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Research Article

Molecular Cloning of the Extracellular Lipases of *Bacillus Amyloliquefaciens* Isolated from Agrifood Wastes

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Background: The lipase enzyme (EC: 3.1.1.3) is one of the most important catalysts in food, dairy, detergent, and textile industries.

Objective: This study was performed to identify, isolate and characterize of lipase producing bacterial strain from agrifood wastes and to identify and characterize of their lipase genes.

Materials and Methods: In the present study, two lipase-producing isolates were identified from the effluent of Golbahar meat products and Soveyda vegetable oil factories using in silico and *in vitro* approaches.

Results: The results of morphological, biochemical, and molecular characterizations showed that both lipase-producing isolates belong to the *Bacillus amyloliquefaciens* species. Phylogenetic analysis confirmed the results of phenotypic, biochemical, and molecular characterizations. The results showed differences between LipA and LipB in the Golbahar and Soveyda isolates. Three different amino acids (residues 14, 100, and 165) were observed in LipA and one different amino acid (residue 102) was detected in LipB extracellular lipases. The protein molecular weight of the two extracted lipases ranged from 20 to 25 kDa. The identified extracellular lipases also had different physicochemical features. The maximum lipase activity of the Golbahar and Soveyda isolates was observed at 45 °C and at the pH of 8, but the Golbahar isolates exhibited higher lipase activity compared to the Soveyda isolates. The Golbahar and Soveyda isolates exhibited different activities in the presence of some ions, inhibitors, denaturing agents, and organic solvents and the Golbahar isolates showed better lipase activity than the Soveyda isolates.

Conclusions: In this study, two extracellular lipase-producing isolates of *B. amyloliquefaciens* were identified from different food industries, and their characteristics were investigated. The results of various investigations showed that the lipases produced by the Golbahar isolate have better characteristics than the lipases of the Soveyda isolate. The Golbahar lipases have a suitable temperature and pH activity range and maintain their activity in the presence of some ions, inhibitors, denaturing agents, and organic solvents. After further investigation, the Golbahar isolate lipase can be used in various industries. In addition, this lipase can be used enzyme engineering processes and its activity can be arbitrarily changed by targeted mutations. The results of this study can increase our knowledge of extracellular lipases and may turn out to have industrial applications.

Keywords: Bacillus amyloliquefaciens, Biocatalyst, Biotechnology, Enzyme activity, Extracellular lipase

1. Background

Living organisms have specialized in a wide variety of biochemical reactions during the course of their evolution to survive and adapt to environmental conditions. Almost all of these biochemical reactions are carried out by a group of biological substances called enzymes (1, 2). High catalytic power and adjustable activity are the most important features that distinguish the function of enzymes in biological processes. Due to these characteristics as well as the existence of different isomeric forms, the use of enzymes has increased in industry (1-4).

Lipases (triacylglycerol acyl-hydrolases, EC 3.1.1.3) are a group of hydrolase enzymes (2, 3, 5, 6). Lipases possess a conserved folding pattern of α/β-hydrolase which consists of a beta-sheet surrounded by alpha helices. The catalytic center of lipases consists of three conserved amino acids (Ser, Glu/Asp and His) which are functionally similar to serine proteases, with differences in spatial orientation (7, 8). The mechanism of action of lipases includes the hydrolysis of longer glyceride chains and their conversion into diacylglycerides, monoglycerides, glycerol, and fatty acids (9-12). Lipases can also perform reverse hydrolysis reactions to form various esters from alcohol and fatty acids (glycerides), and they are also able to catalyze esterification reactions in low amounts of water or in the presence of organic solvents. The unique ability of lipases to react at the interface between polar and non-polar phases distinguishes them from esterase enzymes (13-15). Lipases are the third most traded enzymes globally after proteases and amylases, and it is predicted that their annual worldwide sales will reach around 590.2 million dollars by 2023 (16, 17). Considering the high capacity of lipases as multifunctional bio-catalysts, they are suitable candidates to meet the needs of various industries such as bio-diesel, food and beverage, leather and textile, detergent, pharmaceuticals, and medicine (5, 9, 18-21).

So far, numerous lipases have been identified and characterized from different species of bacteria, fungi, plants, and animals (2, 6).

However, it has been demonstrated that bacterial lipases are more efficient than plant and animal lipases (2, 3, 14). Bacterial lipases are more important compared to lipases from other organisms due to their great capacity to perform various catalytic processes, lack of side reactions during catalysis, production with greater performance and stability, and high bacterial growth rates (18, 19, 21-24).

Bacterial lipases exert their effects in a wide range of pH levels (from 4 to 11) and are most active in neutral and alkaline pH levels. These lipases are to a great extent thermally stable and are active in organic solvents without the need for cofactors (5, 25). Based on substrate specificity, bacterial lipases are classified into three categories: non-specific, region-specific, and fatty acid-specific. Non-specific lipases randomly catalyze fat hydrolysis and produce free fatty acids and glycerol. Region-specific bacterial lipases act only on primary ester bonds and, thus, produce free fatty acids. However, fatty acid-specific lipases interact specifically with certain fatty acids such as saturated and unsaturated fatty acids (5, 25, 26).

Bacterial strains such as *Pseudomonas alcaligenes*, *P. aeruginosa*, *P. fragi*, *P. fluorescens*, *Bacillus subtilis*, *B. nealsonii*, *B. licheniformis*, and *B. amyloliquefaciens* are considered as the most important lipase-producing bacteria genera due to the high efficiency over a broad of range of temperatures and pH levels (17).

Previous studies have shown that species of the *Bacillus* genus are the most important lipase-producing bacteria and show high efficiency in harsh industrial conditions (2, 9, 11, 18, 19, 27, 28). The lipase-producing bacteria belonging to the genus *Bacillus* are gram-positive and often grow in culture media containing carbon, nitrogen, phosphorus, and mineral salts with pH in the neutral range (5, 7, 8) and at temperatures between 20 °C and 70 °C (7). It has been shown that natural oils, including olive oil, cotton seed oil, mustard oil, sesame oil, soybean oil, fish oil, and corn oil (alone or in combination) are the best carbon sources for lipase-producing *Bacillus* species (5, 29). Microorganisms that produce lipase enzymes are found in various environments such as industrial effluents, leather processing waste, effluents of vegetable oil processing industry, wastewater of dairy industries, effluents from food industries, and other oily environments. These places are suitable to isolate the lipase-producing bacteria (30). In a study, a lipase-producing strain of *B. amyloliquefaciens* was isolated from lipid-rich food waste and it was shown that the lipase production of this strain is induced by vegetable oils (31). In another study, two lipase-producing strains of *B. stearothermophilus* and *B. licheniformis* were isolated from milk powder production lines and

their lipase enzymes showed high stability in heat treatments applied during commercial milk powder manufacture (32). In a similar study, a highly thermo-tolerant and methanol-tolerant lipase-producing strain of *B*. *circulans* was isolated from cooking oil (10). Chakraborty and Paulraj (2008) isolated a lipase-producing strain of *B*. *licheniformis* from sardine oil and characterized its extracellular lipase enzyme (33). Therefore, due to the importance of lipase-producing bacteria as valuable biological resources, the present study was conducted with two objectives including: the first step to isolate of lipase-producing bacteria from the effluents of meat products and vegetable oil factories, and the second step to identify extracellular lipases with beneficial properties from identified bacteria.

