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ORIGINAL PAPER

Bacterial chitinase biochemical properties, immobilization on zinc oxide (ZnO) nanoparticle and its effect on *Sitophilus zeamais* as a potential insecticide

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Abstract
It has been planned to minimize the yield and quality impairment of the seed corn, which is strategically important in the world, by pests under storage conditions with a biological product produced with a biotechnological approach. In this context, the present study aimed to control the maize weevil *Sitophilus zeamais*, known as a warehouse pest, using a nanoformulation. In the study, the chitinase enzyme from *Lactobacillus coryniformis* was purified first using ammonium sulfate precipitation and then by using the HiTrap Capto DEAE column, and the molecular mass of the purified enzyme was determined to be ~ 33 kDa, and the optimum pH and the values as pH 6.0 and 65–75 °C, respectively. Five different doses of nanoformulation (2, 4, 6, 8 and 10 mg/L) were applied to corn grains by the spraying method with three repetitions so that the insect can ingest the formulation through feeding. The effects of the applications on the death rate and mean time of death of *Sitophilus zeamais* were determined. According to these findings, it was concluded that the best practice was nanoformulation with 6 mg/L, considering both the mortality rate (100%) and the average death time (2.4 days). Chitinase from *L. coryniformis* is a promising candidate for corn lice control and management.

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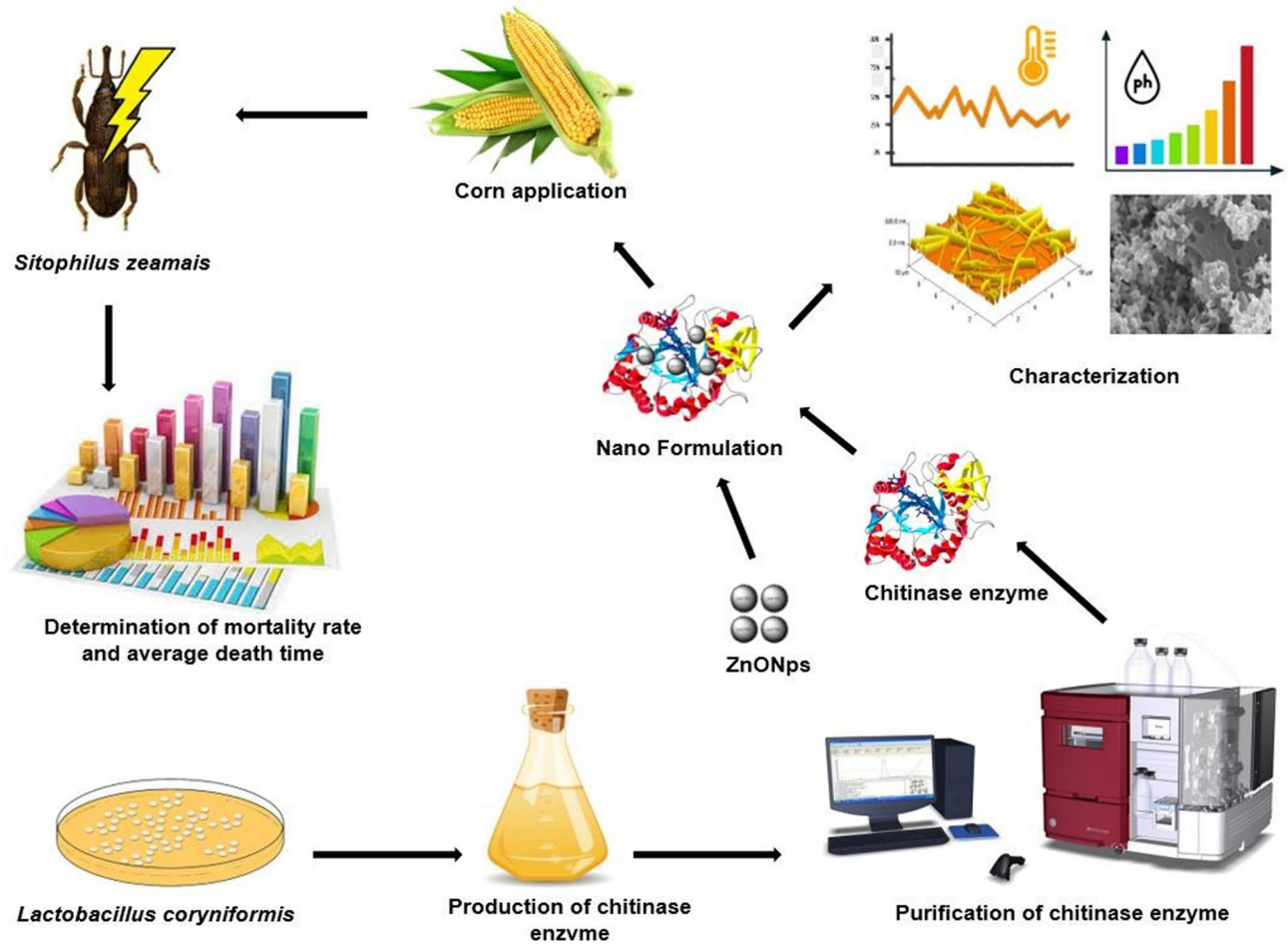
Graphic abstract



Keywords *Lactobacillus coryniformis* · Chitinase · Zinc oxide · *Sitophilus zeamais* · Corn

Introduction

One of the most important objectives of biotechnological methods used in agriculture is to disrupt the normal properties of the pest and minimize the harm level using some natural or artificial substances that are effective on the biology, physiology, and behavior of pest species. For future generations, a productive and ecologically convenient farming model that grows healthy food, while maintaining environmental integrity, is a major focus of interest (Heap 2014).

It is of great importance to keep products of strategic importance, such as corn, in stock. After the production stage, corn must be stored until planted. However, in this process, some warehouse pests cause great losses in the product in terms of quality and weight. Pests in stored cereals cause product losses of up to 10% (Ertürk et al. 2017). One of these pests, *Sitophilus zeamais* (*S. zeamais*) is one of the species causing the highest damage to the stored cereals (Ojo and Omolove 2016). It has been reported that the seed weight loss for corn stored as a result of the damage of *S. zeamais* is between 20 and 90% (Muzemu et al. 2013).

A wide variety of chemicals are used to control pests in agricultural production. Regular contact of people with a hight amount of pesticides can cause various diseases including cancer, sudden kidney failure, respiratory syndrome, and cardiovascular disorders (Xavier et al. 2004). As a result of its use, it causes the ecosystem to become unstable and increase the resistance in pests against insecticides (Patil and Jadhav 2015). Regarding an obvious need for an alternative, biopesticides offer an alternative to synthetic pesticides due to low toxicity, low environmental pollution, and other advantages (Kumar et al. 2021). Organophosphate (OP) insecticides such as malathion have

been widely used as grain protectants to protect stored products in many countries throughout the world (Boyer et al. 2012).

Chitinases are hydrolytic enzymes responsible for the degradation of chitin. Based on the amino acid sequence, chitinolytic enzymes are classified in the CAZY database (Lombard et al. 2014) into the glycosyl hydrolase (GH) families 18, 19, and 20. GH18 includes bacterial, fungal and animal chitinases, while GH19 consists of plant chitinases and some Streptomyces chitinases. (Hart et al. 1995). GH20 consists of Vibrio harveyi, Dictyostelium discoideum and Beta-N-Acetyl hexosaminidase from humans (Dahiya et al. 2006).

Chitinases are used in biocontrol against various fungal pathogens and insects to reduce the use of synthetic fungicides and insecticides. They can serve as sustainable and environmentally friendly alternatives to pesticides. Chitinase-producing microorganisms are a potential alternative to chemicals as part of the ecosystem (Singh et al. 2021). Interest in chitin-degrading enzymes has increased over the years and microbial chitinases have been the most attractive and promising candidates for the control of plant pests (fungi and insects). Microbial chitinases have significant application potential in various agricultural and biotechnological industries, especially in biological control (Pasin et al. 2021).

The reason for the use of Lactic Acid Bacteria (LAB), which are included in the GRAS (Generally Recognized as Safe) list, in the production of chitinase (E.C 3.2.1.14) is that it can be used safely in health, environmental and industrial applications. Since these enzymes break down chitins, if they are applied to the insect or injected into the intestine of the insect larvae, it is thought that the larvae cannot feed and the application will lead to significant damage to the peritrophic membrane structure (Binod et al. 2007).

Except for the isolation of enzymes from natural sources, immobilization techniques should be used to increase their efficacy. With the immobilization process, it is possible to provide the economic usability of enzymes (Kurnaz-Yetim et al. 2019). Enzyme immobilization techniques can be carried out in various ways, such as: entrapment of the enzyme in the gel, enzyme encapsulation, covalent binding of the enzyme to the support material, adsorption of the enzyme on the surface of the solid. Enzyme immobilization technique that has been developed recently is by means of adsorption on the surface of solids or membranes. Enzymes can be more durable or less easily damaged when immobilized in a membrane, where the enzyme is separated in the pores of the membrane (Supernak-Marczewska and Zielinski 2020; Hamsina et al. 2021).

