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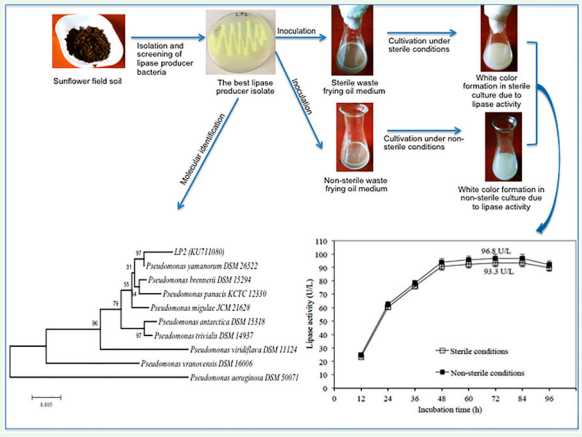
Waste frying oil hydrolysis and lipase production by cold-adapted *Pseudomonas yamanorum* LP2 under non-sterile culture conditions

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ABSTRACT

Non-sterile culture technique is currently used in some microbial processes. However, there is no study on the use of this technique in the production of microbial lipases and hydrolysis of waste frying oils. This study was conducted to hydrolyse waste frying oils and produce lipase under non-sterile culture conditions using locally isolated cold-adapted bacteria. Of 75 bacterial isolates, the psychrotolerant *Pseudomonas yamanorum* LP2 (Genbank number: KU711080) was determined to have the highest lipase activity. It was found that a combination of restricted nutrient availability, low temperature and high inoculum volume prevented microbial contaminants under non-sterile conditions. The most favourable parameters for lipase production under both sterile and non-sterile conditions were 15°C temperature, pH 8, 30 mL/L inoculum volume, 40 mL/L waste frying oil concentration, 10 mL/L Tween-80 and 72 h incubation time. The maximum lipase activities in sterile and non-sterile media were determined as 93.3 and 96.8 U/L, respectively. The present process designed for enzyme production and waste oil hydrolysis can reduce the cost of cultivation medium as well as energy consumption and workload. The potential of cold-adapted bacteria to produce lipase and hydrolyse waste oils under non-sterile culture conditions was first tested in the current study.



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Pseudomonas yamanorum LP2; cold-adapted; lipase; non-sterile culture; waste frying oil

1. Introduction

Enzymes are important biological macromolecules that perform important functions in living organisms. Today, nearly 4000 enzymes are known, and about 200 of these enzymes are in commercial use. The majority of industrial enzymes are obtained from microorganisms such as bacteria, yeasts and filamentous fungi. Following proteases and carbohydrases, lipases are reported to be the third-largest group based on total sales volume [1–3].

Lipases can perform very specific chemical reactions such as interesterification, hydrolysis, esterification and alcoholysis. Therefore, they are very popular in different industries [4–6]. Since microbial lipases are inducible, various lipidic carbon sources, such as canola oil, soybean oil, sesame oil, coconut oil, castor oil, sunflower oil and olive oil, are used as a substrate in the microbial production of these enzymes [7–10].

Recently, investigators have reported that waste-frying oils can also be used as a substrate in the

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production of microbial lipases [11,12]. Frying oil is extensively employed for frying of foods either in home, restaurants or in the food industry. During the frying process, many physical and chemical changes in oil structure occur. These changes can make the frying oil unsuitable for human consumption. On the other hand, waste oil resulting from the frying process causes some serious environmental problems. For example, one litre of oil poured into natural waters may pollute 500,000 L of water [13,14]. Therefore, usage of waste frying oil as a substrate in the production of microbial lipases is considered as an alternative approach in reducing environmental problems as well as the cost of microbial media.

Cold-adapted microorganisms (psychrophilic and psychrotolerant) surviving at low temperatures are utilized as important sources of cold-active lipases [15,16]. For example, it has been documented that psychrophilic and/or psychrotolerant bacterial strains belonging to genera such as *Acinetobacter, Bacillus, Moraxella, Psychrobacter, Staphylococcus* and *Pseudomonas* are a good producer of cold-active lipases [15,17]. The lipases of cold-adapted microorganisms show higher activity at low temperatures than those of mesophilic and thermophilic microorganisms [18]. Therefore, cold-active lipases can be used as a detergent additive for cold washing, which prevents wear of textile fibres and reduces energy consumption. Cold-active lipases also function as an important biocatalyst in the production of optically active amines that are used as intermediate in the preparation of pharmaceuticals. They are also used for the preparation of esters in organic synthesis reactions. In the food industry, they are applied for the synthesis of lipophilic antioxidants and flavouring compounds [15–21].

