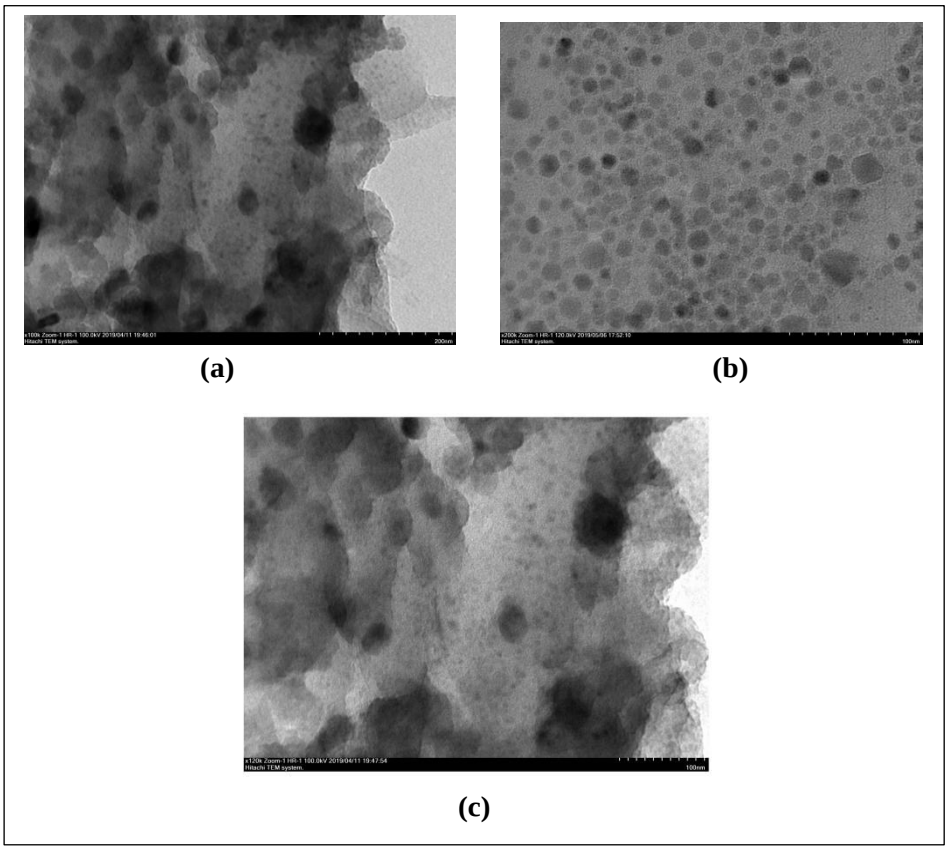

Figure 1. TEM micrograft of (a) ZnSe, (b) CuSe, and (c) CuZnSe nanoparticles at 100 nm

Graphene-Based Nanocomposite

Graphene oxide nanoparticles (GONPs) exhibit potential for incorporation into heavy metal detection sensors (Li et al., 2013b), electrodes (Chen et al., 2012), and diverse biomedical applications (Li et al., 2013a). The integration of copper into graphene composites has yielded remarkable mechanical features, such as elevated tensile and compressive strengths, enhanced hardness, and superior electrical conductivity (Wang et al., 2017). This attribute facilitates a reduced thermal energy expenditure during the annealing of nanoparticle-based films. Annealing is a crucial process in the production of high-quality films, even with vacuum deposition methods (Khanna et al., 2007; Hulkoti & Taranath, 2014).

Metal-doped GO nanoparticles find applications in many electronic and optical fields. GO: Cu, GO:Ag, GO:Cu:In, and GO:Se nanoparticles have been introduced to the literature for the first time by us using the bacterial synthesis method (Çakıcı et. al., 2019; Çakıcı, 2019; Çakıcı, 2020) Figure 2 shows the TEM micrographs of GO:Cu, GO:Ag, GO:Cu:In, and GO:Se nanoparticles at 100 nm.