Nanotechnology and nanoscale materials have attracted a remarkable interest in basic and applied sciences including health, energy, environment, and agriculture for the past few years due to their unique structure and physicochemical properties. Nanotechnology is an application developed in the field of pest management of insects (Athanassiou et al. 2017). Zinc oxide nanoparticles (ZnO NPs) are widely used due to their antibacterial, antifungal, UV filtering properties, and high catalytic and photochemical activities (Chen et al. 2017). Chitinase enzyme immobilized to chitosan shows higher hydrolytic activity than free enzyme (Kidibule et al. 2021). According to the results of enzyme activity measurement under different pH and temperature conditions in various studies, it was determined that the immobilized chitinase enzyme showed better activity and stability than the free enzyme (Sosnowska et al. 2018; Mohammadzadeh et al. 2020; Gomaa 2021).

In the present study, the chitinase enzyme was purified from *L. coryniformis* strain 3N11, immobilized to zinc oxide (ZnO) nanoparticles and a nanoformulation was obtained. The efficacy of the formulation created as a bioinsecticide against storage pest *S. zeamais*, which causes significant yield losses in agriculture, was determined. It was concluded that the obtained formulation could damage the insect's intestine and integrating parts containing chitin and could therefore be used in insecticide control.

Materials and method

Bacterial strain

L. coryniformis strain 3N11 has been isolated from traditional cheese and described in NCBI (accession number: MH121153). It is protected under the code of 3N11 in the N. DIKBAŞ culture collection at Atatürk University, Faculty of Agriculture, Department of Agricultural Biotechnology.

Determination of *L. coryniformis* chitinase enzyme activity

Lactobacillus coryniformis strain 3N11 was taken using a loop and inoculated into MRS agar by the streak plate method. It was left to incubate for 48 h at 35 °C, then inoculated into MRS Broth, the bacteria were transferred into the liquid culture medium and incubated at 35 °C for 48 h in the incubator. The bacterial concentration was measured using a turbidimeter and adjusted to 10^8 CFU/mL (Magnusson and Schnürer 2001). The enzyme activity of the bacterium was measured every 6 h to find the development range in which *L. coryniformis* strain 3N11 showed the highest activity, and enzyme purification processes were continued from the development interval in which the measurements showed the highest activity (Ong et al. 2017).

Purification of chitinase enzyme

The bacteria set at 10^8 CFU/mL in the turbidimeter were centrifuged at + 4 °C for 10 min at 9000 rpm, the supernatant and the pellet were separated. For the chitinase enzyme, the enzyme activity of the precipitates was measured in each ammonium sulfate saturation range (0–80%) (Sarıbuğa et al. 2014). In the AKTA Avant device, salts were removed using a desalting column. Akta-Avant (HiTrap Capto DEAE Colon) device was used to purify the chitinase enzyme produced by *L. coryniformis* strain 3N11 and an anion exchange chromatography (AEC) procedure was performed (Xi et al. 2018). Chitinase enzyme (SIGMA-CAS Number: 9001-s06-3) was used as standard.

Measurement of enzyme activity

The activity of the chitinase enzyme was determined for the precipitates obtained in each range and for the supernatants obtained after precipitation. Thus, the chitinase activity range (% 0–80) was determined. 200 µL of enzyme and 500 µL of substrate solution were reacted at 37 °C for 30 min. After adding 750 µL of coloring solution (3,5-Dinitrosalicylic acid) to the tubes, the reaction was stopped by keeping it in a water bath set at 80 °C for 10 min. 1300 µL of distilled distilled water was placed on the samples removed from the water bath. Measurement was made in the spectrophotometer at 540 nm and the activity was determined (Vaidya et al. 2003).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The electrophoresis was carried out in tris–glycine-SDS running buffer at 20 mA/gel constant current for 90 min. by using 4% stacking and 10% resolving gel (Laemmli 1970). After completion of running, proteins were stained with oriole (BioRad) and electrophoretograms were analyzed with gel analysis software.

Determination of optimum pH and temperature value of enzyme

Substrate solutions with 0.05% chitin were prepared using buffers with different pH values (pH 2.0–11.0). Sodium acetate (pH 2.0–3.0), Sodium citrate (pH 4.0–6.0), Tris (pH 7.0–9.0), and Sodium carbonate (pH 10.0–11.0) buffers were used. It was measured at 540 nm on a spectrophotometer. The range of maximum activity of the enzyme was determined (Sarıbuğa et al. 2014). Temperatures of 15, 25, 35, 45, 65, 75, 85 °C was chosen to determine the temperature at which the enzyme exhibits optimum activity. The temperatures of the reaction were adjusted in the water bath. Measurements were made every 30 min for 3 h to determine the temperature stability (Sarıbuğa et al. 2014).

Immobilization of chitinase to zinc oxide (ZnO) nanoparticles

The immobilization of the chitinase enzyme on zinc oxide nanoparticle (Zinc oxide nanopowder, < 50 nm particle size (BET), > 97% can number 1314–13-2) was carried out by adsorption in the batch system by modifying the Preety and Hooda (2018) method. For this purpose, 3 mL of ZnO nanoparticles prepared as 100 mg/L and 1 mL of chitinase enzyme were taken and mixed in a magnetic stirrer at 100 rpm for 2 h to immobilize the enzyme to the nanoparticle.

The effect of pH and temperature on immobilized (ZnO) chitinase enzyme

Sodium acetate (pH 2.0–3.0), Sodium citrate (pH 4.0–6.0), Tris (pH 7.0–9.0), and Sodium carbonate (pH 10.0–11.0) buffers were used to determine the optimum pH. The samples were measured at 540 nm on a spectrophotometer. The range of maximum activity of the enzyme was determined (Sarıbuğa et al. 2014; Sachdeva and Hooda 2015). To determine the thermal stability, the enzyme activity was measured by exposing the immobilized enzyme to temperatures between 15 and 85 °C at 10 °C intervals. The temperature value at which the immobilized enzyme shows optimum activity was determined (Prasad and Palanivelu 2014).

Characterization of support bound chitinase

All measurements were made in DAYTAM (Eastern Anatolia High Technology Application and Research Center).

FTIR (fourier transform infrared spectrophotometer) analysis

FTIR spectra of ZnO Nanoparticle and Chitinase enzyme bound to ZnO Nanoparticle were obtained using FTIR spectrophotometer (Varian FTS 7000, ADB). The samples (0.1 g) were mixed homogeneously with KBr (0.1 g) and pelleted and the FTIR spectrum was taken (Preety and Hooda 2018).

XRD (x-ray diffraction) analysis

The XRD pattern of the chitinase enzyme was taken using a CuKα beam source Rigaku D-max 2200 X-ray diffractometer with a wavelength of 1.5405 Å, with a scanning speed of 4 degrees/min and a scanning step of 0.01 degrees, with an angle of $2\theta = 10–80°$ (Preety and Hooda 2018).

SEM (scanning electron microscope) analysis

SEM images were obtained with Phillips XL-305 FEG device in order to evaluate the surface morphology and structure of the ZnO Nanoparticle and the Chitinase enzyme immobilized to the ZnO Nanoparticle (Kasthuri et al. 2007).

AFM (atomic force microscope) analysis

In order to evaluate the structure of the immobilized enzyme, AFM images were obtained with the Hitachi 5100 N device (Preety and Hooda 2017).

Production and application of nanoformulation doses

Adult *S. zeamais* individuals were obtained from Atatürk University, Faculty of Agriculture, Department of Plant Protection (recently completed the pupa period). The trial was carried out according to the randomized block design according to the Pests Preservative Standard Drug Trial Method of the Ministry of Food, Agriculture, and Livestock (Anonymous 2020). Accordingly, five different Nanoformulation doses (2, 4, 6, 8, 10 mg/L) were applied to 250 cm^3 glass jars with 50 g of corn in each of them by spraying. It was applied to corn kernels to allow insects to ingest their bodies while feeding.

As a control group, enzyme (0.94 U/mL), bacteria (10^8 CFU/mL), water (10 mL), ZnO (10 mg/L), and Malathion (200 mL/100 m^2) were applied. The application was made with three repetitions for each dose. After 24 h following the application of the nanoformulation to corn kernels, 25 (including control) 1–7-day-old insects were placed in each jar ($25 \times 10 \times 3$). These jars were placed in the laboratory with their labels (25 ± 1 °C–$60\% \pm 5\%$). The samples placed accordingly were checked daily and the numbers of dead and live insects depending on time were recorded in prepared tables. Insect deaths were counted at the same hour for twenty-eight days (Anonymous 2020).

Using the data obtained, % Mortality rate and average death time were determined by the following formulas.

Mortality rate (%) $= [\left(\frac{n}{N}\right) \times 100]$; n the number of insects died at the end of the experiment, N total number of insect in formule.

Average death time (days) $= (\frac{\sum n_i t_i}{\sum n_i})$ n_i: number of deaths on day t_i, t_i: the counting day.