Non-sterile culture technique is preferably used in fermentation studies because it reduces electricity and time consumption as well as labour. For instance, it has been reported that this technique can be employed in the production of some valuable substances such as citric acid and microbial lipids [22–24]. However, there is no study on the lipase production potential of cold-adapted bacteria under non-sterile culture conditions.

With regard to the facts mentioned above, this study was performed to reveal the potential of locally isolated cold-adapted bacteria to hydrolyse waste frying oils and produce lipase under non-sterile conditions.

2. Materials and methods

2.1. Isolation of lipase producing or oil-degrading cold-adapted bacteria

Soil samples that remained under snow or ice for a long time (about 6 months) were used as the source of isolation of microorganisms adapted to cold. For this purpose, the samples taken from different soils (campus, field and forest soils) around Erzurum city (Turkey) during winter season were brought to the laboratory in sterile containers. The samples were then employed for the isolation of cold-adapted bacteria with lipase production potential.

During the isolation experiments, soil samples were serially diluted with sterile-saline water (0.9%). Then, 0.2 mL of the prepared suspension was spread on Petri dishes containing the isolation medium. The isolation medium composed of 20 mL/L tributyrin, 3 g/L ammonium sulphate, 1.5 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, 0.01 g/L $CaCl_2$, 0.01 g/L NaCl, 0.003 g/L $FeSO_4$, 20 g/L agar and 1 ml/L trace elements solution (8 mg/L $ZnSO_4$, 6 mg/L $MnSO_4$ and 0.3 mg/L $CuSO_4$) (pH 8.0). The inoculated Petri dishes were incubated at 4°C. After 15-day incubation, bacterial colonies growing on tributyrin agar plates were sub-cultured and purified on tributyrin agar plates and then transferred to trypticase soy agar (TSA) slants.

2.2. Screening of lipase producer cold-adapted bacteria

At this stage, the isolates were compared in terms of their ability to produce extracellular lipase a low temperature at 15°C. For this purpose, screening experiments were performed in the waste olive oil broth medium. In contrast to the isolation medium, waste frying olive oil was used as a lipase substrate instead of tributyrin when preparing the screening medium. Furthermore, a solidifying agent (agar) was not added into the screening medium.

Pre-culture of each isolate was prepared in a 250-mL flask containing 100 mL of standard trypticase soy broth (TSB) medium for 48 h, and 1 mL of pre-culture was then employed for the inoculation of the screening medium or production medium. The optical density$_{600nm}$ of pre-culture of each isolate was adjusted to McFarland's standard 2.0. After the flasks were inoculated with pre-culture, they were incubated in a shaking incubator at 200 rpm and 15°C. At the end of 48 h incubation period, the cultures were centrifuged, and the cell-free supernatants obtained were used to measure lipase activity. Among bacterial isolates, the isolate (LP2) identified as the best lipase producer was used in the later stages of the study.

2.3. Lipase production and waste oil degradation under sterile and non-sterile culture conditions

Lipase production was performed in 250-mL flasks containing 100 mL of sterile or non-sterile production

medium (screening medium). While non-sterile culture conditions were being established, the flasks were not sterilized before inoculation. Furthermore, non-sterile flasks were not sealed with cotton plugs and remained open to the environment during the cultivation period. For the preparation of the sterile medium, the flasks containing 100 mL of the production medium were sealed with a cotton plug and then sterilized at 121°C for 15 min. The sterilized flasks were then cooled to room temperature. After sterile and non-sterile production media were inoculated with the pre-culture, they were incubated in a shaker at 200 rpm. Tween-80 was added into the media together with bacterial inoculum. The initial optimization experiments were done to determine the most favourable culture pH (pH 4–10) and temperature (10°C–40°C). Then, the influence of inoculum volume (10–50 mL/L), oil (waste frying olive oil) concentration (10–70 mL/L), Tween-80 concentrations (1–15 mL/L) and incubation time (up to 96 h) on lipase production was investigated. During the experiments, non-inoculated flasks were used as control.

