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ISOLATION, SCREENING, AND MOLECULAR IDENTIFICATION OF LIPASE-PRODUCING BACTERIA FROM DIFFERENT BACTERIAL SOURCES

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ABSTRACT

The need for enzymes is growing across a number of industries due to their several advantages, such as their minimal environmental toxicity. Lipases are one of the most valuable biotechnological enzymes. As a result, studies on lipases have become significantly popular in the field of enzymology in recent years. Several attempts have been made to isolate different bacterial isolates that can produce the lipase enzyme. The current study aims to isolate diverse bacterial isolates from different sources: soil contaminated with oil, animal wounds, and contaminated culture media. A qualitative screening for lipase-producing isolates was conducted using tween 80 agar. The results showed that a total of 124 bacterial isolates were obtained from different sources, including 41 isolates that can produce the lipase enzyme. Upon the lipase activity assay, the 20 highest lipase-producing isolates were identified phenotypically. The best potential lipase producers were further identified using 16S rRNA sequencing as *Brevibacillus sp.* strain HC1 and *Brevibacillus sp.* strain HS5, with the accession numbers OR048061 and OR048060, respectively. This study highlights the significance of using bacteria as a microbial source for lipase enzyme production for future industrial and biotechnological applications.

Keywords: Lipase enzyme, Lipase-producing bacteria, Tween 80 agar, Lipase activity assay, 16S rRNA sequencing

INTRODUCTION

Enzymes are very effective biocatalysts that increase the rate of different reactions. Therefore, the use of enzymes for industrial-scale catalysis in different applications have been extensively investigated (Chapman *et al.*, 2018 and Al-Ghanayem and Joseph, 2020). The need for enzymes is rising across several industrial sectors because of their special properties, such as high specificity and reactivity. By 2025, the global enzyme market may reach 13 to 14 billion US dollars, based on recent studies (Al-Ghanayem and Joseph, 2020).

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Enzymes are classified based on their catalytic activity into six categories of enzymes: lyases, ligases, hydrolases, oxidoreductases, and transferases (Gürkök, 2019). After proteases and carbohydrases, lipases are the third most valuable industrial enzymes on the global market (Gurkok and Ozdal, 2021). Lipases (E.C.3.1.1.3) are enzymes that catalyze the hydrolysis of oils and fats into free fatty acids, diglycerides, monoglycerides, and glycerol (Hemlata *et al.*, 2016). Lipases are serine-hydrolases that belongs to the superfamily of alpha-beta hydrolases. A cofactor is not necessary for lipases to function (Gürkök, 2019).

Although many species of plants, animals, fungi, yeast, and bacteria can produce lipases, the enzymes from microbial origins are the most intriguing in biotechnological processes due to their distinctive properties, such as their continuous production regardless of season, high yield, high activity, stability, and ease of molecular alterations (Cherif *et al.*, 2011 and Salihu and Alam, 2015). Almost 90% of the overall industrial enzyme market is made up of enzymes derived from microorganisms (Phukon *et al.*, 2020). Many different habitats, such as soil contaminated with oil and various industrial wastes, have been shown to include lipase-producing microbes. A favorable habitat for the isolation of microorganisms, such as bacteria that produce lipase enzymes, may be provided by the oily environment (Mobarak-Qamsari *et al.*, 2011).

Mesophilic bacteria have provided the bulk of bacterial lipases utilized in commercial applications, including Staphylococcus sp., Bacillus sp., Pseudomonas sp., and Burkholderia sp. (Abol-Fotouh *et al.*, 2021). The Brevibacillus genus is one of the gram-positive genera that are found in a variety of environmental habitats. This genus is considered a good source of several enzymes of significant biotechnological use due to several advantages, such as their high growth rate (Panda *et al.*, 2014).

Lipases from microbial sources are traditionally produced through submerged fermentation since they are extracellular enzymes, and this method allows for more precise control of the production conditions than solid-state fermentation (Hemlata *et al.*, 2016). Lipases are mostly inducible enzymes that are produced in the presence of inducers such as oils, fatty acids, tri-acylglycerols, and bile salts. This carbon source has been the main factor in lipase enzyme expression (Bora and Kalita, 2008).