Statistical analysis

Data for the mortality rate and mean death time of *S. zeamais* were analyzed by one-way analysis of variance (ANOVA) for a completely randomized desing using the PROC GLM procedure in SAS 9.3 (SAS Institute 2011). The differences between the mean values were determined by the Tukey's HSD multiple comparison test (95% confidence).

Results

Purification of chinase enzyme

It was determined that *L. coryniformis* strain 3N11 showed the highest enzyme activity as 1.48 U/mL at the end of the 2nd day (Fig. 1). The range of 0–20% (0.89 U/mL) and 20–40% (0.94 U/mL) of the chitinase enzyme from the precipitates obtained after ammonium sulfate precipitation was performed (Fig. 2), and 20–40% (0.94 U/mL), was loaded into the HiTrap Capto DEAE column (Akta Avant) and the enzyme was purified (Fig. 3). In Fig. 3, the overlapping graphic image of the ammonium sulphate precipitation intervals (0–20, 20–40) and the standard chitinase enzyme in the AKTA Avant device is given. In this figure, the evaluation was made based on the standard chitinase enzyme peak expressed in brown. The blue peak refers to the 0–20, the pink peak to the 20–40 ammonium sulphate precipitation range. It was observed that the enzyme gave the highest peak at UV:280 nm 'in the 20–40 ammonium sulfate precipitation range.The study was continued from the enzyme obtained in the 20–40% range with high activity. The purified enzyme had a molecular mass of ~ 33 kDa as a result of SDS-PAGE (Fig. 4).

Characterization of support bound chitinase

The FTIR spectrum of ZnO, which was immobilized with the chitinase enzyme in Fig. 5, was bound to amide I

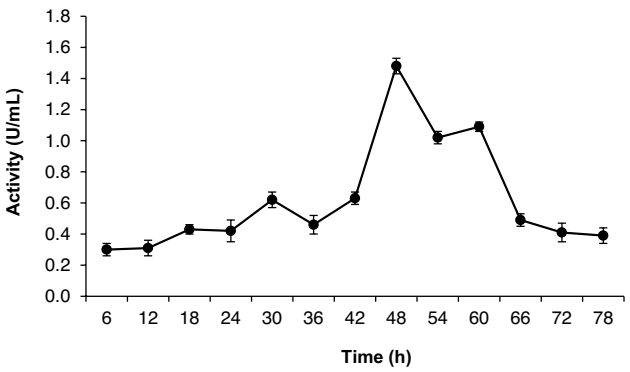


Fig. 1 Enzyme activity graph according to the development curve of *L. coryniformis* strain 3N11. Bars show the average percentage per experiment ± SD

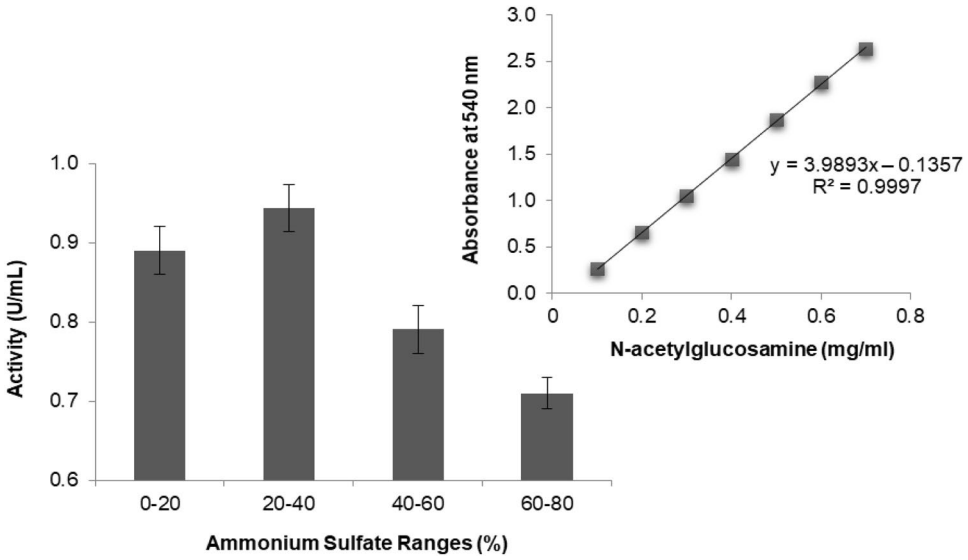


Fig. 2 Chitinase enzyme activity of ammonium sulfate ranges. Bars show the average percentage per experiment ± SD

connection at 1641 cm^{-1} caused by CO tensile vibrations of the peptide bond, at 1575 and 1301 cm^{-1} as a result of the NH bending vibration/CN tensile vibration, respectively. This confirms the presence of the enzyme with the emergence of peaks corresponding to amide II and amide III linkages and amide V linkage at 800 cm^{-1} within the out-of-plane NH bending vibration. The structural model of chitinase immobilized into ZnONPs used in the study was characterized by XRD. In Fig. 6, the diffraction peaks observed in XRD diffractograms at 31.7°, 34.4°, 36.5°, 47.7°, 56.6°, 62.8°, 66.5°, 67.9°, 69.4° ve 72.7° at 2θ scale correspond to the reflections of Zinc oxide at (100), (002), (101), (102), (110), (103), (200), (112), (201), (004), and (202). These values, which were very close to the values in the card numbered JCPDS No.74-0576, also clearly showed that ZnO has grown in the wurtzite phase with a two-dimensional hexagonal P63mc space group before and after being immobilized with the with chitinase enzyme. The fact that the diffraction peaks of ZnO were numerous and intense also indicated that the synthesized products had high crystalline properties, and that no peak of impurities was found, and that the crystal structure of ZnO was not degraded. The morphological structure of chitinase immobilized ZnO (Fig. 7a) and pure ZnO (Fig. 7b) nanoparticles were examined by taking SEM images at different magnifications. From these images, it is clear that the biocomposite was attached to the surface of spherical ZnO particles with an average size of 18 µm, about 8 µm wide chitinase enzyme (Fig. 7a). In higher magnification (×43000), it was interestingly observed that the prepared biocomposites were composed of nanoparticles, each micro ZnO sphere with a width ranging from 10 to 80 nm. Also, high-resolution SEM images showed very clearly that these nanoparticles also covered the flat surface of the porous enzyme. In addition to SEM, "Atomic Force Microscope (AFM)" was used to determine the topological appearance of ZnO particles before and after modification with the enzyme, and it was determined that the average particle size was approximately 60 nm using the three-dimensional profile of the surface in the nanoscale. AFM images were also used to determine the degree of roughness before (Fig. 8a) (Ra: 129 nm) and after (Fig. 8b) (Ra: 7.1 nm) immobilization with chitinase. The surface topologies examined using AFM supported that the layer structure consisting of ZnO particles underwent a significant change after the enzyme modification. It was seen that the highly rough surface of the layer consisting of naked ZnO particles forms a more regular layer structure with the addition of enzymes. This was associated with the decrease in the degree of agglomeration of ZnONPs after modification with the chitinase enzyme.

Optimum pH and temperature values of immobilized and free chitinase enzyme

The activity values measured against different pHs were plotted and the pH range where the highest activity was observed was determined as 6.0 for both the immobilized and free enzyme (Fig. 9). The temperature value at which the highest activity was observed was 65–75 °C for both immobilized and free enzyme, and the results are given in Fig. 10.

Effect of applications on *S. zeamais*

The effect of nanoformulation doses on insect mortality and mean time of death was studied (Table 1). Examining the effects of the applications on the mortality rate, it was determined that the effect on the mortality rate was very significant ($F = 142.01$, $df = 9$, $p = 0.000$), and the mortality rates