2. Objective

The objective of the study was to identify, isolate and characterize of lipase producing bacterial strain from agrifood wastes and to identify and characterize of their lipase genes. The selection of the best lipase-producing bacterial strains and determination of their optimal growth conditions (temperature, pH and incubation time), as well as activity measurement of extracellular lipases produced by these strains in the presence of metal ions, denaturing agents and organic solvents, were the other objectives of this study.

3. Material and Methods

3.1. Sampling and Isolation of Lipase-Producing Bacteria

To isolate and identify lipase-producing bacteria, water and sludge samples were collected from the effluent of Golbahar meat products and Soveyda vegetable oil factories in Lotrestan, Iran. The samples were immediately processed on the same day of collection. The samples were inoculated into a nutrient enriched broth (NB) culture medium containing 1% olive oil in 50 mL polypropylene tubes and were incubated at 28 and 37 °C with shaking for 48 hours. After incubation, the enriched samples were streaked on a lipase screening medium including nutrient agar (NA), rhodamine B and olive oil to obtain lipase-producing single colonies. The cultures were incubated at 28 and 37 °C for 48 h, best strains were screened as lipase producers by the yellow-orange zone formed around colonies under UV light. Finally, selected colonies were subjected to further investigation.

Table 1. Phenotypic and biochemical identification of lipase-producing bacteria.

Test	Isolate Golbahar	Isolate Soveyda
Morphology	Rod-shaped	Rod-shaped
Gram	+	+
Catalase	+	+
Oxidase	+	+
Motility	+	+
Indole	-	-
Tryptophanase	-	-
H_2S	-	-
O/F	Aerobic, Facultative anaerobic	Aerobic, Facultative anaerobic
Glucose fermentation	+	+
Utilization of citrate	+	+
Spore	+	+

3.2. Phenotypic and Biochemical Identification of Lipase-Producing Bacteria

Phenotypic and biochemical tests were used for the identification of lipase-producing bacteria (**Table 1**). The morphology of bacterial strains was analyzed using a light microscope (Olympus, Japan). The phenotypic and biochemical tests included gram staining, spore-forming, oxidase, catalase, aerobic or anaerobic growth, citrate utilization, sulfur, indole, motility, and triple sugar iron. To produce more accurate results, all tests were performed in triplicate.

3.3. Molecular Identification of Lipase-Producing Bacteria

Genomic DNA from pure cultures of lipase-producing bacteria was extracted using SinaClon DNA extraction kit (SinaClon, Iran). The quality and quantity of the extracted DNA were evaluated by NanoDrop (Thermo Fisher Scientific, USA) and agarose gel electrophoresis (AGE). Furthermore, the extracted DNA was used as a template for the polymerase chain reaction (PCR) using Takara Ex *Taq* DNA polymerase (Takara, Japan) and universal 16S rDNA primers (**Table S1**).

The PCR mixture with the final volume of 50 µL contained 50 ng of genomic DNA, 0.25 µL of Takara Ex *Taq* DNA Polymerase (Takara, Japan), 5 µL of 10X Ex *Taq* buffer, 4 µL of 2.5 mM dNTPs, 10 pmol of each of the forward and reverse primers. The reaction was carried out on a Bio-Rad thermocycler (Bio-Rad, USA). The cycling steps included initial denaturation for 10 min at 95 °C, 35 cycles of denaturation for 30 sec at 94 °C, primer annealing for 30 sec at 55 °C, and extension for 1.5 min at 72 °C with a final extension of 15 min at 72 °C. The amplification products were analyzed by electrophoresis in 1% (w/v) agarose gel and evaluated under UV light.

3.4. PCR Amplification of Lipase Encoding Genes

Lipase encoding genes were amplified using specific primers (**Table S1**) and the extracted genomic DNA was used as template. Specific primers of the lipase genes were designed based on nucleotide sequences of lipase gene retrieved from NCBI GeneBank (CP038028.1) using Allele ID 6.0 (Premier Biosoft, USA).

PCR was performed using Takara Ex *Taq* DNA Polymerase (Takara, Japan) and lipase specific primers (**Table S1**). PCR mixture with the final volume of 50 µL contained 50 ng of genomic DNA, 0.25 µL of Takara Ex *Taq* DNA Polymerase (Takara, Japan), 5 µL of 10X Ex *Taq* buffer, 4 µL of 2.5 mM dNTPs, 10 pmol of each of the forward and reverse primers. The reaction was carried out on a Bio-Rad thermocycler (Bio-Rad, USA). The cycling steps included initial denaturation for 10 min at 95 °C, 35 cycles of denaturation for 30 sec at 94 °C, primer annealing for 30 sec at 58 °C, and extension for 60 sec at 72 °C with a final extension of 15 min at 72 °C. The PCR products were electrophoresed on a 1% (w/v) agarose gel and evaluated under UV light.

3.5. Cloning and Sequencing of PCR Products

The PCR products were electrophoresed on a 1% (w/v) agarose gel and evaluated under UV light. The size of the PCR bands was determined using the 1Kbp molecular marker. After evaluating the size, PCR bands were excised from the agarose gel and purified using a gel extraction kit (Thermo Fisher Scientific, USA). The extracted and purified PCR bands were then ligated into pTZ57R/T cloning plasmid using T/A cloning kit (Thermo Fisher Scientific, USA). The cloned PCR bands were sequenced by Metabion (Metabion, Germany) using M13 universal primers. The sequencing results were evaluated by Vector NTI 11.0 (Invitrogen, USA) and BLASTn of NCBI (34).

3.6. Bioinformatic Analysis of Lipase Encoding Genes

The open reading frames (ORFs) of lipase genes were determined using Vector NTI 11.3 (Invitrogen, USA), NCBI ORF-finder, and NCBI CDD (35). Protein functional domains in lipase proteins were predicted using NCBI CDD and the InterProScan software (36, 37). The signal peptides and subcellular localization of lipase proteins were predicted by SignalP 6.0 and CELLO2GO tools (38, 39). The physicochemical features of lipase proteins were predicted using the ProtParam tool (40).

3.7. Multiple Sequence Alignment and Phylogenetic Analysis

Multiple sequence alignment (MSA) was carried out using Vector NTI 11.0. The phylogenetic relationship was constructed by the MEGA 6.0 software (41). The phylogenetic tree was constructed by the maximum likelihood algorithm and evaluated by the bootstrap method (1000 replications).