To determine the extent of possible contamination in non-sterile media, 0.1 mL sample withdrawn from the culture was spread on Petri dishes containing TSA medium after diluted with sterile saline water. The inoculated Petri dishes were then left to the incubation at 15°C for 48 h. Colony morphology (shape, texture, colour and size) of bacteria developing on TSA was examined. In order to determine whether bacterial colonies were different from each other, a sample taken from each colony was spread on a glass slide, and then the slides were examined under a microscope to determine the shape, size and motility of bacterial cells. Briefly, the presence of different bacteria (unwanted bacterial contamination) during optimization studies was determined by colony morphology and microscopic examination. On the other hand, in the last step (optimization of the incubation period) the presence of possible contamination was also investigated by using 16S RNA sequence analysis. For this purpose, 16S RNA sequence analysis of each bacterial colony on TSA was performed, as described in materials and methods section 2.5. In the end, colonies were counted, and the degree of contamination was expressed by the symbols −, +, ++ and +++. No contamination (−), less contamination (+), moderate contamination (++) and high contamination (+++).

2.4. Assay of lipase activity

At the end of the specified incubation period, 10 mL of sample drawn from the culture was centrifuged at 5000 rpm for 5 min. The prepared supernatant was employed for lipase assay. The activity assay was done according to the method of Hung et al [25]. The reaction mixture contained 1 mL of 0.05 M phosphate buffer (pH 8), 0.1 mL of enzyme source (supernatant) and 1 mL of 0.013 M p-nitrophenyl palmitate (p-NPP) in ethanol. It was mixed for 5 min at 30°C. To terminate the reaction, 2 mL of 0.5 M Na_2CO_3 was added to the mixture. Then, the mixture was subjected to the centrifugation at 10,000 rpm for 10 min. Finally, the absorbance of the mixture was read at 410 nm using a spectrophotometer. The mixture containing 0.1 mL of the bacteria-free medium instead of the enzyme source (supernatant) was used as the blank. One unit (U) of lipase activity was designated as the amount of enzyme required to hydrolyse 1 µmol/min of p-NPP under the assay conditions. A molar extinction coefficient (ε410) of 13,290 M^{-1} cm^{-1} for p-nitrophenol was used.

2.5. Identification of the best lipase producer strain

Extraction of total genomic DNA from the best producer strain (LP2) was carried out according to the method of Baltaci and Adiguzel [26]. 16S rRNA gene was selectively amplified from purified genomic DNA using oligonucleotide primers [27F (5′-AGA GTT TGA TCC TGG CTC AG- 3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′)]. Then, the PCR product was cloned to a pGEM-T vector system (pGEM-T, Promega, the UK), and the clone was sequenced (Macrogen, Amsterdam, the Netherlands). The obtained sequence was compared with the complementary sequences within GenBank and Eztaxon (http://blast.ncbi.nlm.nih.gov/blast.cgi and http://www.eztaxon.org) server [26,27].

2.6. Statistical analysis

Each experiment was repeated at least three times. The variance analysis was performed according to the one-way ANOVA test using SPSS 13.0 for Microsoft Windows, and the averages were compared with the Duncan test at a confidence level of 0.05.

3. Results and discussion

3.1. Isolation, screening and identification of lipase producer cold-adapted bacteria

It is known that some amino-acids, nucleotides, vitamins, or fatty acids function as growth factors for some microorganisms [28,29]. Therefore, medium components such as yeast extract and peptone, providing these growth factors, are widely added to the culture media of these microorganisms. But, it is well known that since yeast

extract and peptone are very expensive, their addition into the growth medium is not an economical approach. Conversely, since the nutritional requirements of some microorganisms are simple, these microorganisms can be easily cultivated in a minimal medium containing inorganic nitrogen, phosphorus, carbon and trace elements. [30–33]. Considering this knowledge, we decided to isolate a bacterial strain, being capable of growing on a simple and cheap medium. In this context, in the current study, a minimal isolation medium consisting of only simple salts and a carbon source (tributyrin) was designed.