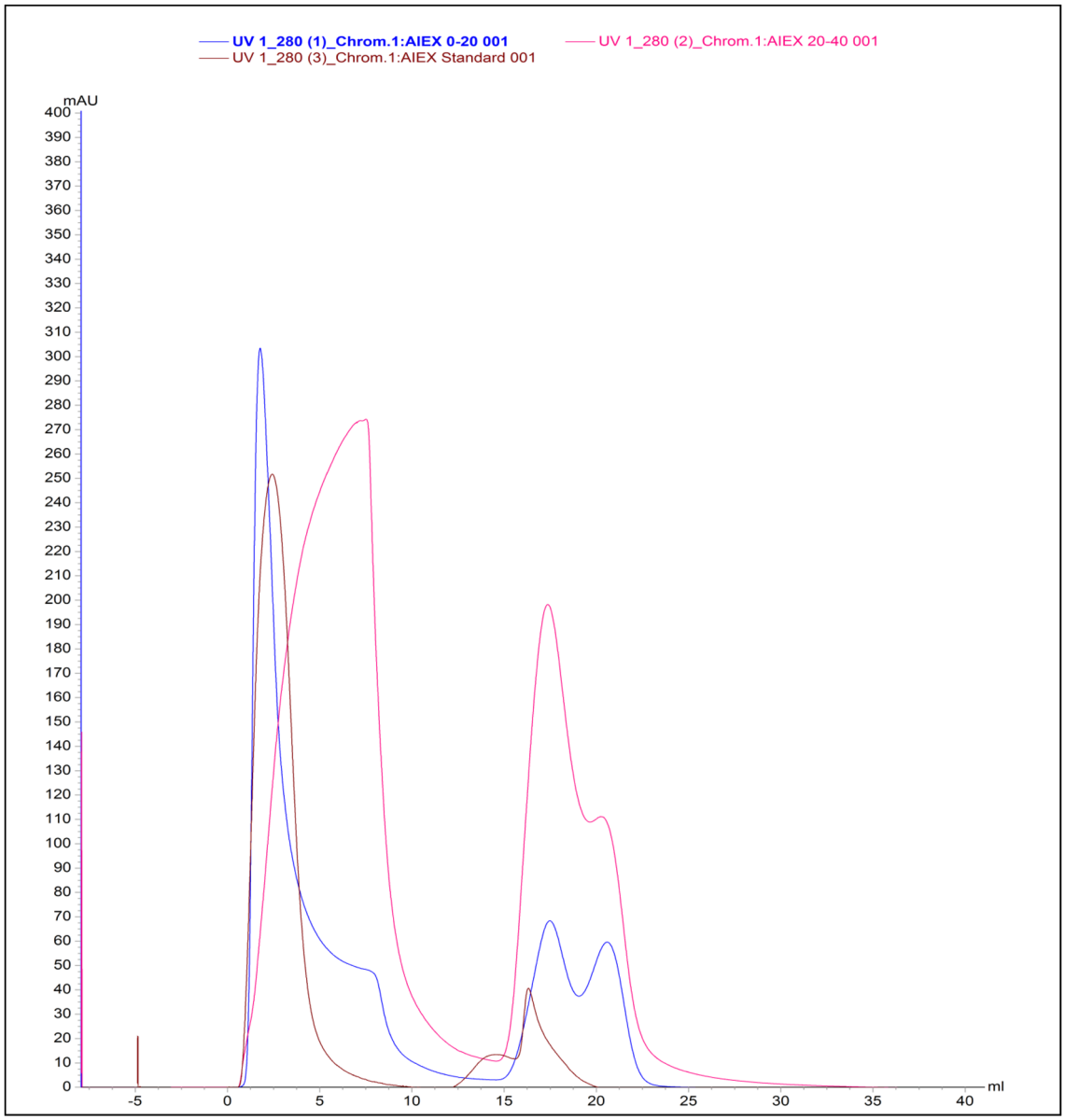


Fig. 3 Overlapping graphic display of ammonium sulfate precipitation ranges (0–20, 20–40) of chitinase enzyme and standard chitinase enzyme in AKTA Avant device. Red peak: 20–40; blue peak: 0–20, brown peak: standard chitinase enzyme

varied between 0.0 and 100%, depending on the application (Fig. 11). The highest mortality rate was determined in 6 mg/L (100.0) and malathion (100.0) applications, followed by 4 mg/L (98.7), 8 mg/L (97.3), 2 mg/L (96.0), ZnONP (96.0) ve and 10 mg/L (94.7) applications in decreasing order, and the difference between them was not found to be statistically significant ($p > 0.05$). However, the difference between these applications and enzyme (80.0), bacteria (78.7) and water (0.0) applications was found to be statistically significant ($p < 0.05$). The difference between enzyme and bacteria application and water application was found to be significant ($p < 0.05$). On the other hand, the effect of the applications on the average death time of the pest was very significant ($F = 1061.10$, $df = 9$, $p = 0.000$) and the death time varied between 1 and 120 days, depending on the application (Fig. 12). The shortest mean time to death was observed in malathion (1.0) application, followed by 8 mg/L (2.3), 6 mg/L (2.4), 2 mg/L (3.1), 10 mg/L (4.4), and 4 mg/L (4.4) applications in ascending order, and the difference between them was not found to be statistically significant ($p > 0.05$). However, the difference between malathion application and enzyme (7.1), ZnONP (7.6), bacteria (12.7) and water (120.0) applications was statistically significant ($p < 0.05$). Likewise, a significant difference ($p > 0.05$) was observed

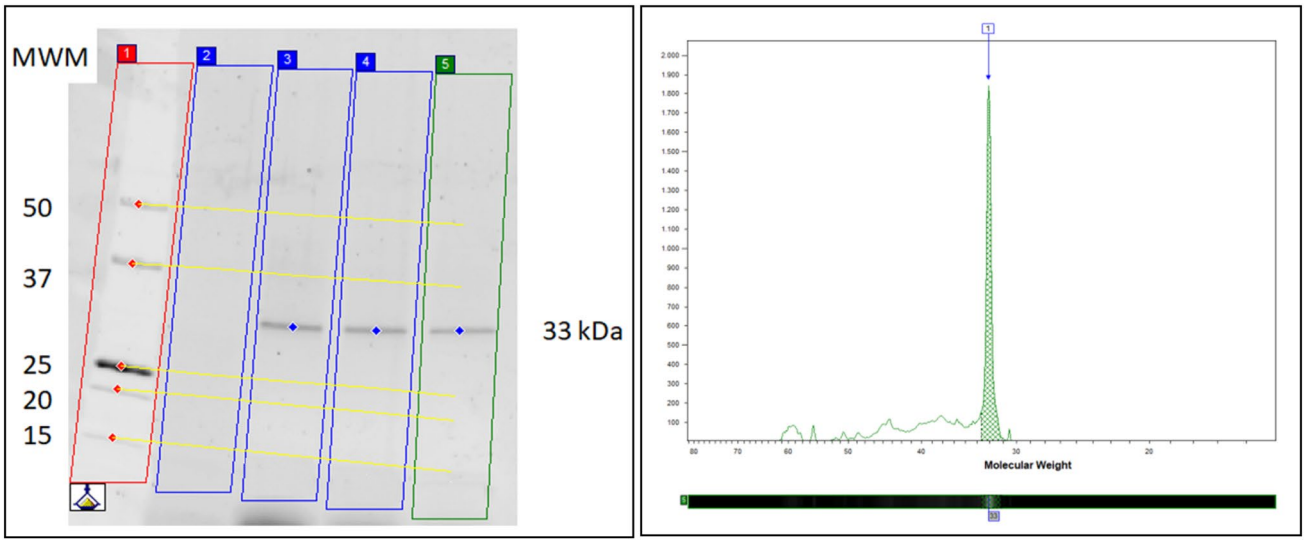


Fig. 4 SDS-PAGE analysis of *L. coryniformis* chitinase purified by HiTrap Capto DEAE column. Lane 1 was loaded with the standard marker mix and lane 3,4,5 was loaded with Purified enzyme. MWM: Marker Molecular weight

between 2 mg/L, 4 mg/L, 6 mg/L, 8 mg/L, and 10 mg/L applications and water and bacteria applications.

Discussion

In the present study, chitinase enzyme (GH18) with a molecular mass of ~ 33 kDa was first precipitated with ammonium sulfate and then purified from *L. coryniformis* strain 3N11 and characterized using the HiTrap Capto DEAE column after precipitation. Subsequently, the optimum pH and temperature values for the activity were found to be pH 6 and 65–75 °C, respectively. Similar to our study, Shivakumar et al. (2014), partially purified a new 14 kD alkalophilic low molecular mass chitinase enzyme from *Bacillus subtilis* JN032305 and determined the optimum temperature for the enzyme to be 50 °C and the optimum pH to be 9.0. Also, Lee et al. (2007), cloned the gene encoding a chitinase from *Bacillus* sp. DAU101 to *Escherichia coli*. The enzyme was purified using the GST-fusion purification system, optimal pH and temperature values were determined to be 7.5 and 60 °C, respectively. Yahiaoui et al. (2019), purified a new strain of extracellular chitinase from *Paenibacillus timonensis*. The molecular weight of the enzyme was determined to be 70 kDa, the optimum pH and temperature were determined to be 4.5 and 80 °C, respectively. Using the same method, Mohamed et al. (2019), purified endochitinase from the *Melghiribacillus thermohalophilus* strain and determined the molecular weight of the enzyme as 45,201.10 Da, pH as 3.5 and temperature as 90 °C. Kudan and Pichyangkura (2009) purified chitinase from *Bacillus licheniformis* and determined the molecular weight of purified enzyme as 72 kDa, the optimum pH and temperature values were pH 6–8 and 50 °C, respectively. Our study continued using a similar acidic enzyme. Loni et al. (2014), purified alkaline chitinase enzyme from *Paenibacillus pasadenensis* NCIM 5434, the molecular weight of the purified enzyme was approximately 35 kDa, the optimum pH and temperature values were determined to be pH 10 and 37 °C, respectively. Du et al. (2020), purified chitinase from *Paenibacillus* sp. and determined the molecular weight of the purified enzyme, the optimum pH and temperature of the enzyme as Sodium 30 kDa, pH 4.5 and 50 °C, respectively. Xie et al. (2021) purified the chitinase enzyme from *Penicillium oxalicum* k10, which they isolated from the soil. The optimum pH value of the purified enzyme was determined as 5 and the optimum temperature value was determined as 40 °C. The results of the present study were similar to the results of some studies in the literature (Lee et al. 2007; Kudan and Pichyangkura 2009; Loni et al. 2014; Shivakumar et al. 2014). Temperature and pH values differ in some studies (Mohamed et al. 2019; Yahiaoui et al. 2019; Du et al. 2020; Xie et al. 2021).