3.8. Optimization of Growth Conditions of Lipase-Producing Bacteria

To determine the optimum growth temperature, 0.5 McFarland standard of the identified bacteria was cultured in a nutrient-rich broth and incubated at 25, 30, 35, 40, 45, 50, 55, and 60 degrees Celsius for 24 h. After 24 h, the absorbance of the cultures was measured at OD 600 nm. To determine the optimum growth pH, 0.5 McFarland standard of the identified bacteria was cultured in a nutrient-rich broth with pH levels of 3, 4, 5, 6, 7, 8, 9, 10, and 11 for 24 h. After 24 h, the absorbance of the cultures was measured at OD 600 nm. To determine the optimum incubation time for growth, 0.5 McFarland standard of the identified bacteria was cultured in a nutrient-rich broth and incubated for 6, 12, 24, 48, and 72 h. Then, the absorbance of the cultures was measured at OD 600 nm. To determine the optimum carbon source, 0.5 McFarland standard of the identified bacteria was cultured in M9 medium containing different carbon sources, including olive oil, corn oil, sucrose, and glucose. Then, the absorbance of the cultures was measured at OD 600 nm. To determine the optimum nitrogen source, 0.5 McFarland standard of identified bacteria was cultured in M9 medium containing different nitrogen sources, including, tryptone, peptone, yeast extract, ammonium chloride, and ammonium nitrate. Then, the absorbance of the cultures was measured at OD 600 nm.

3.9. Purification of Extracellular Lipases and Lipase Activity Assay

To better evaluate the activity of enzymes, extracellular lipases were purified from the culture media of the bacteria. For this purpose, two liters of optimized culture medium was inoculated separately with 100 mL of each lipase-producing strain and kept in a shaker for 24 hours under optimal conditions. After 24 h, the cultures were centrifuged at 13000 rpm for 10 min at room temperature and the supernatants containing the extracellular lipase proteins were kept in a -20 °C freezer. The supernatant proteins were concentrated using standard ammonium sulfate precipitation and the protein pellets were filtered through a sterile 0.45 µm syringe filter (42). The filtered proteins were dissolved in a minimum amount of buffer containing a 10 mM sodium phosphate buffer, pH 7.0, and dialyzed against the same buffer for 24 h at room temperature. The dialyzed proteins were loaded onto a Sephadex G-50 column (3×150 cm) according to the manufacturer's instructions. The protein fraction containing extracellular lipases was determined by lipase activity assay. The purity and concentration of protein was determined using the SDS-PAGE and Bradford methods (43). The extracellular lipase activity assay was performed with p-nitrophenyl laurate (pNPL) as substrate according to the Winkler and Stuckmann method (44). Briefly, 10 mL of isopropanol containing 30 mg of pNPL was mixed with 90 ml of phosphate buffer (0.05 M), containing 100 mg of gum arabic and 207 mg of sodium deoxycholate. A 2.4 mL of this substrate solution mixed with 0.1 mL of protein fraction containing extracellular lipases. After incubation at 37 °C, the absorbance of solution was measured at OD 410 nm using spectrophotometer.

3.10. The Effects of Temperature and pH on Extracellular Lipase Activity

To determine the effect of temperature, the activity and stability of the extracellular lipases were determined at various temperatures ranging from 25 °C to 60 °C for 1 h. The extracellular lipase activity assay was performed with pNPL as substrate according to the Winkler and Stuckmann method (44). To determine the effect of pH on extracellular lipase activity, equal amounts of the purified extracellular proteins of the identified bacteria were kept at pH levels of 3, 4, 5, 6, 7, 8, 9, 10, and 11 for 1 h. Then, the lipase activity assay was performed with pNPL as substrate according to the Winkler and Stuckmann method (44).

3.11. The Effects of Different Metal Ions on Extracellular Lipase Activity

To determine the effect of different metal ions on extracellular lipase activity, equal amounts of purified extracellular proteins of the identified bacteria were kept with 10 mM of metal ion salts including, NaCl, $CaCl_2$, $ZnCl_2$, KCl, $AlCl_3$, $MnCl_2$, $HgCl_2$, $FeCl_3$, and $CuCl_2$. The reaction mixture was incubated at optimum temperature and pH for 1 h and the lipase activity assay was performed with pNPL as substrate according to the Winkler and Stuckmann method (44).

3.12. The Effects of Different Inhibitory and Denaturing Agents on Extracellular Lipase Activity

To determine the effect of different inhibitory and denaturing agents on extracellular lipase activity, equal

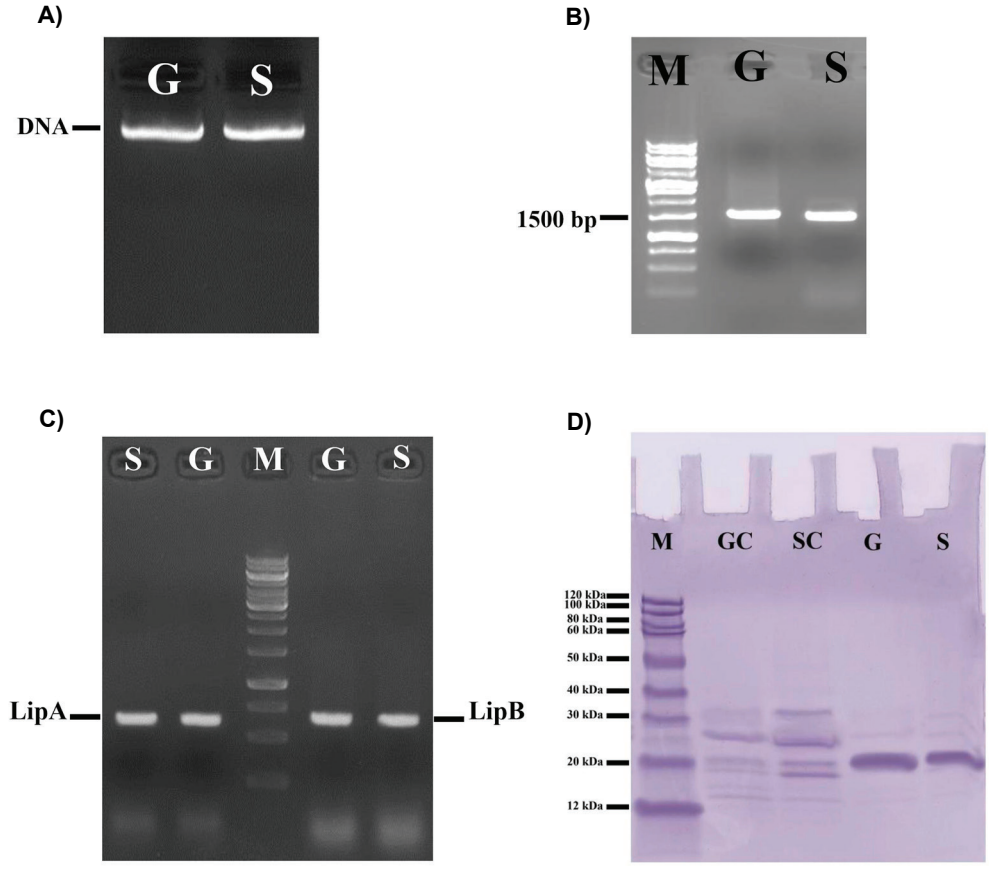

Figure 1. Agarose gel electrophoresis and SDS-PAGE results. **A)** DNA extracted from the Golbahar and Soveyda isolates. **B)** The PCR products of 16S rDNA of the Golbahar and Soveyda isolates. **C)** The PCR products of LipA and LipB genes in Golbahar and Soveyda isolates. **D)** SDS-PAGE of 20 to 25 kDa protein fractions containing extracellular lipases. M: 1Kbp marker and standard protein marker; G: Golbahar isolate; S: Soveyda isolate.

amounts of purified extracellular proteins of the identified bacteria were kept with 1% of the inhibitory and denaturing agents including, EDTA, SDS, Urea, and β-mercaptoethanol. The reaction mixture was incubated at optimum temperature and pH for 1 h and the lipase activity assay was performed with pNPL as substrate according to the Winkler and Stuckmann method (44).