Alkaline-active enzymes used in the detergent industry are usually obtained from microorganisms that grow at neutral or alkaline pH values [34,35]. Taking into account of this knowledge, the pH of the isolation medium was adjusted to an alkaline value (pH 8.0). On the other hand, it has been widely reported that cold-active lipases are usually obtained from microorganisms distributed in cold habitats [15–17]. Therefore, soil samples collected during the cold winter season were chosen as isolation sources of target microorganisms. Besides, the inoculated petri dishes were incubated at a low temperature of 4°C in order to isolate cold-adapted ones rather than mesophilic and thermophilic microorganisms. In brief, preliminary experiments focused on the isolation of bacteria having the growth potential on simple nutritional components at alkaline pH and low temperature. After an incubation period of 15 days at 4°C, the colonies growing on the isolation medium were considered to have lipolytic activity. Among these colonies, those with different morphology were selected and purified. In this way, a total of 75 bacterial strains were isolated from different soil samples. When the screening experiments were carried out at a low temperature of 15°C, only 15 isolates were determined to possess high lipase activity (Table 1). Among 15 bacterial strains, the strain LP2 isolated from soil of sunflower field was determined to have the highest lipase activity (58.9 U/L).

According to the 16S rRNA sequencing analysis, LP2 (GenBank accession: KU711080) had approximately 1498 nucleotides (nt) and resembled to *Pseudomonas yamanoruma* (Genbank accession: EU557337) a rate of 99% in GenBank database (Figure 1). The presence of lipase activity in *P. yamanorum* species was first reported by Arnau et al. [36]. However, their studies focused on detecting qualitative lipase activity on tributyrin agar. But, lipase production or waste oil hydrolysis with this species in submerged or solid-state culture has not yet been studied. On the other hand, it is stated in the literature [37–39] that bacteria, fungi and algae can be used in the biosorption of heavy metals. Therefore, we consider that biosorption of heavy metals by *P. yamanorum* strains may be studied in future studies.

Table 1. Screening of lipase producing bacteria.

Isolation source	Isolate code	Activity (U/L)
Campus soil of Ataturk University (Erzurum, Turkey)	LS3	38.5 ± 1.5d
	LS7	50.3 ± 2.2b
Soil of sunflower field (Erzurum, Turkey)	LP2	58.9 ± 3.4a
	LP14	33.5 ± 2.6f
	LP17	48.4 ± 3.1bc
	LP23	44.2 ± 2.9c
Soil of potato field (Erzurum, Turkey)	LO3	32.7 ± 3.1f
	LO14	41.5 ± 2.1cd
	LO15	36.7 ± 2.2e
Soil of pinea forest (Erzurum, Turkey)	LM4	49.6 ± 3.3b
	LM8	37.1 ± 2.5e
	LM9	45.6 ± 1.4c
Soil of wheat field (Erzurum, Turkey)	LN3	36.8 ± 2.0e
	LN8	50.1 ± 1.8b
	LN10	43.5 ± 2.0c

*All values are mean ± standard error of six determinations ($n = 3$). Different lowercase letters in the same column indicate significant differences ($p \leq 0.05$). Screening conditions: Temperature 15°C, incubation time 48 h, pH 8.0, waste olive oil concentration 30 mL/L, Tween 80 concentration 3 mL/L and inoculum volume 10 mL/L. All experiments were performed under sterile culture conditions.

3.2. Design of non-sterile culture conditions for lipase production

Non-sterile culture process may be designed by selecting culture parameters that limit or hinder undesirable microbial contaminants in the medium, but which make possible the growth of target microorganism [22]. For example, Tao et al. [40] showed that low culture pH (pH 4.5) prevented the development of microbial contaminants but allowed ethanol production from Zymomonas mobilis under open fermentation conditions. Another study [41] showed that lactic acid production was accomplished with thermophilic Bacillus in non-sterile culture, where high cultivation temperature was selected. Dietz and Zeng [42] demonstrated that fermentative production of 1,3-propanediol was succeeded under non-sterile working conditions when the mixed cultures were used. Some studies [23,24,43] exhibited that selection of low cultivation temperature suppressed undesired microbial contaminants, thereby making possible the production of citric acid, invertase and chitosan under non-sterile open fermentation conditions. Furthermore, some researchers [22,44] reported that large inoculum size, low temperature or limited nutrient availability impeded the growth of undesired contaminants in non-sterile cultures during lipid production from yeasts. However, lipase production from cold-adapted microorganisms, including *Pseudomonas* strains under non-aseptic working conditions, has not yet been investigated.