MATERIALS AND METHODS

1- Collection of samples

Twenty samples were collected from each of the following sources: oil-contaminated soil, animal wounds, and contaminated culture media. Soil samples contaminated with oil (approximately 10g) were collected in sterile sample containers and kept in a refrigerator (Phukon *et al.*, 2020). Swab samples were collected from different animal wounds presented at the clinic of the veterinary teaching hospital, Faculty of Veterinary Medicine, Assuit University, Egypt. Before collecting the samples, the swabs were dipped in sterilized phosphate buffer saline. The swab samples were then cultured in nutritional broth for 24 hours at 37°C (Sarwar *et al.*, 2021). Contaminated culture media samples were also collected and screened for lipase-producing bacteria.

2- Isolation of bacteria and screening for lipase-producing isolates (Qualitative screening method)

Isolation of the bacteria from different samples was done on nutrient agar at 37 °C for 24 hours. Preliminary screening of these bacterial isolates was performed by the plate-based screening method. This was carried out by inoculating the different isolates on tween 80 agar plates. The plates were incubated for 48 hours at 37 °C. The lipase-producing bacteria showed an opalescent zone around the colony (Haba *et al.*, 2000 and Ilesanmi *et al.*, 2020).

3- Determination of lipase activity of different lipase-producing isolates (Quantitative screening method)

After the qualitative identification of bacterial isolates that produce lipase enzyme, the isolates were used for lipase production. These isolates were inoculated on lipase-producing liquid media. The liquid culture media contain glucose (10 gram/liter), peptone (10 gram/liter), yeast extract (5 gram/liter), NaCl (5 gram/liter), K_2HPO_4 (0.3 gram/liter), KH_2PO_4 (1 gram/liter), $CaCl_2$ (2 gram/liter), $MgSO_4.7H_2O$ (0.2 gram/liter), $(NH4)_2SO_4$ (2 gram/liter), and olive oil 2% (v/v) at 37ºC for 48 hours with pH 7.0 (Soleymani *et al.*, 2017). The generated supernatant from centrifuging this medium at 10,000 rpm for 10 minutes at 4ºC was used as an enzyme extract for lipase. The lipase activity of the extracellular lipase was estimated using p-nitrophenyl palmitate (pNPP) as a substrate. The reaction consisted of 160 µl of 0.1 M sodium phosphate buffer (pH 7), 20 µl of enzyme extract, and 20 µl of 0.01 M pNPP in isopropyl alcohol. The reaction was then incubated at 30ºC for 30 minutes. Finally, we added 50 µl of 0.1 M sodium carbonate to stop the reaction. The absorbance of the released p-nitrophenol was measured using a microplate reader at 410 nm (Khosla *et al.*, 2017 and Phukon *et al.*, 2020). A calibration curve using p-nitrophenol was prepared as a standard. One unit of lipase enzyme was expressed as the amount of lipase enzyme required to liberate one µmole of p-nitrophenol from pNPP per minute under the assay conditions (Bussamara *et al.*, 2010). The experiments were carried out in triplicate, and the mean and standard deviation of the data were provided.

4- Phenotypic identification of lipase-producing bacterial isolates

Using conventional phenotypic identification techniques, the 20 bacterial isolates with the highest lipase production were identified. First, a gram stain was used for direct microscopic examination. Various biochemical reactions were also conducted such as catalase, oxidase, indole production, citrate utilization, and urease. In addition, the ability of the bacteria to ferment carbohydrates was tested on triple sugar iron agar (Collee *et al.*, 1996, de Melo Oliveira *et al.*, 2013, AS and HA, 2019 and ABED *et al.*, 2021).

5- Molecular identification of the best lipase-producing isolates using 16S rRNA sequencing

The best lipase-producing isolates were identified by 16S rRNA gene sequencing. The region of 16S rRNA was amplified using the universal forward primer 27F (5′AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 1492 R (5′-GGTTACCTTGTTACGACTT-3′) (Li and Zhang, 2005 and Ji *et al.*, 2015).

Then, the obtained sequences were deposited in the NCBI database (HARDI *et al.*, 2020 and Gurkok and Ozdal, 2021). These nucleotide sequences were compared to the sequences that are present in the NCBI GenBank. Multiple sequence alignments of our sequences were performed using Multiple Sequence Comparisons by Log-Expectation (MUSCLE). The phylogenetic trees were then constructed by the neighbor joining method using Molecular Evolutionary Genetics Analysis (MEGA 11 software), which is computer software for constructing phylogenetic trees (Abol-Fotouh *et al.*, 2021).