3.13. The Effects of Different Organic Solvents on Extracellular Lipase Activity

To determine the effects of different organic solvents on extracellular lipase activity, equal amounts of purified extracellular proteins of the identified bacteria were kept with 25% of organic solvents, including, chloroform, ethanol, hexane, propanol, and toluene. The reaction mixture was incubated at optimum temperature and pH for 1 h and the lipase activity assay was performed with pNPL as substrate according to the Winkler and Stuckmann method (44).

4. Results

4.1. The Isolation and Phenotypic Characterization of Lipase-Producing Bacteria

The collected samples were examined for the presence of lipase positive bacteria using a screening medium. In total, four and two lipase-producing strains were isolated from effluent of Golbahar meat products and Soveyda vegetable oil factories, respectively. These lipase producer strains formed yellow-orange fluorescent halos on screening medium under UV light.

In the second stage of screening, only one isolate with a clear and large halo was detected in each source and selected for further analyses. The isolates were kept at 4 °C on nutrient agar (NA) plates.

4.2. The Phenotypic and Biochemical Identification of the Lipase-Producing Bacteria

Selected lipase-producing isolates were further characterized by their phenotypic and biochemical properties. Interestingly, the results of phenotypic and biochemical evaluations were the same for both isolates (**Table 1**). Gram staining results showed that the two isolates are gram-positive spore-forming rod-shaped bacteria. The two isolates were catalase positive, oxidase positive, motile, and negative for indole, tryptophanase and H_2S production. They also utilized citrate and fermented glucose. The results of the oxidative-fermentative test showed that the two isolates are aerobic, facultative anaerobic (**Table 1**).

4.3. The Molecular Identification of the Lipase-Producing Bacteria

The quantity and quality of the genomic DNA extracted from the pure cultures of the lipase-producing isolates using NanoDrop and agarose gel electrophoresis analysis showed that the extracted DNA is suitable for PCR amplification (**Fig. 1 and Table S1**). The 16S rDNA genes of lipase-producing isolates were amplified, cloned and sequenced (**Fig. 1**). The near full-length 16S rDNA genes were subjected to BLASTn against the GenBank using the NCBI BLASTn tool. Interestingly, the BLASTn results showed a 99% identity with *Bacillus amyloliquefaciens* species for both lipase-producing isolates. Phylogenetic analysis showed that the lipase-producing isolates belong to the *Bacillus amyloliquefaciens* species and confirmed the results of phenotypic, biochemical, and molecular characterizations (**Fig. 2**).

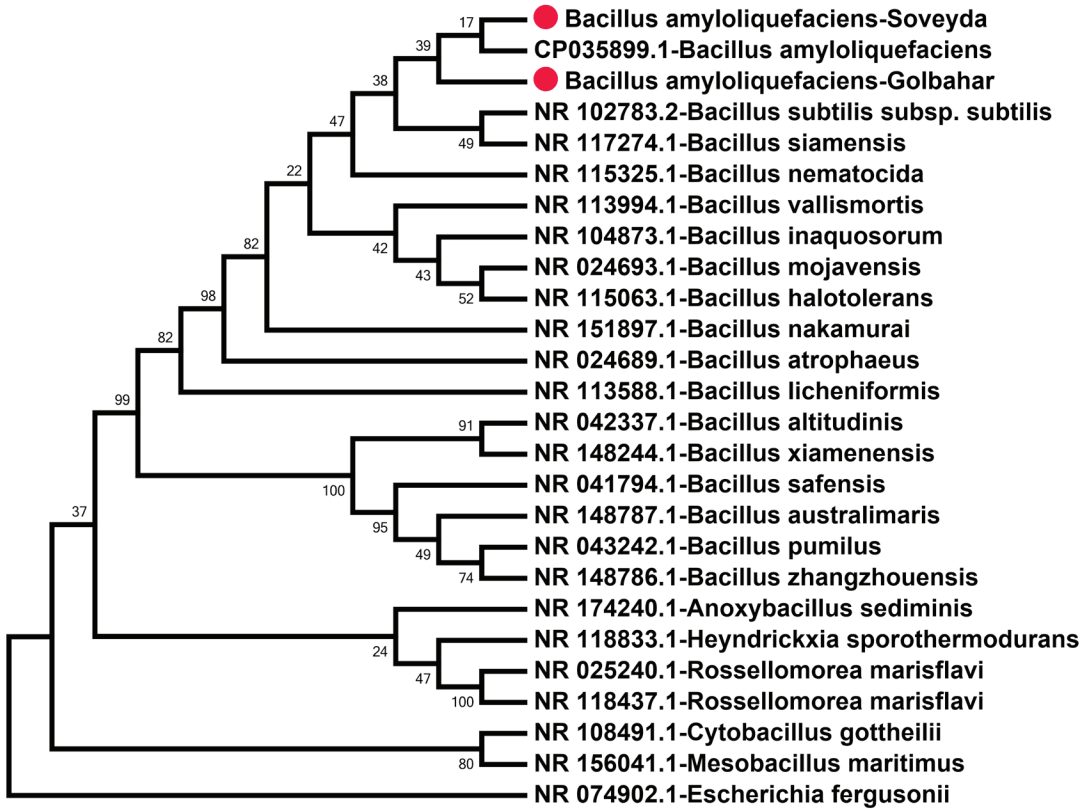

Figure 2. The phylogenetic analysis of the lipase-producing isolates. The phylogenetic tree was constructed by the Neighbor-Joining method and evaluated by the bootstrap method (1000 replications). The lipase-producing isolates are indicated by red circles.

4.4. Molecular and Bioinformatic Analysis of Lipase Encoding Genes

The lipase genes of the lipase-producing isolates were successfully amplified, cloned, and sequenced (**Fig. 1c**). The full-length lipase genes were subjected to BLASTn against the GenBank using the NCBI BLASTn tool. The BLASTn confirmed accurate identification of lipase genes of lipase-producing isolates. Sequence analysis showed coding sequences (CDS) of 645 bp and 615 bp for LipA and LipB genes in both lipase-producing isolates. The CDS of both LipA and LipB genes started with the ATG codon and terminated with the TAA codon in the lipase-producing isolates. The LipA and LipB genes produced 214 and 204 amino acid proteins in the lipase-producing isolates, respectively. The signal peptide prediction showed 30 and 32 amino acid secretory signal peptides in LipA and LipB proteins, respectively. The analysis of the physicochemical properties revealed that the molecular mass ranged from 21.695 to 22.844 kDa, the isoelectric pH was in the range of 9.33 to 9.68, the instability index varied from 19.06 to 23.2, the aliphatic index was between 97.01 and 98.83, and the GRAVY value ranged from -0.057 to 0.082 for the lipase genes (**Table 2**).