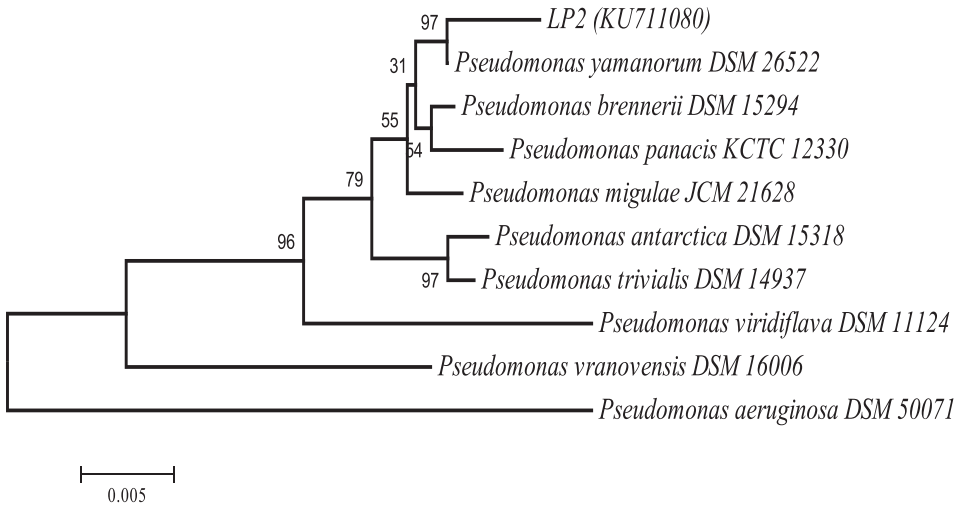


Figure 1. Neighbour-joining phylogenetic tree on the basis of 16S rRNA gene sequence data of *Pseudomonas yamanorum* strain LP2. Bootstrap values based on 1000 replications are listed as percentages at branching points. The accession numbers are given in parenthesis. Only bootstrap values >50% are shown at nodes. The scale bar represented 1% divergence. Neighbour-joining phylogenetic tree on the basis of 16S rRNA gene sequence data of *P. yamanorum* strain LP2. Bootstrap values based on 1000 replications are listed as percentages at branching points. The accession numbers are given in parenthesis. Only bootstrap values >50% are shown at nodes. The scale bar represented 1% divergence.

Considering the results of previous studies [22,23,43,44], we considered that a combination of limited nutrient availability, low temperature and high inoculum would prevent unwanted contaminants, but enable lipase production from *P. yamanorum* LP2 in a non-sterile culture. We have predicted that microbial contaminants with low lipase activity cannot effectively use olive oil as a carbon source, unlike P. yamanorum LP2 with high lipase activity. Furthermore, we considered that microbial contaminants do not show good growth potential in this medium since the medium does not contain rich nutritional components such as peptone and yeast extract. Namely, most of the microbial contaminants may be eliminated due to the restricted nutritional composition of the production medium. The rest of the microbial contaminants may be eliminated by means of the selection of low temperature and/or high inoculum. For instance, it has been reported that low ambient temperature prevents mesophilic or thermophilic contaminants in the non-sterile environment, but allows the growth of cold-adapted microorganisms [45].

Table 2. Effect of initial pH on lipase production by P. yamanorum LP2 under sterile and non-sterile culture conditions.

	Sterile conditions	Non-sterile conditions	
pH	Activity (U/L)	Activity (U/L)	Contamination
4	10.9 ± 1.1A	11.9 ± 1.3e	–
5	19.7 ± 1.8D	21.2 ± 1.2d	–
6	25.8 ± 2.9C	26.9 ± 3.1c	–
7	51.3 ± 2.0B	44.2 ± 2.4b	+
8	58.9 ± 2.9A	54.9 ± 2.1a	+
9	24.2 ± 2.1C	22.7 ± 1.9d	+
10	12.4 ± 1.3E	11.9 ± 1.2e	–

*All values are mean ± standard error of six determinations ($n = 3$). Different letters in the same column (uppercase letters for sterile conditions but lowercase letters for non-sterile conditions) indicate significant differences ($p \leq 0.05$). Culture conditions: Temperature 15°C, incubation time 48 h, waste olive oil concentration 30 mL/L, Tween 80 concentration 3 mL/L and inoculum volume 10 mL/L. Contamination degree: no contamination (–), less contamination (+), moderate contamination (++) and high contamination (+++).