Structural and morphological analyzes such as FTIR, XRD, AFM, and SEM obtained for these enzyme-based biocomposites prepared by us proved that the chitinase enzyme was successfully attached to the ZnO surface. The data reported in the literature support our results (Zhang and Tan 2001; Ramanaviciene et al. 2006; Lin and Li 2009; Ahmad and Sardar 2014; Preety and Hooda 2017, 2018; Jameel et al. 2020).

The optimum pH and temperature values of the immobilized enzyme were investigated and the pH and temperature values at which the optimum activity was observed

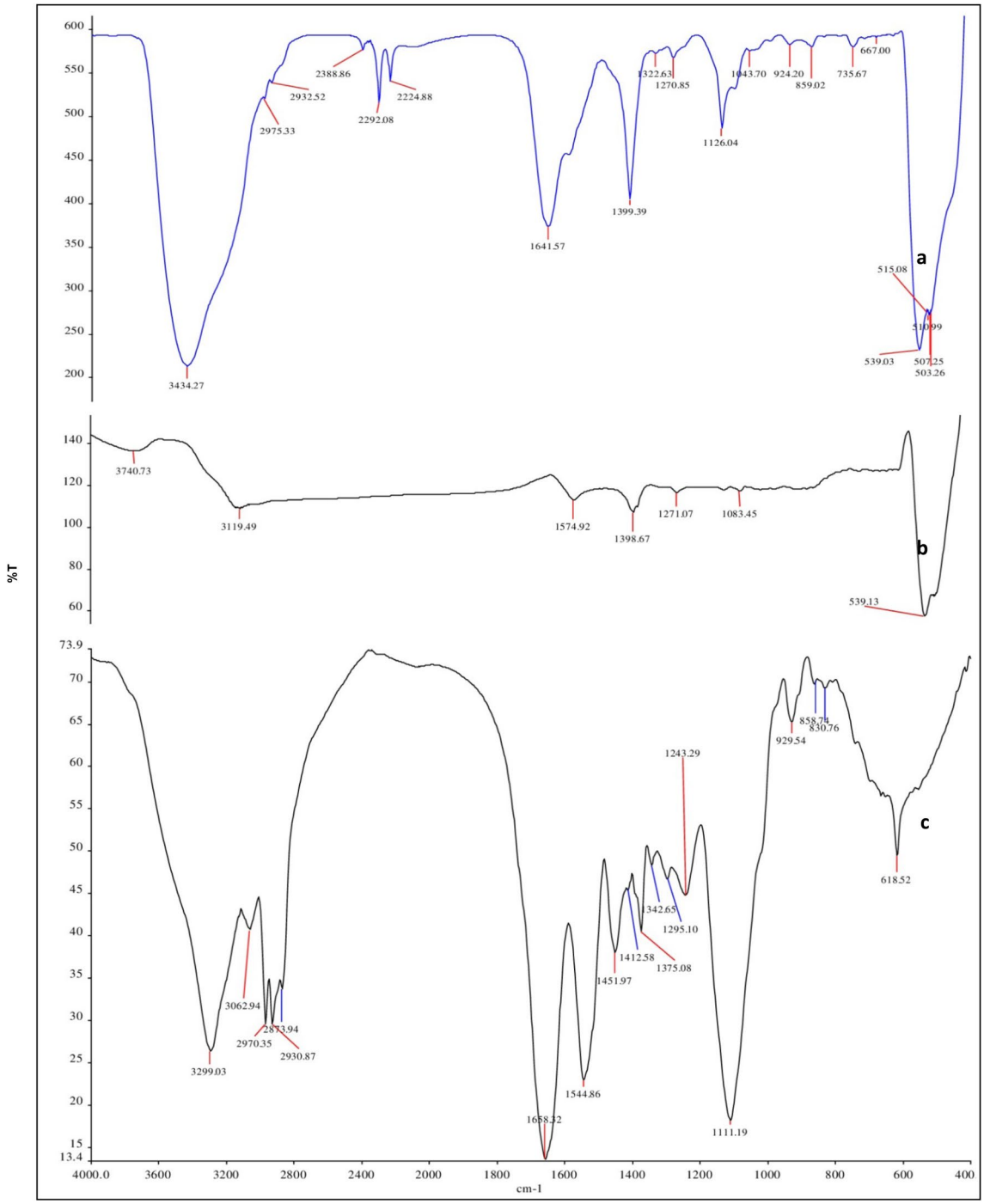


Fig. 5 **a** Enzyme + ZnO-NPs FTIR image. **b** ZnO-NPs FTIR image **c** Chitinase enzyme FTIR image

are 6.0 and 65 °C, respectively. When the immobilized and free enzyme were compared, it was determined that the immobilized enzyme had a higher activity than the free enzyme at different pH and temperature values. The results prove that the immobilized enzyme is more stable than the free enzyme. Different studies also support this finding (Sosnowska et al. 2018; Ismail et al. 2019; Mohammadzadeh et al. 2020; Gomaa 2021).

Many studies have been conducted and positive results have been obtained regarding the use of chitinase enzyme and ZnO particles as a bioinsecticide (Patil and Jadhav 2015; Jameel et al. 2020; Laribi-Habchi et al. 2020). It was

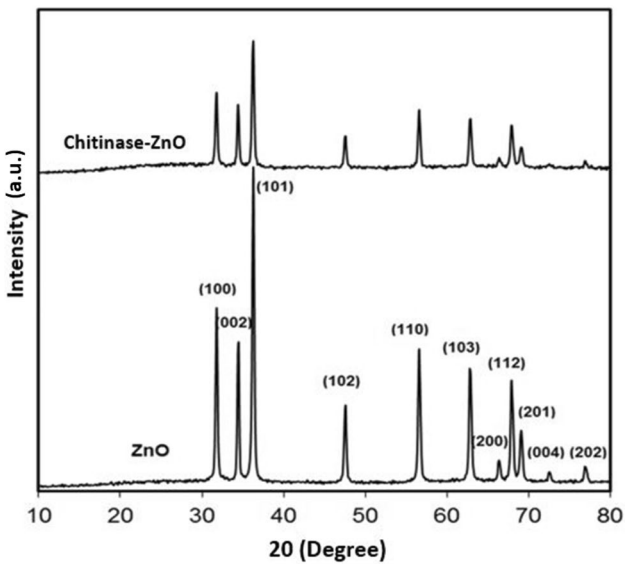


Fig. 6 ZnO-NPs and Enzyme + ZnO-NPs XRD spectra

determined in different studies that the chitinase enzyme damages the insect by showing the hydrolytic effect on the insect cuticle and peritrophic membrane (Patil and Jadhav 2015; Laribi-Habchi et al. 2020). Moharram et al. (2021) reported that the tested chitinase together with other enzymes can help the penetration of the insect exoskeleton and the enzymes can act synergistically. In addition, enzyme applications; They stated that it can help control insect pests and pathogens that attack productive crops and provide potential economic benefits in agriculture. In this context, they think that it would be beneficial to develop screening methods and bioproducts based on them for the identification of new isolates. Zhu et al. (2021) in their study; OfChi-h, a glycoside hydrolase family (GH18) chitinase specific to lepidopterans from the agricultural insect pest Ostrinia furnacalis, is a promising candidate for pest control and management. Delfini et al. (2021) stated in his study that the use of chitinases can contribute to reducing the dose of toxic chemicals. Pour et al. (2021) reported that the secretion of extracellular enzymes (hydrolytic and chitinolytic) caused the death of Glyphodes pyloalis larvae and affected insect host immunity, and stated that they could provide new strategies for pest management. It was thought that the