Multiple sequence alignment (MSA) was used to investigate the possible differences between the extracellular lipases of the two lipase-producing isolates. The MSA results showed differences between LipA and LipB in the Golbahar and Soveyda isolates. The MSA showed three different amino acids at positions 14, 100 and 165 of in the LipA extracellular lipase (**Fig. 3A**). In position 14, glutamic acid (polar) and valine (non-polar) had different physicochemical properties. This was also observed in position 100 where aspartic acid (polar) and glycine (non-polar) had different physicochemical properties. In position 165, glycine (non-polar) and alanine (non-polar) had the same physicochemical properties.

The MSA also showed one different amino acid at residue 102 in the LipB extracellular lipase (**Fig. 3B**). In position 102, alanine (non-polar) and aspartic acid (polar) had different physicochemical properties.

4.5. The Optimization of The Growth Conditions of the Lipase-Producing Bacteria

The same growth parameters were obtained for both lipase-producing isolates in growth optimization experiments. The lipase-producing isolates had the highest growth rate at 35 °C compared to the other tested temperatures. The pH of 7 compared to the other tested pH levels was suitable for the growth of lipase-producing isolates. The highest growth rate for the lipase-producing isolates was obtained at the incubation time of 24 h. The lipase-producing isolates had the highest growth rate on olive oil and sucrose carbon sources. The peptone in comparison with the other tested nitrogen sources was more effective in enhancing the growth of the lipase-producing isolates and it was shown that the highest growth rate can be achieved in the presence of peptone.

4.6. The Purification of the Extracellular Lipases

In order to ensure the accurate separation of the

Table 2. The physicochemical properties of lipase genes

Gene Name	Protein Length	Signal length	Subcellular Localization	MW (KDa)	pI	Instability index	Aliphatic index	GRAVY
LipA-G	214 aa	30 aa	Extracellular	22.844	9.49	23.2	97.01	-0.057
LipB-G	204 aa	32 aa	Extracellular	21.695	9.46	20.94	97.99	0.082
LipA-S	214 aa	30 aa	Extracellular	22.77	9.68	19.6	98.83	0.004
LipA-S	204 aa	32 aa	Extracellular	21.739	9.33	20.21	97.5	0.056

GRAVY: Grand average of hydropathy, MW: Molecular weight, aa: Amino acids, G: Golbahar isolate, S: Soveyda isolate, pI: Isoelectric pH

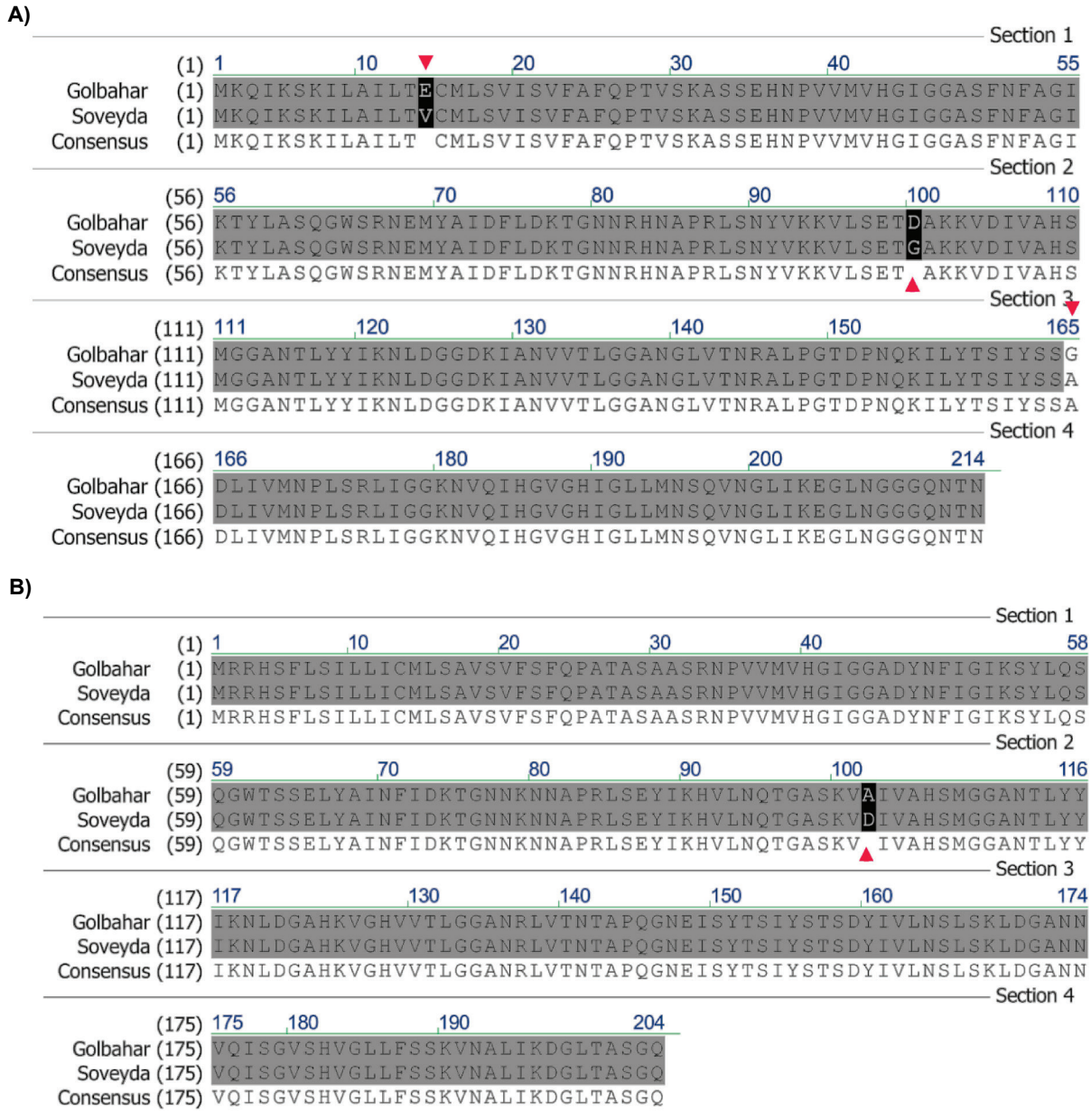

Figure 3. **A)** The MSA analysis of the LipA extracellular lipase in both lipase-producing isolates. The position of amino acid change is indicated by red triangles. **B)** The MSA analysis of the LipB extracellular lipase in two lipase-producing isolates. The position of amino acid change is indicated by red triangles.

protein fraction containing extracellular lipases, all protein fractions obtained from the gel filtration were examined for lipase activity. The highest lipase activity was observed in the 20 to 25 kDa fraction, while no lipase activity observed in other fractions. These results indicate the correct identification of the extracellular lipases (**Fig. 1d**).