RESULTS

1- Isolation of bacteria and screening for lipase-producing isolates (Qualitative screening method):

The isolation of bacteria from different samples was done on nutrient agar medium. Then, the isolated bacteria were examined for lipase production using tween 80 agar medium. The bacterial isolates that can produce lipase enzyme were identified by the formation of an opalescent zone around the bacterial colony, as shown in Figure 1.

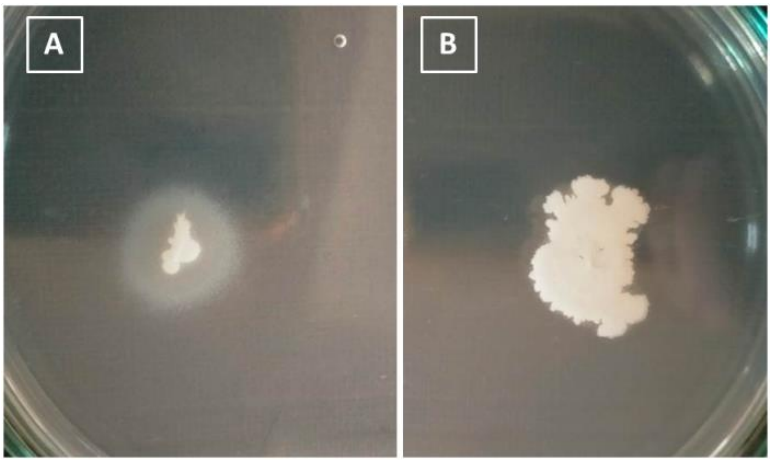

Figure 1: Screening for bacterial isolates that can produce the lipase enzyme on tween 80 agar medium. A: positive isolate; B: negative isolate.

In this study, a total of 124 bacterial isolates were isolated from the different samples. These bacterial isolates were screened for their ability to produce extracellular lipase enzyme. It was observed that 41 bacterial isolates showed a positive result for lipase production (Table 1).

Table 1: The result of the screening study for the lipase-producing bacteria isolated from different sources.

Source	No. of samples	No. of total bacterial	No. of lipase-producing	Percentage
Oil-contaminated soil	20	74	23	31.1% (23/74)
Animal wound	20	20	8	40% (8/20)
Contaminated culture media	20	30	10	33.3% (10/30)
Total	60	124	41	33.1% (41/124)

From this table, it is obvious that the majority of the lipase-producing bacterial isolates were obtained from oil-contaminated soil samples.

2- Determination of lipase activity of different bacterial isolates that produce the lipase enzyme (Quantitative screening method):

The lipase-producing isolates obtained from the previous qualitative method were further screened quantitatively using para-nitrophenyl palmitate (pNPP). A liquid lipase production medium supplemented with olive oil was used to produce the lipase enzyme from the bacterial isolates. After centrifugation, the supernatants were assayed for lipase activity. The lipase activity assay of different lipase-producing bacterial isolates is expressed in Table 2.

Table 2: Lipase activity assay (U/ml) of different lipase-producing bacterial isolates from different sources.

Serial No. of the bacterial isolate	Bacterial isolate code	Source of the bacterial isolate	Activity assay of the bacterial isolate (U/ml)
1	C1	Contaminated culture media	25.76±2.137
2	C2	Contaminated culture media	1.560±0.4621
3	C3	Contaminated culture media	1.386±0.05777
4	C6	Contaminated culture media	1.733±0.4044
5	C7	Contaminated culture media	10.92±1.502
6	C10	Contaminated culture media	13.81±0.2311
7	C12	Contaminated culture media	0.8087±0.05777
8	C15	Contaminated culture media	6.528±1.271
9	C19	Contaminated culture media	2.195±0.1733
10	C20	Contaminated culture media	15.42±0.2311
11	S1.1	Oil contaminated soil	15.65±3.351
12	S2.2	Oil contaminated soil	0.9820±0.1155
13	S3.1	Oil contaminated soil	8.232±1.704
14	S3.3	Oil contaminated soil	1.502±0.6354
15	S4.2	Oil contaminated soil	1.386±0.6354
16	S5.1	Oil contaminated soil	25.82±2.542
17	S5.4	Oil contaminated soil	8.781±0.05777
18	S6.3	Oil contaminated soil	8.723±1.155
19	S7.1	Oil contaminated soil	0.4621±0.1733
20	S7.2	Oil contaminated soil	0.5199±0.1155
21	S8.1	Oil contaminated soil	10.63±2.715
22	S8.5	Oil contaminated soil	1.098±0.01
23	S9.1	Oil contaminated soil	1.849±0.1733
24	S9.3	Oil contaminated soil	5.372±1.040
25	S11.2	Oil contaminated soil	1.617±0.1733
26	S12.1	Oil contaminated soil	1.444±0.1155
27	S13.2	Oil contaminated soil	1.329±0.3466
28	S14.1	Oil contaminated soil	0.5777±0.1733
29	S15.1	Oil contaminated soil	1.964±0.1733
30	S15.3	Oil contaminated soil	1.675±0.2311
31	S17.1	Oil contaminated soil	14.41±4.535
32	S19.2	Oil contaminated soil	17.99±1.069
33	S20.1	Oil contaminated soil	21.84±0.1733
34	W1	Animal wound	1.040±0.2888
35	W5	Animal wound	2.080±0.05777
36	W7	Animal wound	1.791±0.6932
37	W12	Animal wound	5.199±1.098
38	W13	Animal wound	17.88±3.379
39	W14	Animal wound	2.946±0.1155
40	W16	Animal wound	2.022±0.3466
41	W19	Animal wound	1.906±0.2311