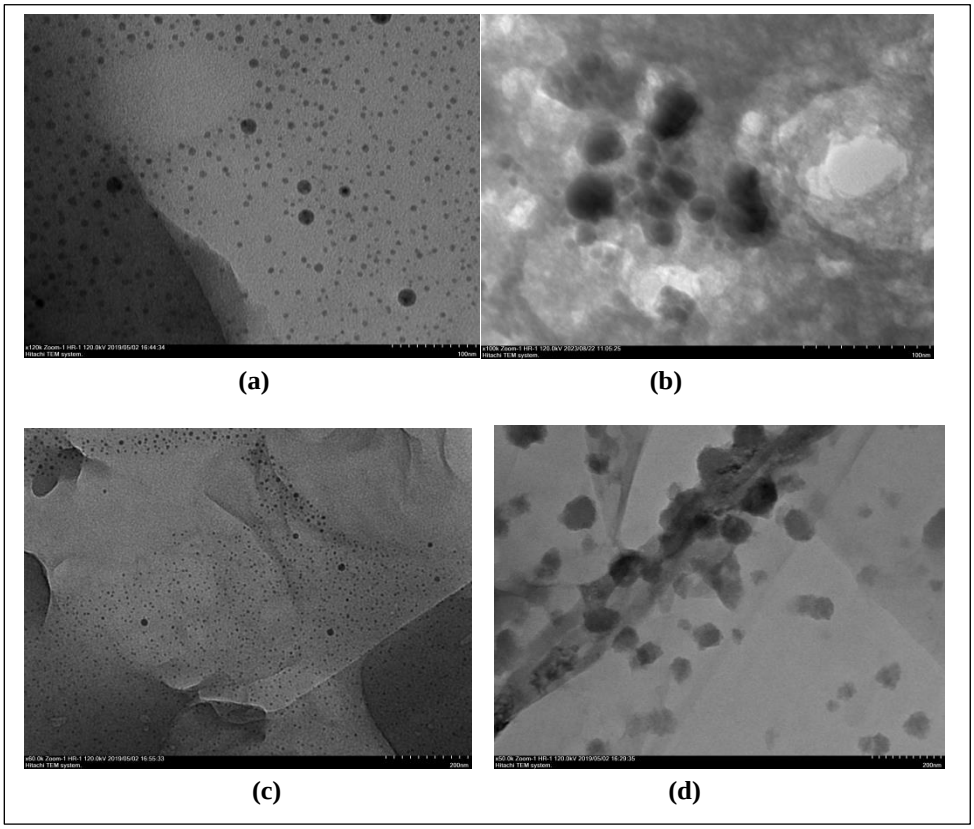

Figure 2. TEM micrograft of (a) GO:Cu, (b) GO:Ag, and (c) GO: Cu :In (d) GO: Se nanoparticles at 100 nm

Conclusion

This study indicates that the green production of nanoparticles, particularly selenide-based nanomaterials, has considerable benefits compared to conventional chemical and physical approaches. The utilisation of biological organisms, including bacteria, fungus, and plants, in nanoparticle

synthesis offers a sustainable, economical, and environmentally friendly strategy, while also producing nanoparticles with distinctive features that are challenging to get by traditional techniques. Selenide-based nanoparticles, such as ZnSe, CuSe, and CdSe, have exhibited potential uses across several domains, including semiconductors, sensors, biomedical devices, and photovoltaics.

Among these applications, semiconductor nanoparticles, such as those used in gas sensor technologies, have gained attention due to their high sensitivity, stability, and versatility. Nanoparticles like ZnO, SnO2, and CuSe exhibit remarkable properties, making them highly effective in detecting gases such as CO, NO2, and H_2S. For instance, ZnSe and CuSe nanoparticles, synthesized through green methods, can be integrated into gas sensors to detect harmful emissions with enhanced precision. Their high surface-area-to-volume ratio and tunable electronic properties make them ideal candidates for applications in environmental monitoring and industrial safety.

Moreover, the use of green synthesis in producing semiconductor materials for gas sensors reduces the environmental impact of sensor fabrication and improves sustainability in nanomaterials research. The ability to synthesize these nanoparticles in a controlled manner allows researchers to tailor sensor performance, improving response time, selectivity, and overall efficiency. Future advancements in this field will likely focus on expanding the range of detectable gases and enhancing the sensitivity of semiconductor nanoparticles through biogenic and eco-friendly methods.

REFERENCES

Çakıcı, T. (2019). Production and characterization of GO: Se nanoparticles produced by biosynthesis method and current-voltage characteristics of the Ag/GO: Se/p-Si device developed by using GO: Se nanoparticles. *Avrupa Bilim ve Teknoloji Dergisi, (17),* 1367-1374.