4.7. The Effects of Temperature and pH on Extracellular Lipase Activity

The lipase activity of the Golbahar lipase-producing isolate gradually increased with increasing temperature from 25 °C and reached its maximum at 45°C and then gradually decreased (**Fig. 4A**). Moreover, the lipase activity of this isolate gradually increased

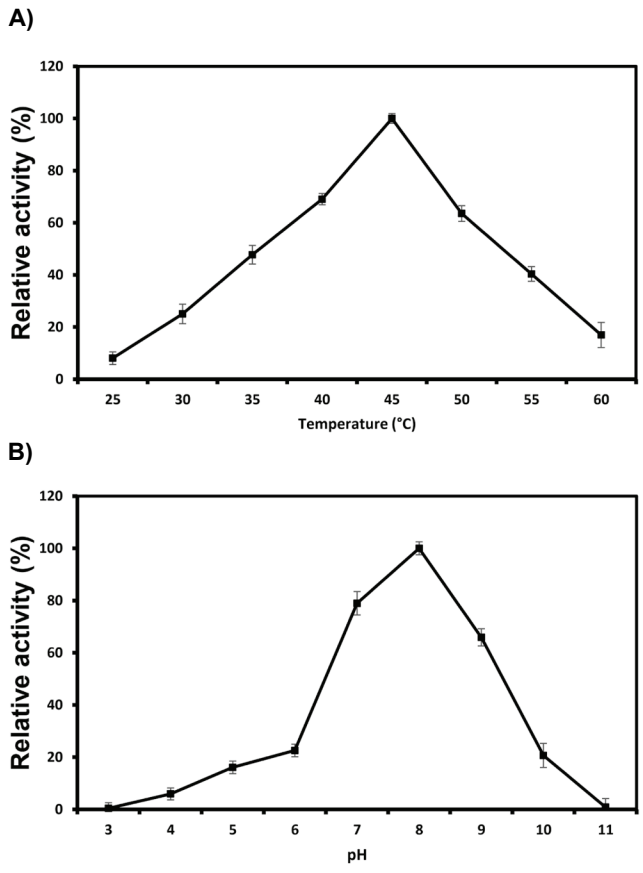

Figure 4. The effects of temperature and pH on the activity of the Golbahar isolate extracellular lipase. Relative lipase activity under different **A)** temperatures and **B)** pH levels.

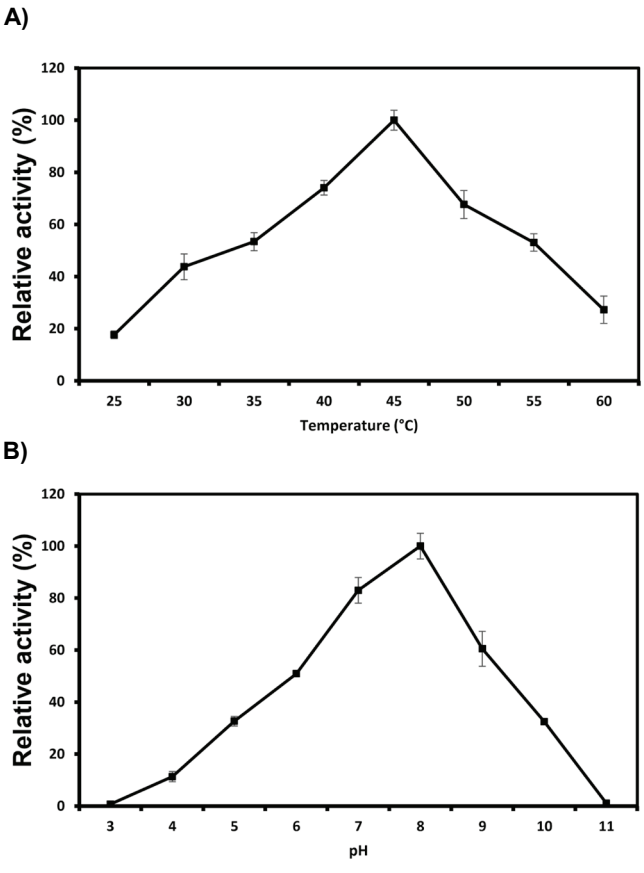

Figure 5. The effects of temperature and pH on the activity of the Soveyda isolate extracellular lipase. Relative lipase activity under different **A)** temperatures and **B)** pH levels.

with increasing pH from 3 and reached its maximum at pH 8 and then gradually decreased (**Fig. 4B**).

The lipase activity of the Soveyda lipase-producing isolate gradually increased with increasing temperature from 25 °C and reached its maximum at 45 °C and then gradually decreased (**Fig. 5A**). Additionally, the lipase activity of this isolate gradually increased with increasing pH from 3 and reached its maximum at pH 8 and then gradually decreased (**Fig. 5B**).

There were differences in lipase activity in optimized conditions between the lipases of the Golbahar and Soveyda isolates. The Golbahar isolate exhibited greater lipase activity compared to the Soveyda isolate (**Fig. 6**).

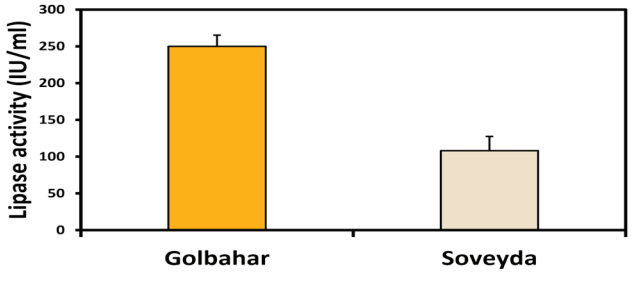

Figure 6. The comparison of the lipase activities of lipase-producing isolates.