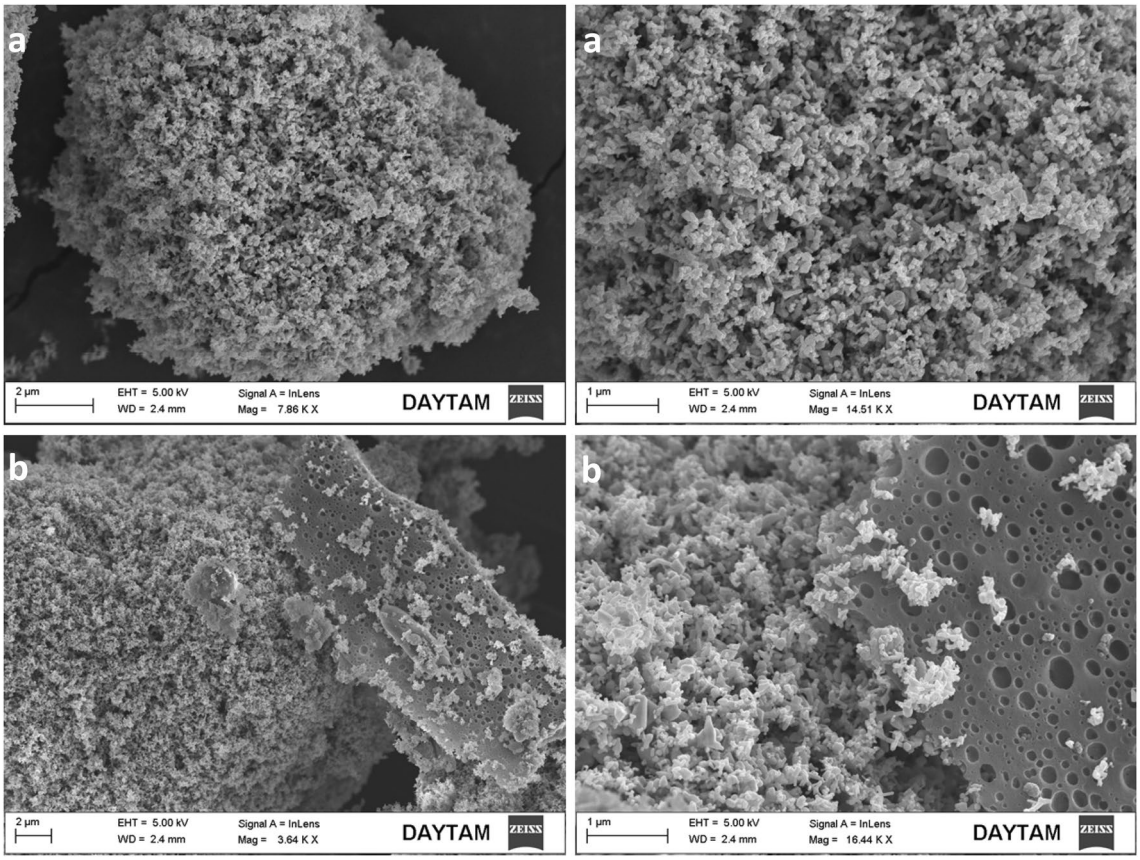


Fig. 7 **a** ZnO-NPs SEM image (2–1 µm). **b** Enzyme + ZnONPs SEM image (2–1 µm)

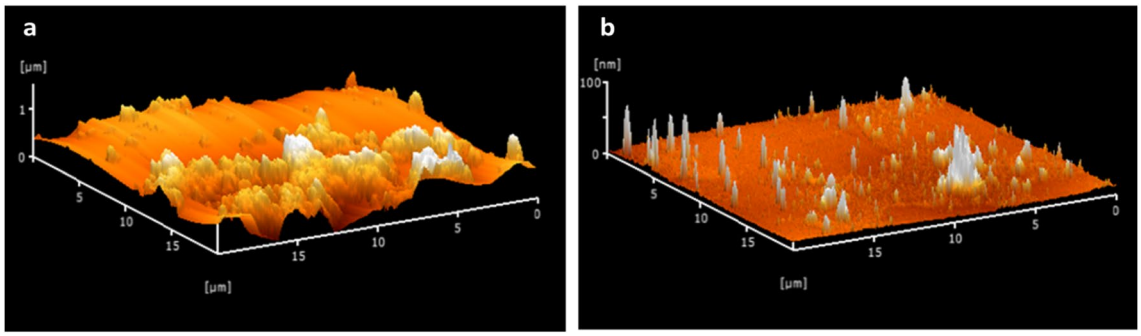


Fig. 8 **a** ZnO-NPs AFM image. **b** Enzyme + ZnO-NPs AFM image

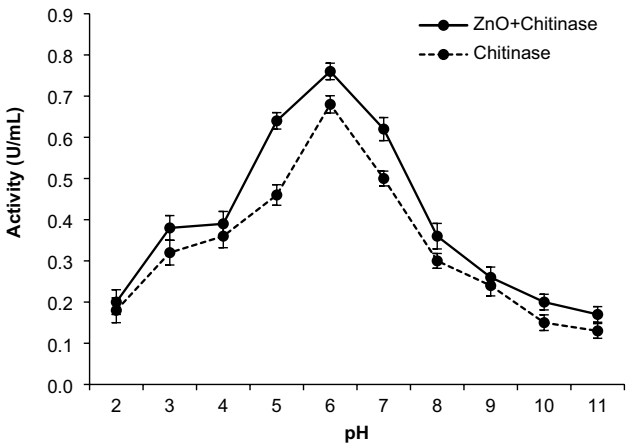


Fig. 9 The effect of pH on immobilized and free chitinase enzyme. Bars show the average percentage per experiment ± SD

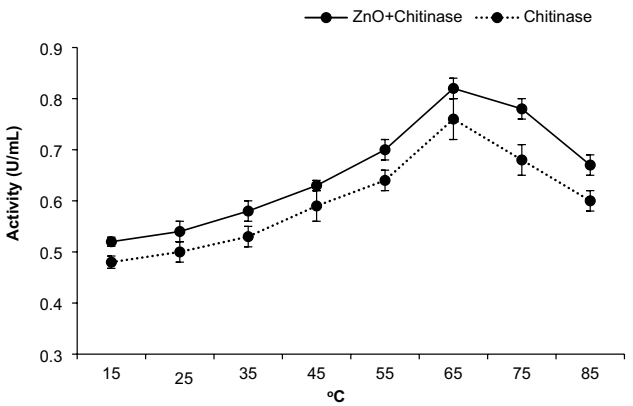


Fig. 10 The effect of temperature on immobilized and free chitinase enzyme. Bars show the average percentage per experiment ± SD

nanoformulation (ZnONps + Chitinase), whose stability and effectiveness was increased with ZnONPs, affected the morphological (chitin-containing) structure of the insect and, therefore, has a lethal effect.

Table 1 Mortality rates and average death times by the application

Application	Mortality rate (%)	The average death time (days)
2 mg/L	96.0 ± 4.0^{a}	3.1 ± 1.0^{cd}
4 mg/L	98.7 ± 1.3^{a}	4.4 ± 1.6^{cd}
6 mg/L	100.0 ± 0.0^{a}	2.4 ± 0.4^{cd}
8 mg/L	97.3 ± 2.7^{a}	2.3 ± 1.3^{cd}
10 mg/L	94.7 ± 1.3^{a}	4.4 ± 1.0^{cd}
Enzyme (0.94 U/mL)	80.0 ± 4.6^{b}	7.1 ± 0.4^{bc}
Bacteria (10^8 CFU/mL)	78.7 ± 3.5^{b}	12.7 ± 2.2^{b}
Water (10 mL)	0.0 ± 0.0^{c}	120.0 ± 0.0^{a}
ZnO (10 mg/L)	96.0 ± 2.0^{a}	7.6 ± 1.1^{bc}
Malathion (200 mL/100 m^2)	100.0 ± 0.0^{a}	1.0 ± 0.0^{d}
Total	84.1	16.5

Each value comprises the means of three replicates ± SE. In the same column, data followed by the same letters are not significantly different ($p > 0.05$)

Considering the effect of formulations on the mortality rate of *S. zeamais*, it was determined that the most effective applications with 100% mortality were malathion and 6 mg/L. When the death times were examined, it was determined that malathion caused insect death in a shorter time compared to our effective dose (6 mg/L). Based on these findings, it was concluded that the best practice is 6 mg/L, considering the mortality rate, mean time to death, and the use of low-dose nanoformulation. According to the results obtained, it was observed that there was a significant relationship between the average death time and the average mortality rate, and the average death time decreased as the mortality rate increased.

The nanoformulation is an effective biopesticide that shows strong biological activities against plant pest insects in sustainable agriculture, it is biodegradable, environmentally friendly, and has no negative effects on human health and can be used as an alternative to chemicals in industry. When we consider the increasing costs and harms of

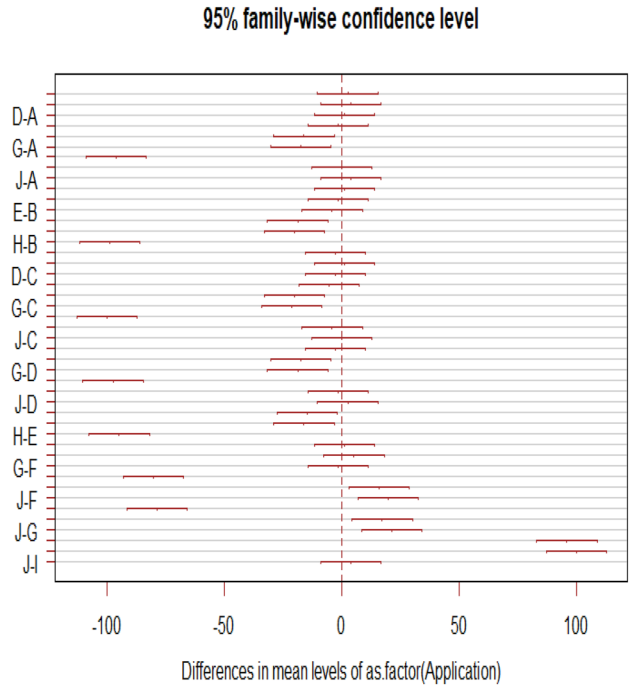


Fig. 11 Multiple comparison results of mortality rates according to Tukey HSD test (at 5% significance level). **a** 2 mg/L, **b** 4 mg/L, **c** 6 mg/L, **d** 8 mg/L, **e** 10 mg/L, **f** Enzyme, **g** Bacteria, **h** Water, **i** ZnO, **j** Malathion