The activity assay results were expressed as means ± SD.

3- Phenotypic identification of the highest lipase-producing bacterial isolates:

The twenty highest lipase-producing isolates were selected and subjected to phenotypic identification as presented in Table 3.

Table 3: Phenotypic identification of the highest lipase-producing isolates.

Serial No. of the bacterial isolate	Bacterial isolate code	Source of the bacterial isolate	Activity assay of the bacterial isolate (U/ml)	Phenotypic identification of the bacterial isolate
1	C1	Contaminated culture media	25.76±2.137	*Bacillus sp.*
2	C7	Contaminated culture media	10.92±1.502	*Bacillus sp.*
3	C10	Contaminated culture media	13.81±0.2311	*Bacillus sp.*
4	C15	Contaminated culture media	6.528±1.271	*Bacillus sp.*
5	C19	Contaminated culture media	2.195±0.1733	*Bacillus sp.*
6	C20	Contaminated culture media	15.42±0.2311	*Bacillus sp.*
7	S1.1	Oil-contaminated soil	15.65±3.351	*Staphylococcus aureus*
8	S3.1	Oil-contaminated soil	8.232±1.704	*Staphylococcus aureus*
9	S5.1	Oil-contaminated soil	25.82±2.542	*Bacillus sp.*
10	S5.4	Oil-contaminated soil	8.781±0.05777	*Klebsiella sp.*
11	S6.3	Oil-contaminated soil	8.723±1.155	*Klebsiella sp.*
12	S8.1	Oil-contaminated soil	10.63±2.715	*Bacillus sp.*
13	S9.3	Oil-contaminated soil	5.372±1.040	*E-coli sp.*
14	S17.1	Oil-contaminated soil	14.41±4.535	*Staphylococcus aureus*
15	S19.2	Oil-contaminated soil	17.99±1.069	*Pseudomonas*
16	S20.1	Oil-contaminated soil	21.84±0.1733	*Bacillus sp.*
17	W5	Animal wound	2.080±0.05777	*Staphylococcus aureus*
18	W12	Animal wound	5.199±1.098	*Staphylococcus aureus*
19	W13	Animal wound	17.88±3.379	*Pseudomonas*
20	W14	Animal wound	2.946±0.1155	*Pseudomonas*

4- Molecular identification of the highest lipase-producing isolates using 16S rRNA sequencing

The highest lipase-producing bacterial isolates were identified based on the 16S rRNA sequence. These obtained sequences were submitted to GeneBank with the accession numbers OR048061 and OR048060. Then they identified using BLAST as *Brevibacillus sp.* strain HC1 and *Brevibacillus sp.* strain HS5, respectively. *Brevibacillus sp.* strain HC1 was isolated from a contaminated culture medium, and *Brevibacillus sp.* strain HS5 was isolated from a soil sample contaminated with oil. The phylogenetic trees of these two bacterial isolates were constructed with the aid of MEGA 11 software and presented in Figures 2 and 3.