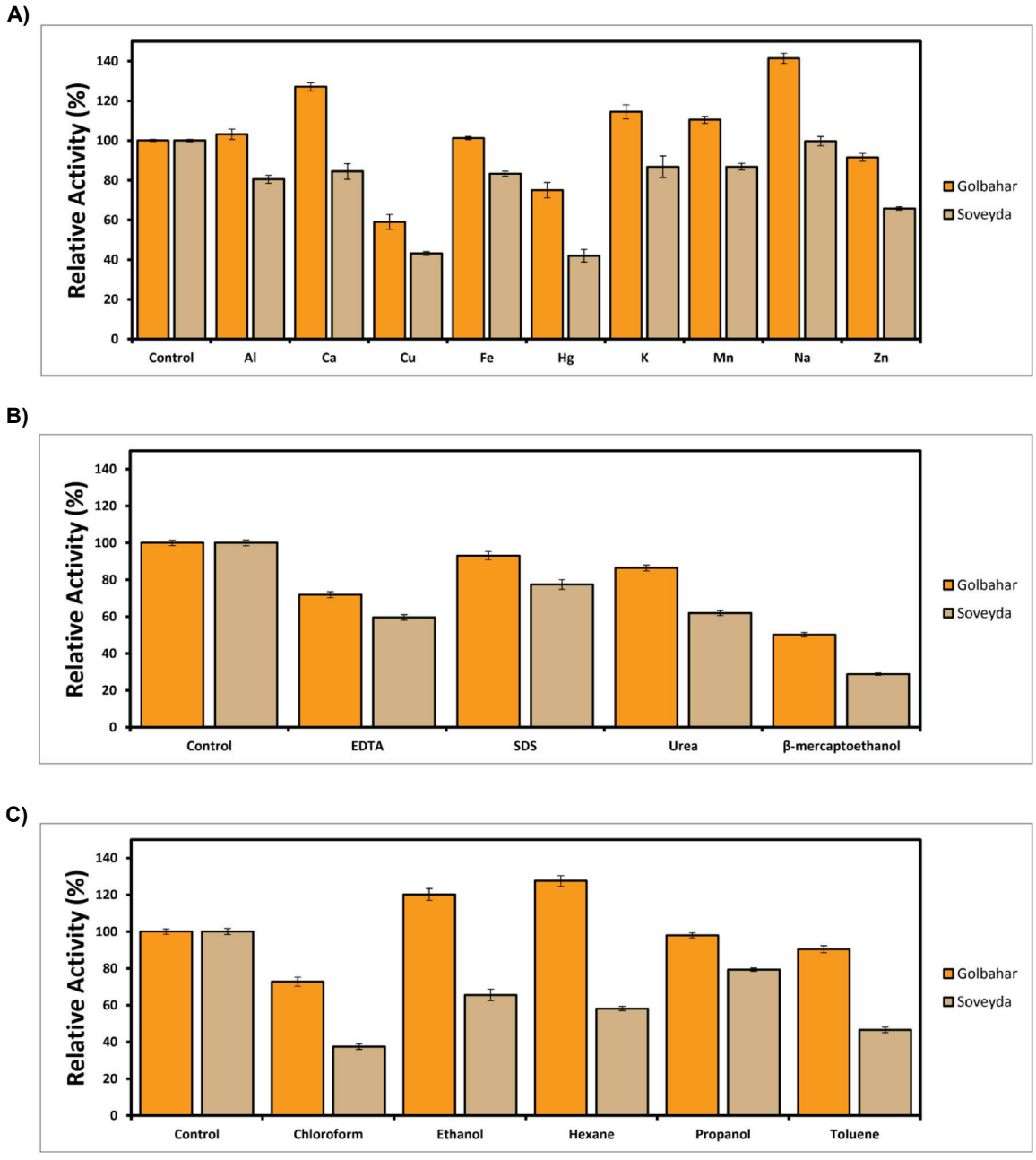

Figure 7. **The lipase activity in the presence of metal ions, inhibitory and denaturing agents and organic solvents in comparison to the control. A)** The effects of different metal ions on extracellular lipase activity. **B)** The effects of different inhibitory and denaturing agents on extracellular lipase activity. **C)** The effects of different organic solvents on extracellular lipase activity.

4.8. The Effects of Different Metal ions on Extracellular Lipase Activity

Various metal ions showed different effects on the lipase activity of the lipase-producing isolates (**Fig. 7A**). The Golbahar lipase showed higher activity than the Soveyda lipase in the presence of various ions. The activity of the Golbahar lipase in the presence of Al^{3+} ion did not show difference compared to the control, while the activity of the Soveyda lipase decreased in the presence of this ion. In the presence of Ca^{2+} ion, the activity of the Golbahar lipase increased compared to the control, while the activity of the Soveyda lipase

decreased. The activity of both Golbahar and Soveyda lipases decreased in the presence of Cu^{2+} ion. The lipase activity of the lipase-producing isolates showed a similar trend to Al^{3+} ion in the presence of Fe^{2+} ion. The Hg^{2+} ion showed similar effects to Cu^{2+} ion on the lipase activity of the lipase-producing isolates (**Fig. 7B**). The K^{+}, Mn^{2+}, and Na^{+} ions increased the activity of the Golbahar lipase, while they did not affect the activity of the Soveyda lipase. The Zn^{2+} ion exhibited effects on the lipase activity of the lipase-producing isolates similar to the Cu^{2+} and Hg^{2+} ions (**Fig. 7A**).

4.9. The Effect of Different Inhibitory and Denaturing Agents on Extracellular Lipase Activity

The various inhibitory and denaturing agents exhibited different effects on the lipase activity of the lipase-producing isolates (**Fig. 7B**). The lipase activity of the lipase-producing isolates decreased in the presence of different inhibitory and denaturing agents. The lipase activity of the Soveyda isolate showed a greater decrease compared to the Golbahar isolate in the presence of different inhibitory and denaturing agents. The β-mercaptoethanol (2ME) and SDS showed the highest and lowest inhibitory effect on the lipase activity of both lipase-producing isolates, respectively (**Fig. 7B**).

4.10. The Effects of Different Organic Solvents on Extracellular Lipase Activity

Various organic solvents showed different effects on the lipase activity of the lipase-producing isolates (**Fig. 7C**). The activity of the Golbahar and Soveyda lipases decreased in the presence of chloroform. Ethanol and hexane increased the activity of the Golbahar lipase, but decreased the activity of the Soveyda lipase. Propanol did not affect the activity of the Golbahar lipase. However, it decreased the activity of the Soveyda lipase. Toluene showed similar effects to chloroform on the lipase activities of both lipase-producing isolates (**Fig. 7C**).

5. Discussion

Lipases are an important group of enzymes that are capable to hydrolyze ester bonds in triacylglycerol molecules. Lipase enzymes exist widely in nature and are found in animals, plants, fungi, and microorganisms (17, 22, 23). However, most industrial lipases have microbial origins (bacteria and fungi). Due to their unique features, bacterial lipases have attracted more and more attention from researchers (45-48). Therefore, the present study was conducted to isolate and identify lipase-producing bacteria from different sources and to identify their lipase genes.

Samples collected from wastes of different meat products and vegetable oil companies were screened, revealing the presence of lipase-producing strains from both sources. In total, four and two lipase-producing strains were isolated from the effluents of the Golbahar meat products and the Soveyda vegetable oil factories, respectively. In the second stage of screening, only one isolate with a clear and large halo was detected in each source and selected for further analyses. The same phenotypic, biochemical, and molecular analyses were carried out on both isolates. Interestingly, the BLASTn results confidently identified both isolates as *B. amyloliquefaciens* species with a great similarity. The phylogenetic analysis recognized the lipase-producing isolates as belonging to the *B. amyloliquefaciens* species and confirmed the results of the phenotypic, biochemical and molecular characterizations. So far, the *B. amyloliquefaciens* species have been isolated from different lipid-rich sources. In a study, *B. amyloliquefaciens* was isolated from dairy products (49). In the other study, *B. amyloliquefaciens* was isolated from soybean paste (50). In a similar study, *B. amyloliquefaciens* was detected in lipid-rich food waste (31). Due to the presence of high amounts of lipids in food factories, lipase-producing bacteria are very likely to be present in these environments.

The lipase genes were successfully identified from the lipase-producing isolates. Sequence analysis showed coding sequences (CDS) of 645 bp and 615 bp for LipA and LipB genes in both lipase-producing isolates. The LipA and LipB genes produced 214 and 204 amino acid proteins in the lipase-producing isolates, respectively. Signal peptide prediction showed 30 and 32 amino acid secretory signal peptides in LipA and LipB proteins, respectively.