As seen from the data presented in Table 1, pH 8.0 resulted in maximum lipase activity in both sterile (58.9° U/L) and non-sterile (54.9 U/L) culture medium. The bacterium also showed noteworthy lipase activity at pH 9 and 10. These results implied that *P. yamanorum* LP2 might be a novel source of alkaline-active lipases. The experiments also demonstrated that when the initial pH was between 7 and 10, the lipase activities in the non-sterile culture medium were lower than those in the sterile culture medium. This situation might be due to the presence of undesired bacterial contamination. That is, unwanted contaminants might have depleted or reduced some nutritional components (minerals, nitrogen source, etc.), which were required for cell growth and/or lipase production. Besides, contaminants might have produced some compounds restricting the lipase synthesis in the test bacterium. Conversely, adjustment of initial pH to levels below 7.0 completely prevented undesired bacterial contaminations, thereby causing the obtaining of higher lipase activity in the non-sterile medium compared to the sterile medium (Table 2). Although pH levels below 7.0 prevented bacterial contaminants under non-sterile culture conditions

at 15°C, they caused lower lipase activities compared to pH 8.0. Based on these results, the subsequent experiments were carried out at pH 8.0, whose highest lipase activity was achieved.

As seen from the data presented in Table 3, the maximum lipase activity in both media was reached at 20°C followed by 15°C. However, the lipase activities obtained at 15°C and 20°C were very close to each other. Moreover, the bacterium showed the lipase production potential in a wide temperature range from 10°C to 40°C. These results indicate that *P. yamanorum* LP2 had psychrotolerant property, since psychrophiles cannot grow at temperatures above 20°C, but psychrotrophs (psychrotolerants) may tolerate a wider temperature range from 0°C to about 30°C [46]. Furthermore, a newly published study [36] demonstrated that the species *P. yamanorum* had psychrotolerant property.

The present experiments also revealed that temperature was an important factor for the elimination of microbial contaminants under non-sterile culture conditions. Moderate level of contamination was detected in the cultures at 20°C and 25°C. Contamination reached the highest level at 30°C and 35°C under non-sterile culture conditions. Less contamination was detected at 15°C. Although the incubation temperature of 10°C completely prevented contaminants, it caused too low the lipase activity (29.8 U/L). The maximum lipase activity (55.8 U/L) in a non-sterile medium was observed at 20°C, but the subsequent experiments were performed at 15°C, at which less contamination was detected. At this point, it can be considered that cooling of the fermentation tanks to 15°C or below may cause energy consumption. However, we believe that this problem can be solved using tap water, having a temperature of 15°C or lower. This is because, although the tap water temperature may vary depending on the time of year and the location where the water temperature is measured, its average is 10°C–15°C. Furthermore, this process may find industrial application in countries or locations with a climate where low temperatures dominate.

As seen from Table 4, there was less contamination in the non-sterile medium at inoculum volumes of 10 and 20 mL/L, and inoculum volumes over 20 mL/L completely prevented undesired bacterial contamination. On the other hand, higher lipase activities were reached in the non-sterile medium compared to the sterile medium when inoculum over 20 mL/L was used. The lipase activities in sterile (74.2 U/L) and non-sterile (76.8 U/L) media reached the maximal levels at an inoculum volume of 30 mL/L. But, higher inoculum volume decreased lipase activity in both media, probably due to the rapid depletion of some nutrients supporting the lipase activity. In addition, very high inoculum volume might have reduced lipase activity by rapidly consuming oxygen. The similar decreases for enzyme activities in high inoculum volumes were also reported in the previous studies [47–49].

Data summarized in Table 5 indicate that oil concentration significantly affected lipase activities in the media. This result is consistent with those reported in previous studies [50,51]. The maximum lipase activity in both sterile (75.7 U/L) and non-sterile (78.2 U/L) media could be attained at a waste frying oil concentration of 40 mL/L. Concentrations over 40 mL/L gradually decreased the lipase activity, probably due to the presence of some inhibitory compounds inside waste frying oil. This is because it has been documented that olive oil contains some antimicrobial compounds such as tyrosol, hydroxytyrosol dihydroxy phenyl glycol and oleuropein [52]. Furthermore, it is well known that many physical and chemical changes in oil structure occur during the frying process [13]. Therefore, it was

Table 3. Effect of temperature on lipase production by P. yamanorum LP2 under sterile and non-sterile culture conditions.