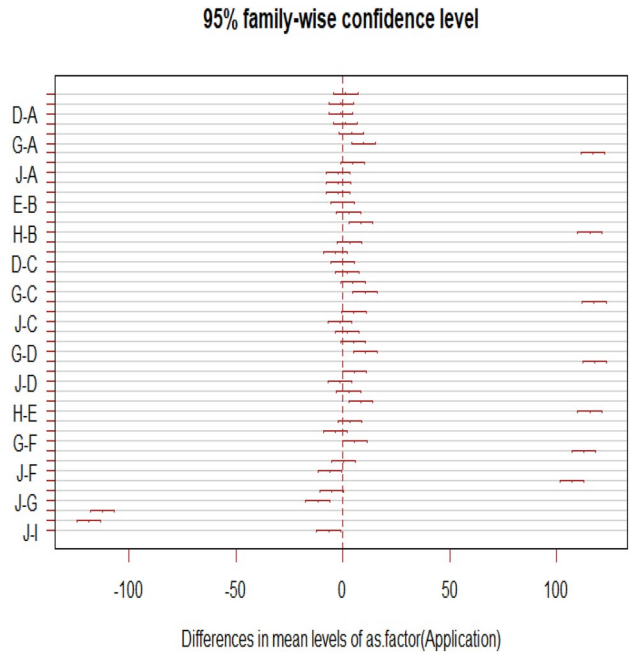


Fig. 12 Multiple comparison results of average death times according to Tukey HSD test (at 5% significance level). **a** 2 mg/L, **b** 4 mg/L, **c** 6 mg/L, **d** 8 mg/L, **e** 10 mg/L, **f** Enzyme, **g** Bacteria, **h** Water, **i** ZnO, **j** Malathion

pesticides every year, it can be thought that with the technologies developed in recent years, enzyme purification steps will decrease day by day and they will remove the burden on the cost and create a strong alternative.

The present study is original regarding the production of the source of chitinase, the use of a new purification method, and obtaining a formulation that can be used in insecticide control. A highly active and stable enzyme produced by *L. coryniformis* strain 3N11 at an acidic pH (6.0) and high temperature (65–75 °C), which has a molecular mass of ~ 33 kDa was used. The nanoformulation obtained in this study prevented the development of the insect and caused its death. It has a lethal effect on harmful *S. zeamais* in grain crops and has the potential to be used as a representative of biopesticides in agricultural bioprocesses. The formulation obtained as a result of the study can provide a large input in agriculture and support the economy.

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Authors contribution ND and SU conceived and designed research. ND, SU, GT and TÖÖ conducted experiments. ND, TÖÖ, GT and RK analyzed data. ND and SU wrote the manuscript. All authors read and approved the manuscript.

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Data availability All data and materials are available from the corresponding author.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This research did not involve any studies with human participants or vertebrate research subjects.

Consent for publication All authors have read and approved the final version.

References

Ahmad R, Sardar M (2014) Immobilization of cellulase on TiO_2 nanoparticles by physical and covalent methods: a comparative study. Indian J Biochem Biophys 51:314–320

Anonymous (2020) Plant pests standard drug trial methods. https://www.tarimorman.gov.tr/TAGEM/Belgeler/yayin/Ambar%20Zararl%C4%B1lar%C4%B1%20Standart%20%20%C4%B0la%C3%A7%20Deneme%20Metotlar%C4%B1.pdf. Accessed 20 July 2020

Athanassiou CG, Kavallieratos NG, Benelli G, Losic D, Usha Rani P, Desneux N (2017) Nanoparticles for pest control: current status

and future perspectives. J Pest Sci 91:1–15. https://doi.org/10.1007/s10340-017-0898-0

Binod P, Sukumaran RK, Shirke SV, Rajpu JC, Pandey A (2007) Evaluation of fungal culture filtrate containing chitinase as a biocontrol agent against *Helicoverpa armigera*. J Appl Microbiol 103:1845–1852. https://doi.org/10.1111/j.1365-2672.2007.03428.x

Boyer S, Zhang H, Lempérière G (2012) A review of control methods and resistance mechanisms in stored-product insects. Bull Entomol Res 102:213–229. https://doi.org/10.1017/S0007485311000654

Chen Y, Ding H, Sun S (2017) Preparation and characterization of ZnO nanoparticles supported on amorphous SiO_2. Nanomaterials 7:217. https://doi.org/10.3390/nano7080217

Dahiya N, Tewari R, Hoondal GS (2006) Biotechnological aspects of chitinolytic enzymes: a review. Appl Microbiol Biotechnol 71:773–782. https://doi.org/10.1007/s00253-005-0183-7

Delfini CD, Villegas LB, Martínez MA, Baigorí MD (2021) Extracellular antifungal activity of chitinase-producing bacteria isolated from guano of insectivorous bats. Curr Microbiol 78:2787–2798. https://doi.org/10.1007/s00284-021-02555-0

Du J, Duan S, Miao J, Zhai M, Cao Y (2020) Purification and characterization of chitinase from *Paenibacillus* sp. Appl Biochem Biotechnol 164:77–88. https://doi.org/10.1002/bab.1889

Ertürk S, Yılmaz A, Fırat-Akdeniz T, Alkan M (2017) Fumigant effect of trans-anethole and carbon dioxide mixture against to *Rhyzopertha dominica, Tribolium castaneum* and *Sitophilus oryza*. Plant Prot Bull 57:391–400. https://doi.org/10.16955/bitkorb.320138

Gomaa EZ (2021) Microbial chitinases: properties, enhancement and potential applications. Protoplasma 258(4):695–710. https://doi.org/10.1007/s00709-021-01612-6

Hamsina H, Tang M, Hasani EIR (2021) Activity test, selectivity, stability of chitinase on amobil chitosan membranes. Int J Multicult Multirelig Underst 8:453–464. https://doi.org/10.18415/ijmmu.v8i7.2750

Hart PJ, Pfluger HD, Monzingo AF, Hollis T, Robertus JD (1995) The refined crystal structure of an endochitinasefrom Hordeum vulgare L. seeds at 1.8 Å resolution. J Mol Biol 248:402–413. https://doi.org/10.1016/S0022-2836(95)80059-X

Heap I (2014) Global perspective of herbicide-resistant weeds. Pest Manag Sci 70:1306–1315. https://doi.org/10.1002/ps.3696

Ismail SA, Hassan ME, Hashem AM (2019) Single step hydrolysis of chitin using thermophilic immobilized exochitinase on carrageenan-guar gum gel beads. Biocatal Agric Biotechnol 21:101281. https://doi.org/10.1016/j.bcab.2019.101281

Jameel M, Shoeb M, Khan MT, Ullah R, Mobin M, Farooqi MK, Adnan SM (2020) Enhanced insecticidal activity of thiamethoxam by zinc oxide nanoparticles: a novel nanotechnology approach for pest control. ACS Omega 5:1607–1615. https://doi.org/10.1021/acsomega.9b03680

Kasthuri N, Hayworth K, Lichtman J, Erdman N, Ackerley CA (2007) New technique for ultra-thin serial brain section imaging using scanning electron microscopy. Microsc Microanal 13(S02):26–27. https://doi.org/10.1017/S1431927607078002

Kidibule PE, Costa J, Atrei A, Plou FJ, Fernandez-Lobato M, Pogni R (2021) Production and characterization of chitooligosaccharides by the fungal chitinase Chit42 immobilized on magnetic nanoparticles and chitosan beads: selectivity, specificity and improved operational utility. RSC Adv 11:5529–5536. https://doi.org/10.1039/d0ra10409d

Kudan S, Pichyangkura R (2009) Purification and characterization of thermostable chitinase from *Bacillus licheniformis* SK-1. Appl Biochem Biotech 157:23–35. https://doi.org/10.1007/s12010-008-8328-7

Kumar J, Ramlal A, Mallick D, Mishra V (2021) An Overview of some biopesticides and their importance in plant protection for commercial acceptance. Plants 10:1185. https://doi.org/10.3390/plants10061185

Kurnaz-Yetim N, Hasanoğlu Özkan E, Sarı N (2019) Enzyme immobilization and optimization on polymeric nanospheres. Suleyman Demirel Univ J Sci 14:97–104. https://doi.org/10.29233/sdufeffd.479246

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685. https://doi.org/10.1038/227680a0

Laribi-Habchi H, Bouacem K, Allala F, Jabeur F, Selama O, Mechri S, Yahiaoui M, Bouanane-Darenfed A, Jaouadi B (2020) Characterization of chitinase from *Shewanella inventionis* HE3 with bio-insecticidal effect against granary weevil, *Sitophilus granarius Linnaeus* (Coleoptera: Curculionidae). Process Biochem 97:222–233. https://doi.org/10.1016/j.procbio.2020.06.023