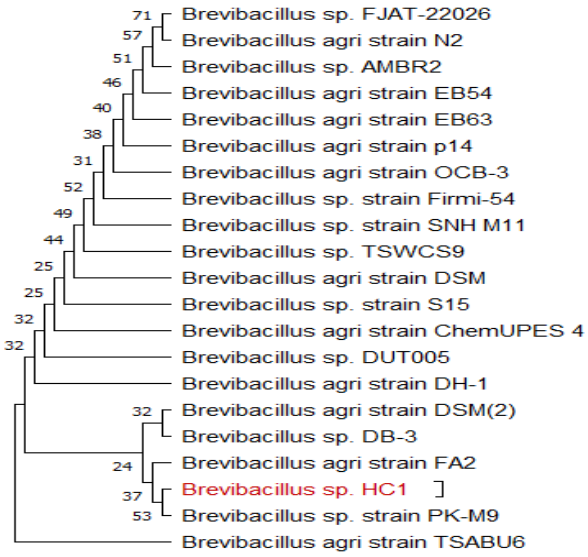

Figure 2: A phylogenetic tree based on the sequence of the 16S rRNA gene shows the evolutionary relationships of *Brevibacillus sp*. strain HC1.

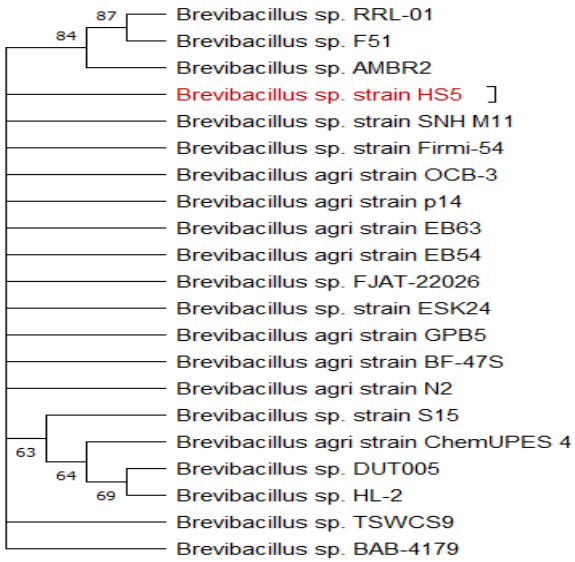

Figure 3: A phylogenetic tree based on the sequence of the 16S rRNA gene shows the evolutionary relationships of *Brevibacillus sp*. strain HS5.

DISCUSSION

A constantly expanding number of review publications on the molecular biology, biochemical characterization, and industrial uses of lipases from prokaryotic and eukaryotic origins demonstrate the great interest in lipases (Padma Priya and Vasudevan, 2018). Lipases have the ability to hydrolyze triacylglycerol. They originate from several sources, including microorganisms. They are nearly everywhere (Ilesanmi *et al.*, 2020).

In our study, the samples were collected from soil contaminated with oil, animal wounds, and contaminated culture media. Bacterial isolates found in oil-contaminated soil samples showed higher lipase activity in comparison to those found in other samples. This difference might be due to the fact that bacteria from other sources are not accustomed to the presence of oils and their analogs, whereas bacteria isolated from soil samples are, as found by Ilesanmi *et al.* (2020). Sirisha *et al.* (2010), Anbu *et al.*

(2011), Aly *et al.* (2012) and Lee *et al.* (2015) isolated several bacterial species that can produce the lipase enzyme from oil-contaminated soil samples.

In the present study, bacterial lipases were produced through submerged fermentation using olive oil as a substrate (Chauhan *et al.*, 2013). In general, the production of lipase enzyme is carried out using a carbon source, such as fatty acids, oils, tweens, or glycerol (Cherif *et al.*, 2011). Many oils have been used as carbon sources to produce lipase enzymes from microbial strains, such as olive oil, sunflower oil, almond oil, and palm oil (Bharathi and Rajalakshmi, 2019).

In our study, we determined the lipase activity of the different bacterial isolates using p-NPP as a substrate, as done by Grbavčić *et al.* (2011) and Chauhan *et al.* (2013). The lipase-producing isolates that showed high lipase activities were selected and identified phenotypically. Our phenotypic identification revealed that most of these isolates belonged to the Bacillus genus. Some of them belonged to the Pseudomonas, Staphylococcus, Klebsiella, and E. coli genera. These findings harmonise with the precious literature. The majority of the bacterial strains that produce lipases have been reported from the following genera: Bacillus, Geobacillus, Burkholderia, Enterococcus, Staphylococcus, and Pseudomonas (Javed *et al.*, 2018).