Temperature (°C)	Sterile conditions Activity (U/L)	Non-sterile conditions Activity (U/L)	Non-sterile conditions Contamination
10	28.7 ± 2.1C	29.8 ± 2.4d	–
15	58.9 ± 3.9A	54.9 ± 2.8a	+
20	61.6 ± 3.2A	55.8 ± 3.0a	++
25	60.7 ± 4.1A	53.3 ± 2.5a	++
30	55.8 ± 3.4A	46.3 ± 2.2b	+++
35	43.2 ± 2.2B	37.8 ± 2.0c	++
40	17.8 ± 1.1D	15.6 ± 0.8e	+

*All values are mean ± standard error of six determinations ($n = 3$). Different letters in the same column (uppercase letters for sterile conditions but lowercase letters for non-sterile conditions) indicate significant differences ($p \leq 0.05$). Culture conditions: incubation time 48 h, initial pH 8.0, waste olive oil concentration 30 mL/L, Tween 80 concentration 3 mL/L and inoculum volume 10 mL/L. Contamination degree: no contamination (–), less contamination (+), moderate contamination (++) and high contamination (+++).

Table 4. Effect of inoculum size on lipase production by P. yamanorum LP2 under sterile and non-sterile culture conditions.

Inoculum volume (mL/L)	Sterile conditions Activity (U/L)	Non-sterile conditions Activity (U/L)	Non-sterile conditions Contamination
10	58.9 ± 3.2C	54.9 ± 2.8c	+
20	68.7 ± 3.7B	65.4 ± 3.4b	+
30	74.2 ± 3.9A	76.8 ± 3.7a	–
40	61.8 ± 3.5C	63.6 ± 3.0b	–
50	51.3 ± 2.7D	53.7 ± 2.1c	–

*All values are mean ± standard error of six determinations ($n = 3$). Different letters in the same column (uppercase letters for sterile conditions but lowercase letters for non-sterile conditions) indicate significant differences ($p \leq 0.05$). Culture conditions: Temperature 15°C, incubation time 48 h, initial pH 8.0, waste olive oil concentration 30 mL/L and Tween 80 concentration 3 mL/L. Contamination degree: no contamination (–), less contamination (+), moderate contamination (++) and high contamination (+++).

Table 5. Effect of waste olive oil on lipase production by P. yamanorum LP2 under sterile and non-sterile culture conditions.

Oil concentration (mL/L)	Sterile conditions	Non-sterile conditions	
	Activity (U/L)	Activity (U/L)	Contamination
10	27.1 ± 1.8D	28.1 ± 2.0d	–
20	46.2 ± 2.2C	47.4 ± 2.4c	–
30	74.2 ± 3.6A	74.8 ± 3.3ab	–
40	75.7 ± 3.5A	78.2 ± 3.6a	–
50	74.3 ± 3.0A	76.3 ± 3.2a	–
60	72.5 ± 3.1AB	73.9 ± 3.4ab	–
70	68.5 ± 2.9B	70.4 ± 3.5b	–

*All values are mean ± standard error of six determinations ($n = 3$). Different letters in the same column (uppercase letters for sterile conditions but lowercase letters for non-sterile conditions) indicate significant differences ($p \leq 0.05$). Culture conditions: Temperature 15°C, incubation time 48 h, initial pH 8.0, waste olive oil concentration 30 mL/L, Tween 80 concentration 3 mL/L and inoculum volume 30 mL/L. Contamination degree: no contamination (–), less contamination (+), moderate contamination (++) and high contamination (+++).

considered that new compounds arising after the frying process might have also restricted the lipase activity in the test bacterium.

As seen from Table 6, the incorporation of Tween-80 to the media resulted in a significant enhancement in lipase activity. For example, the lipase activity was 67.4 U/L in non-sterile medium containing 1 ml/L Tween-80, whereas it was 48.1 U/L in the control medium (Tween 80-free). The maximum lipase activity in both media was reached at a Tween 80 concentration of 10 mL/L. At this optimal concentration, the lipase activities in sterile and non-sterile media were found as 90.7 and 93.9 U/L, respectively. This positive effect could be ascribed to the increased permeability of cell membranes due to the surfactant effect of Tween-80, as reported in the literature [53,54]. Higher concentrations of this surfactant gradually decreased the

Table 6. Effect of Tween 80 concentration on lipase production by P. yamanorum LP2 under sterile and non-sterile culture conditions.