Çakıcı, T. (2020). Investigation of GO: Cu nanoparticles produced by green synthesization method and fabrication of Au/GO: Cu/p-Si/al diode. *Journal of Molecular Structure, 1199*, 126945.

Çakıcı, T., Ajjaq, A., Çağırtekin, A. O., Barin, Ö., Özdal, M., & Acar, S. (2023). Surface activation of Si-based Schottky diodes by bacterial biosynthesized AgInSe2 trimetallic alloy nanoparticles with evidenced negative capacitance and enhanced electro-dielectric performance. *Applied Surface Science, 631*, 157522.

Çakıcı, T., Özdal, M., Kundakcı, M., & Kayalı, R. (2019). ZnSe and CuSe NP's by microbial green synthesis method and comparison of IV characteristics of Au/ZnSe/p-Si/Al and Au/CuSe/p-Si/Al structures. *Materials Science in Semiconductor Processing, 103*, 104610.

Çakıcı, T., Özdal, Ö. G., Almousa, N., Yıldız, F., Perişanoğlu, E. K., Khalil, H., ... & Zakaly, H. M. (2023). Bacterial MgSe complex nanoparticle synthesis and electrical characterization of fabricated Ag/MgSe/p-Si hetero-structure under dark and illumination. *Heliyon, 9*(11).

Cui, Y., & Lieber, C. M. (2001). Functional nanoscale electronic devices assembled using silicon nanowire building blocks. Science, 291(5505), 851-853.

Guo, X., & Tao, N. (2004). Single-molecule conductance of conjugated molecular systems. *Nature Nanotechnology, 1*, 163-167.

Hulkoti, N. I., & Taranath, T. C. (2014). Biosynthesis of nanoparticles using microbes—a review. *Colloids and Surfaces B: Biointerfaces, 121*, 474-483.

Khanna, P. K., Gaikwad, S., Adhyapak, P. V., Singh, N., & Marimuthu, R. (2007). Synthesis and characterization of copper nanoparticles. *Materials Letters, 61*(25), 4711-4714.

Liu, Y., Pharr, M., & Salvatore, G. A. (2017). Lab-on-skin: A review of flexible and stretchable electronics for wearable health monitoring. *ACS Nano, 11*(10), 9614-9635.

Malik, M. A., O'Brien, P., & Revaprasadu, N. (1999). A novel route for the preparation of CuSe and $CuInSe_2$ nanoparticles. *Advanced Materials, 11*(17), 1441-1444.

Moya, A., Gabriel, G., Villa, R., & Llobet, E. (2021). Organic electronics in chemical and biological sensing: The next generation of bioelectronic devices. *Chemical Reviews, 121*(5), 2601-2647.

Narayanan, K. B., & Sakthivel, N. (2010). Biological synthesis of metal nanoparticles by microbes. *Advances in Colloid and Interface Science, 156*(1-2), 1-13.

Oremland, R. S., Herbel, M. J., Switzer-Blum, J., Langley, S., Beveridge, T. J., Ajayan, P. M., ... & Curran, S. (2004). Structural and spectral features of selenium nanospheres produced by Se-respiring bacteria. *Applied and Environmental Microbiology, 70*(1), 52-60.

Ramanathan, R., O'Mullane, A. P., Parikh, R. Y., Smooker, P. M., Bhargava, S. K., & Bansal, V. (2019). Bacterial synthesis of gold nanoparticles: Electrochemical and optical properties. *Journal of the American Chemical Society, 131*(7), 2770–2771.

Rasool, U., & Hemalatha, S. (2017). Marine endophytic actinomycetes assisted synthesis of copper nanoparticles (CuNPs): Characterization and antibacterial efficacy against human pathogens. *Materials Letters, 194*, 176-180.

Shah, M., Fawcett, D., Sharma, S., Tripathy, S. K., & Poinern, G. E. J. (2015). Green synthesis of metallic nanoparticles via biological entities. *Materials, 8*(11), 7278-7308.

Turner, A. P. F. (2013). Biosensors: Sense and sensibility. *Chemical Society Reviews, 42*(8), 3184–3196.

Wang, L., Yang, Z., Cui, Y., Wei, B., Xu, S., Sheng, J., ... & Fei, W. (2017). Graphene-copper composite with micro-layered grains and ultrahigh strength. *Scientific Reports, 7*, 41896.