Lee YS, Park IH, Yoo JS, Chung SY, Lee YC, Cho YS, Ahn SC, Kim CM, Choi YL (2007) Cloning, purification, and characterization of chitinase from *Bacillus* sp. DAU101. Bioresour Technol 98:2734–2741. https://doi.org/10.1016/j.biortech.2006.09.048

Lin CC, Li YY (2009) Synthesis of ZnO nanowires by thermal decomposition of zinc acetate dihydrate. Mater Chem Phys 113:334–337. https://doi.org/10.1016/j.matchemphys.2008.07.070

Lombard V, Golaconda Ramulu H, Drula E, Coutinho PM, Henrissat B (2014) The carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Res 42(D1):D490–D495. https://doi.org/10.1093/nar/gkt1178

Loni PP, Patil JU, Phugare SS, Bajekal SS (2014) Purification and characterization of alkaline chitinase from *Paenibacillus pasadenensis* NCIM 5434. J Basic Microbiol 54(10):1080–1089. https://doi.org/10.1002/jobm.201300533

Magnusson J, Schnurer J (2001) *Lactobacillus coryniformis* subsp. coryniformis strain Si3 produces a broad-spectrum proteinaceous antifungal compound. Appl Environ Microbiol 67(1):1–5. https://doi.org/10.1128/aem.67.1.1-5.2001

Mohamed S, Bouacem K, Mechri S, Addou NA, Laribi-Habchi H, Fardeau ML, Jaouadi B, Darenfed AB, Hacène H (2019) Purification and biochemical characterization of a novel acido-halotolerant and thermostable endochitinase from *Melghiribacillus thermohalophilus* strain Nari2AT. Carbohydr Res 473:46–56. https://doi.org/10.1016/j.carres.2018.12.017

Mohammadzadeh R, Arazpour A, Akbari A (2020) Synthesis and characterisation of magnetic κ-carrageenan nanocomposites for chitinase33 enzyme immobilisation. Int J Nano Biomaterials 9(3–4):171–187. https://doi.org/10.1504/IJNBM.2020.112235

Moharram AM, Abdel-Galil FA, Hafez WMM (2021) On the enzymes' actions of entomopathogenic fungi against certain indigenous and invasive insect pests. Egypt J Biol Pest Control 31:51. https://doi.org/10.1186/s41938-021-00397-3

Muzemu S, Chitamba J, Mutetwa B (2013) Evaluation of *Eucalyptus tereticornis*, *Tagetes minuta* and *Carica papaya* as stored maize grain protectants against *Sitophilus zeamais* (Motsch.) (Coleoptera: Curculionidae). Agric Fish 2(5):196–201. https://doi.org/10.11648/j.aff.20130205.13

Ojo JA, Omoloye AA (2016) Development and Life History of *Sitophilus zeamais* (Coleoptera: Curculionidae) on Cereal Crops. Adv Agric 2016:1–8. https://doi.org/10.1155/2016/7836379

Ong LGA, Lam HK, Lim MY, Tan TX (2017) Process optimization on chitinase production by locally isolated *Enterobacter* sp. and *Zymomonas* sp. Int J Chem Eng Appl 8(4):286–289. https://doi.org/10.18178/ijcea.2017.8.4.671

Pasin TM, de Oliveira TB, Scarcella ASDA, Polizeli MDLTDM, Guazzaroni ME (2021) Perspectives on expanding the repertoire of novel microbial chitinases for biological control. J Agric Food Chem 69(11):3284–3288. https://doi.org/10.1021/acs.jafc.1c00219

Patil NS, Jadhav JP (2015) Significance of *Penicillium ochrochloron* chitinase as a biocontrol agent against pest *Helicoverpa armigera*. Chemosphere 128:231–235. https://doi.org/10.1016/j.chemosphere.2015.01.038

Pour SA, Zibaee A, Rostami MG, Hoda H, Shahriari M (2021) Mortality and immune challenge of a native isolate of *Beauveria bassina* against the larvae of *Glyphodes pyloalis* Walker (Lepidoptera: Pyralidae). Egypt J Biol Pest Control 31:37. https://doi.org/10.1186/s41938-021-00386-6

Prasad M, Palanivelu P (2014) A novel method for the immobilization of a thermostable fungal chitinase and the properties of the immobilized enzyme. Biotechnol Appl Biochem 61(4):441–445. https://doi.org/10.1002/bab.1179

Preety HV (2017) Increasing the efficiency of immobilization and chitin determination using copper oxide nanoparticles. Talanta 168:246–256. https://doi.org/10.1016/j.talanta.2017.03.038

Preety HV (2018) A novel polyurethane/nano ZnO matrix for immobilization of chitinolytic enzymes and optical sensing of chitin. Int J Biol Macromol 106:1173–1183. https://doi.org/10.1016/j.ijbiomac.2017.08.114

Ramanaviciene A, Schuhmann W, Ramanavicius A (2006) AFM study of conducting polymer polypyrrole nanoparticles formed by redox enzyme–glucose oxidase–initiated polymerisation. Colloids Surf B Biointerfaces 48(2):159–166. https://doi.org/10.1016/j.colsurfb.2006.02.002

SAS Institute (2011) The SAS system for Windows. Release 9.3. SAS Inst; Cary, NC

Sachdeva V, Hooda V (2015) Immobilization of nitrate reductase onto epoxy affixed silver nanoparticles for determination of soil nitrates. Int J Biol Macromol 79:240–247. https://doi.org/10.1016/j.ijbiomac.2015.04.072

Sarıbuğa E, Dikbas N, Nadaroglu H, Şenol M, Cetin B (2014) Partial purification, characterization of phytase enzyme from *Lactobacillus acidophilus* Bacteria and determination of it's some kinetic properties. J Pure Appl Microbiol 8:91–96

Shivakumar S, Karmali AN, Ruhimbana C (2014) Partial purification, characterization, and kinetic studies of a low-molecular-weight, alkali-tolerant chitinase enzyme from *Bacillus subtilis* JN032305, A potential biocontrol strain. Prep Biochem Biotechnol 44(6):617–632. https://doi.org/10.1080/10826068.2013.844708

Singh R, Upadhyay SK, Singh M, Sharma I, Sharma P, Kamboj P, Saini A, Voraha R, Sharma AK, Upadhyay TK, Khan F (2021) Chitin, chitinases and chitin derivatives in biopharmaceutical, agricultural and environmental perspective. Biointerface Res Appl Chem 11(3):9985–10005. https://doi.org/10.33263/BRIAC113.998510005

Sosnowska ME, Jankiewicz U, Kutwin M, Chwalibog A, Gałązka A (2018) Influence of salts and metal nanoparticles on the activity and thermal stability of a recombinant chitinase from *Stenotrophomonas maltophil*ia N4. Enzyme Microb Technol 116:6–15. https://doi.org/10.1016/j.enzmictec.2018.05.003

Supernak-Marczewska M, Zielinski A (2020) Effects of the origin and deacetylation degree of chitosan on properties of its coatings on titanium. Coatings 10(2):99. https://doi.org/10.3390/coatings10020099

Vaidya R, Roy S, Macmil S, Gandhi S, Vyas P, Chhatpar HS (2003) Purification and characterization of chitinase from *Alcaligenes xylosoxydans*. Biotechnol Lett 25(9):715. https://doi.org/10.1023/A:1023406630791

Xavier R, Rekha K, Bairy K (2004) Health perspective of pesticide exposure and dietary management. Malays J Nutr 10(1):39–51

Xi H, Yu J, Sun Q, Lu J, Gu T, Guo X, Lia B, Chena X, Zhanga K, Kong W, Wu Y (2018) Expression and purification of pneumococcal surface protein a of clade 4 in *Escherichia coli* using hydroxylapatite and ion-exchange column chromatography. Protein Expr Purif 151:56–61. https://doi.org/10.1016/j.pep.2018.06.008

Xie XH, Fu X, Yan XY, Peng WF, Kang LX (2021) A Broad-specificity chitinase from penicillium oxalicum k10 exhibits antifungal activity and biodegradation properties of chitin. Mar Drugs 19(7):356. https://doi.org/10.3390/md19070356

Yahiaoui M, Laribi-Habchi H, Bouacem K, Asmani KL, Mechri S, Harir M, Bendif H, Aïssani-El Fertas R, Jaouadi B (2019) Purification and biochemical characterization of a new organic solvent-tolerant chitinase from *Paenibacillus timonensis* strain LK-DZ15 isolated from the Djurdjura Mountains in Kabylia. Algeria Carbohydr Res 483:107747. https://doi.org/10.1016/j.carres.2019.107747

Zhang P, Tan W (2001) Atomic force microscopy for the characterization of immobilized enzyme molecules on biosensor surfaces. Fresenius J Anal Chem 369(3–4):302–307. https://doi.org/10.1007/s002160000624

Zhu L, Chen L, Shao X, Cheng J, Yang Q, Qian X (2021) Novel inhibitors of an insect pest chitinase: design and optimization of 9-O-aromatic and heterocyclic esters of berberine. J Agric Food Chem 69(27):7526–7533. https://doi.org/10.1021/acs.jafc.0c07401

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