Tween 80 (mL/L)	Sterile conditions	Non-sterile conditions	
	Activity (U/L)	Activity (U/L)	Contamination
0	47.1 ± 2.2F	48.1 ± 2.6f	–
1	66.2 ± 2.8E	67.4 ± 3.3e	–
3	75.7 ± 3.5CD	78.2 ± 3.7c	–
5	79.9 ± 4.4C	82.7 ± 4.5bc	–
7.5	84.9 ± 4.0B	87.8 ± 4.9b	–
10	**90.7 ± 4.4A**	**93.9 ± 5.1a**	–
12.5	85.1 ± 4.5B	87.9 ± 5.1b	–
15	71.9 ± 4.1D	73.1 ± 4.2d	–

*All values are mean ± standard error of six determinations ($n = 3$). Different letters in the same column (uppercase letters for sterile conditions but lowercase letters for non-sterile conditions) indicate significant differences ($p \leq 0.05$). Culture conditions: Temperature 15°C, incubation time 48 h, initial pH 8.0, waste olive oil concentration 40 mL/L and inoculum volume 30 mL/L. Contamination degree: no contamination (–), less contamination (+), moderate contamination (++) and high contamination (+++).

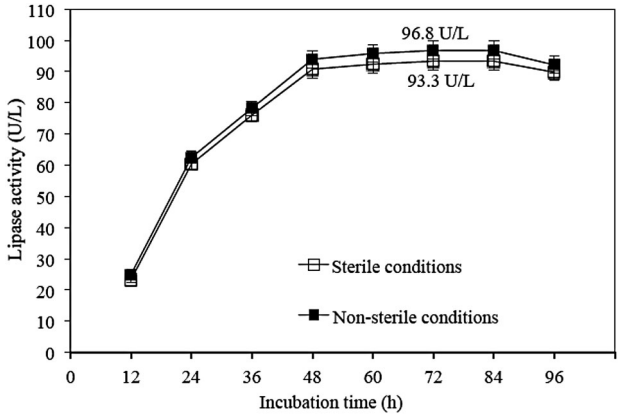


Figure 2. Time profile of lipase production by *P. yamanorum* LP2 under sterile and non-sterile culture conditions. Culture conditions: Temperature 15°C, initial pH 8.0, waste olive oil concentration 40 mL/L, Tween 80 concentration 10 mL/L and inoculum size 30 mL/L.

lipase activity of the bacterium. This situation might be attributed to the loss of cell viability due to excessive increases in membrane permeability. On the other hand, no microbial contamination was observed in the non-sterile medium at any of olive oil and Tween-80 concentrations, when the experiments were performed at the optimal parameters (temperature 15°C and inoculum size 30 mL/L).

So far, several researchers have documented that incubation duration is a crucial factor for the production of microbial lipases [55–57]. As seen from Figure 2, no significant lipase activity in both media was detected within the first 12 h of cultivation. The activities gradually started to increase after 12th h and reached maximal values (93.3 and 96.8 U/L in sterile and non-sterile media, respectively) at 72th h. Obtaining of lower lipase activities in the sterile medium might be due to partial degradation of some nutritional compounds exposed to high temperature during the sterilization process. Lipase activity was stable between 72 and 84 h in both media. This situation might be due to the exhaustion of waste olive oil and/or minerals. On the other hand, incubation times over 84 h decreased lipase activity in both media. This decrease was probably due to the loss of enzyme stability.

In order to determine possible contamination in the culture at the end of 96th h, colonies growing on TSA were examined morphologically and microscopically. Morphological and microscopic analyses indicated that the colonies probably belonged to *P. yamanorum* LP2. When sequence analysis was performed for each colony, it was determined that all the colonies belonged to *P. yamanorum* LP2 precisely. Namely, these analyses revealed that only *P. yamanorum* LP2 developed in the culture under non-sterile conditions.

4. Conclusions

This study revealed that when appropriate culture parameters (large inoculum size, low temperature and nutrient restricted conditions) were selected, the production of lipase from *P. yamanorum* LP2 could be achieved under non-sterile culture conditions. The designed non-sterile culture process can be applied in bioreactors with an ambient temperature in the range of 10°C–15° C. In this way, energy-saving as well as reduction of workload may be possible during microbial lipase production and waste cooking oil hydrolysis. In the next studies, *P. yamanorum* LP2 may be also tested as an alternative bio-system for bioremediation of waste frying oil-contaminated soils, or for the production of the other commercial enzymes such as protease, pectinase, cellulose, tannase and invertase.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Ethical approval

This article does not contain any studies with human participants performed by any of the authors.

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