In a study, an extracellular lipase was identified from *Bacillus tequilensis* that comprised a secretory signal peptide accumulating in the extracellular environment (8). In a similar study, an extracellular lipase was identified from *Bacillus subtilis* (51). Bacterial lipases are generally produced and secreted by the induction of lipase genes. After production, extracellular lipases must be secreted outside the

cell for lipid hydrolysis. Therefore, the presence of secretory signal peptides is necessary to transport lipases out of the bacterial cell (52).

The analysis of physicochemical properties of extracellular lipases revealed that the molecular mass ranged from 21.695 to 22.844 kDa, the isoelectric pH level was in the range of 9.33 to 9.68, the instability index varied from 19.06 to 23.2, the aliphatic index was between 97.01 and 98.83, and the GRAVY value had a range between -0.057 and 0.082. Bacterial lipases typically have a molecular mass between 19 and 60 kDa and, therefore, have variable sizes (52-54). The calculated isoelectric pH (pI) shows the basic properties of the identified extracellular lipases, which is due to the relatively high abundance of basic amino acids in their structures (53, 54). The instability index shows the different stability levels of extracellular lipases as one of the factors affecting the activity of these enzymes (17, 52). The aliphatic index of extracellular lipases indicates their appropriate temperature resistance, which is due to the secondary structures and the high abundance of specific amino acids. This index specifies the relative volume occupied by the aliphatic chains of amino acids and shows the level of hydrophobicity and the stability of proteins (55).

The maximum lipase activity of the Golbahar and Soveyda isolates was observed at 45 °C and the pH of 8, but the Golbahar isolate exhibited higher lipase activity compared to the Soveyda isolate. One of the most important factors affecting the activity of enzymes, including lipases, is temperature, which plays a significant role in their industrial applications. Finding the optimal temperature for enzyme activity is among the most important points that should be considered in the industrial application of enzymes (12, 22). Results show that both isolates identified in this study and their lipase enzymes are mesophilic and have the best activity at temperatures below 50 degrees centigrade. The optimal activity pH is among other factors affecting enzyme activity, which plays a crucial role in their industrial applications. Most lipase enzymes are active in a wide range of pH levels which is suitable for industrial applications (12, 22, 23). Results show that both isolates identified in this study have the highest lipase activity at a pH of 8. This shows that the Golbahar and Soveyda lipases have good stability and activity at alkaline pH levels and can be considered alkaline lipases (24).

The various metal ions showed different effects on the lipase activity of both lipase-producing isolates. The lipase activity of the Golbahar isolate in most cases showed an increasing trend in the presence of different metal ions, except for the Cu and Hg ions, while the lipase of the Soveyda isolate showed a decreasing trend in the presence of different metal ions. Measuring the activity of lipases in the presence of metal ions is necessary because in industrial applications these enzymes are used in solutions containing various metal ions. By binding to the functional domains of enzymes, some metal ions promote the activity of lipases. On the other hand, some metal ions inhibit lipase activity by denaturing the protein structure of enzymes (56). Previous studies have shown that Ca, Mg, Mn, Ni, and Ba can improve lipase activity (57-61). The presence of the Ca ion alone or with other divalent ions such as Mn in the culture medium increases the production and activity of lipase in bacteria. On the other hand, these ions may play the role of cofactor for extracellular lipase enzymes and increase their activity (12, 59, 62, 63). Metal ions, such as Cu, Hg, Zn, and Fe have inhibitory effects on lipase activity. The activity of lipase enzymes in the presence of metal ions depends on the concentration of ions and the sources of enzymes (9, 58, 59, 61, 64, 65).

The various inhibitory and denaturing agents exhibited different effects on lipase activity of the lipase-producing isolates. The lipase activity of both lipase-producing isolates decreased in the presence of different inhibitory and denaturing agents. The lipase activity of the Soveyda isolate showed a greater decrease compared to the Golbahar isolate in the presence of different inhibitory and denaturing agents. The β-mercaptoethanol and SDS showed the highest and lowest inhibitory effects on the lipase activity of both isolates, respectively. Chelating agents may chelate metal ions that are essential for lipase activity and reduce the activity of lipases. Denaturing agents usually have a reducing effect on the activity of lipase enzymes because they denature and disrupt the protein structure of lipases. The β-mercaptoethanol, as a strong protein denaturing agent, strongly decreased the lipase activity of the isolates. But considering that β-mercaptoethanol is not used simultaneously with lipase enzymes, it will not cause any problems for industrial use. On the other hand, other denaturing

agents such as SDS, which have a lower effect on lipase activity, are used together with lipase enzyme in some cases. For example, SDS exists in most detergent powders alongside lipase enzymes. Previous studies have shown the different effects of SDS on the activity of the *Aspergillus oryzae* lipase. In a study, 1% SDS dramatically reduced the activity of lipase enzyme (66). On the other hand, in a similar study, *Ochrobactrum intermedium* lipase was stable in the presence of 1% SDS and its relative activity increased by about 30% (67). The proper activity of lipases in the presence of denaturing agents and detergents is necessary for their use in the detergent industry. Therefore, the identified lipases in this study are suitable candidates for use in detergent and food industries.

The various organic solvents showed different effects on the lipase activity of the identified isolates. The activity of the Soveyda lipase decreased in the presence of all organic solvents, while the Golbahar lipase showed a significant decrease only in the presence of chloroform. Previous studies have also reported the different effects of various organic solvents on lipase activity. In a study, the *Pseudomonas fluorescens* lipase showed proper stability in the presence of octane, xylene, hexane, and heptane (68). In contrast, the activity of *O. intermedium* lipase was reduced in the presence of chloroform and ethanol in a similar study (67).

In general, our results showed different properties and activity levels between LipA and LipB in the Golbahar and Soveyda isolates. This difference in activity may be due to structural changes and gene mutations that cause changes in the protein structures of enzymes. MSA results showed differences between the protein sequences of LipA and LipB in the Golbahar and Soveyda isolates. MSA exhibited different amino acid substitutions in the LipA and LipB extracellular lipases that had different physicochemical properties. These differences may affect the structure and catalytic function of lipases. On the other hand, different lipase activity may be due to different gene expression regulation in the identified lipase-producing isolates (69-71).

6. Conclusion

In this study, two extracellular lipase-producing isolates of *B. amyloliquefaciens* were identified from different food industries, and their characteristics were investigated. The gene sequence encoding extracellular lipases were also identified in these isolates. The results of various investigations showed that the lipases produced by the Golbahar isolate have better characteristics than the lipases of the Soveyda isolate. The Golbahar lipases have a suitable temperature and pH activity range and maintain their activity in the presence of some ions, inhibitors, denaturing agents, and organic solvents.

Therefore, it is a suitable candidate for industrial applications as well as future studies. The purified Golbahar lipases should be subjected to various investigations. The enzyme can be purified from the culture medium of the Golbahar isolate or expressed in different recombinant expression systems via genetic engineering methods. After further investigation, the Golbahar isolate lipase can be used in various industries. In addition, this lipase can be used enzyme engineering processes and its activity can be arbitrarily changed by targeted mutations.

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Conflicts of Interest

The authors have no conflicts of interest to disclose.

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