Numerous species in the genus Bacillus are significant for the industry, and approximately half of the currently manufactured bulk enzymes come from Bacillus strains (Padma Priya and Vasudevan, 2018). Bacillus spp. is considered an inexpensive supply of enzymes because of their widespread distribution, safety at work, simplicity of culturing, and adaptability to genetic modification (Danilova and Sharipova, 2020). The primary sources of lipases in the Bacillaceae family are bacteria from the genera Geobacillus and Bacillus (Guncheva and Zhiryakova, 2011).

According to the performed lipase activity assay, *Brevibacillus sp.* strain HC1 and *Brevibacillus sp.* strain HS5 were identified as the best bacterial isolates as they produced lipase enzymes with high activity of 25.76±2.137 and 25.82±2.542 U/ml respectively. Afzal and Ihsan-Ud-din (2021) revealed that the highest levels of lipase enzyme that can be produced using olive oil as a substrate from *Bacillus subtilis* AF-23, *Bacillus subtilis* AF-3, and *Bacillus subtilis* AF-6 were reported to be 18 U/ml, 20 U/ml, and 21 U/ml, respectively.

16S rRNA gene sequencing provides a powerful tool for the identification and classification of prokaryotes (Hassan *et al.*, 2018). *Brevibacillus sp.* strains HC1 and *Brevibacillus sp.* strain HS5 were shown to be the best lipase-producing isolates.

These results are consistent with earlier research. Several Bacillus species, including *Bacillus cereus* HSS, *Bacillus idriensis* strain LipT27, *B. pumilus* strain LipT29, *B. velezensis* strain LipO41, and *B. safensis* strain LipT52, have been identified as lipase producers in various studies by Hassan *et al.* (2018) and Gurkok and Ozdal (2021). In addition, Jaouadi *et al.* (2013) used *Brevibacillus brevis* strain US575 for lipase production in the leather industry.

CONCLUSION

The current study screened our local environment for lipase-producing bacterial isolates that can be used for lipase enzyme production. The findings revealed that oil-contaminated soil was one of the most important habitats for the isolation of bacterial isolates that can produce lipase enzymes. Upon phenotypic identification of the lipase-producing isolates, the genus Bacillus had the potential for lipase enzyme production with the highest activity. *Brevibacillus sp.* strains HC1 and

Brevibacillus sp. strain HS5 are the most promising bacterial isolates that can be good candidates for the production of lipase enzymes to be used in different applications.

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العزل والفحص والتعرُف الجزيئي للبكتيريا المُنتجة لليباز من مصادر بكتيرية مختلفة

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تتزايد الحاجة إلى الإنزيمات فى العديد من الصناعات نظرًا لمزاياها العديدة، مثل الحد الأدنى من السمية البيئية. فإنزيمات الليباز هي واحدة من أكثر الإنزيمات المستخدمة في التكنولوجيا الحيوية قيمة. ونتيجة لذلك، أصبحت الدراسات التي تجرى على إنزيمات الليباز تحظى بشعبية كبيرة في مجال علم الإنزيمات في السنوات الأخيرة. حيث أجريت عدة محاولات لعزل سلالات بكتيرية مختلفة يمكنها إنتاج إنزيم الليباز. وتهدف الدراسة الحالية إلى عزل عدة سلالات بكتيرية من مصادر مختلفة مثل التربة الملوثة بالزيت، جروح الحيوانات، ووسط الاستزراع الملوث. وتم إجراء فحص نوعي للسلالات المنتجة لليباز باستخدام توين ٨٠ أجار. حيث أظهرت النتائج أنه تم الحصول على إجمالي ١٢٤ سلالة بكتيرية من مصادر مختلفة، بما في ذلك ٤١ سلالة يمكن أن تنتج إنزيم الليباز. وعند اختبار نشاط الليباز، تم التعرف على النمط الظاهري لأعلى ٢٠ سلالة منتجة لليباز وتحديد أفضل منتجي الليباز المحتملين باستخدام التعريف وراثيًا بواسطة تسلسل الجين 16S الرنا الريباسي وهما *Brevibacillus sp.* سلالة HC1 و Brevibacillus sp. سلالة HS5 وسجلوا في بنك الجينات برقمى انضمام (OR048061) و(OR048060)، على التوالى. وتسلط هذه الدراسة الضوء على أهمية استخدام البكتيريا كمصدر ميكروبي لإنتاج إنزيم الليباز لاستخدامه فى التطبيقات الصناعية والتكنولوجيا الحيوية المستقبلية.