Indonesian Journal on Health Science and Medicine
Vol. 3 No. 1 (2026): July
ISSN 3063-8186. Published by Universitas Muhamamdiyah Sidoarjo

https://doi.org/ 10.21070/ijhsm.v3i1.386

Molecular Profiling of Lipase-Producing Bacillus spp. from Basrah Extreme Soils

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Abstract. **General Background:** Lipases are hydrolytic enzymes with broad industrial applications due to their catalytic versatility and environmental relevance. **Specific Background:** Oil-contaminated and extreme environments represent promising reservoirs for robust lipase-producing bacteria. **Knowledge Gap:** Limited molecular characterization has been conducted on indigenous lipolytic *Bacillus* species from extreme sites in Basrah Governorate. **Aim:** This study aimed to isolate, screen, and molecularly identify lipase-producing *Bacillus* spp. from oil-affected soils and related environments. **Results:** Ten samples were collected, and five high-performing isolates were confirmed through Spirit Blue Agar and Rhodamine B assays. Optimal lipase production occurred at 37°C, pH 7, after 48 hours using olive oil as substrate. 16S rRNA sequencing identified the isolates as *Bacillus velezensis*, *Bacillus subtilis*, *Bacillus tequilensis*, *Bacillus licheniformis*, and *Bacillus cereus*, with sequence similarities ranging from 98.88% to 99.82%. **Novelty:** This study provides molecular evidence of diverse indigenous lipolytic *Bacillus* strains from Basrah's extreme habitats. **Implications:** The findings support their prospective application in biofuel production, wastewater treatment, and industrial biocatalysis.

Highlights:
- Dual-media screening strengthened qualitative detection of lipase activity.
- Molecular identification confirmed five high-similarity *Bacillus* strains.
- Extreme Basrah soils serve as reservoirs of industrially relevant lipolytic bacteria.

Keywords:
Lipase-Producing Bacteria, Bacillus Spp., 16S rRNA Sequencing, Oil-Contaminated Soil, Industrial Biotechnology

Published : 18-02-2026

Indonesian Journal on Health Science and Medicine
Vol. 3 No. 1 (2026): July
ISSN 3063-8186. Published by Universitas Muhamamdiyah Sidoarjo

https://doi.org/ 10.21070/ijhsm.v3i1.386
2020.

Introduction

Lipases are a class of enzymes that belong to the hydrolases, also known as lipolysis, glycerol hydrolases (EC 3.1.1.3),or triacylglycerol hydrolases. It belongs to the class of enzymes that catalyze hydrolysis reactions (hydrolases). Lipases stimulate triglycerides to break them down into glycerol and fatty acids when they encounter oil and water[1] . Lipases constitute a broad and major group of biocatalysts and have enormous applications in Biotechnology due to its ease of production, high stability and genetically modification [2]. Lipases have been isolated and purified from animal,plants,fungi,yeasts, and bacteria sources Enveronmentally, lipase enzyme play an important role in processing fatty waste and converting it into economically valuable compounds, contributing to reducing pollution and increasing industrial returns from organic waste[3] . Bacterial lipases are widely used in the food and dairy industries for lipid hydrolysis. They are also used in the detergent industry, in the synthesis of polymers or biodegradable compounds, in various esterification reactions, and in the synthesis of biodiesel[4] [5]. Currently, there is a great demand for bacterial lipases due to their potential industrial applications. Lipases play a vital role in the manufacturing and human services sectors. Microbial lipases have received particular these enzymes are of great industrial importance due to their stability, selectivity, and broad substrate specificity. Microbial enzymes are among the most stable enzymes, and their production is easier and more safer[6] .

Method

1. Sample collection

Soil samples were obtained from four different locations (six samples from each location). The first included decomposed agricultural soil in Qurna District, Al-Sadiq District, and Al-Madinah District. The second was near the sites where car oils were collected. The third included oil soil from West Qurnah 1 Field. The fourth was from the Chinese Drilling Company. 300 g of soil was collected for each sample using a small sterile shovel at a depth of 5-15 cm and placed in tightly sealed plastic bags (with all necessary data recorded). All samples were transferred to the laboratory and store in a refrigerator at 4°C (Table1).

Table1: Shows sample collection sites

Sample source	**Sample location**
Soil	Decomposed agricultural soil
	Chinese drilling tower HH33
	West qurna field 1
	Chinese drilling tower line L86
	Car oil change places
	Chinese drilling tower Line 79.5
Water	Pond water and contaminated with organic

2. Primary isolation of bacteria

Soil samples were isolated by dilution method according to the method [7] . Each dilution was cultured on plates containing Nutrient Agar for 18-24 hours at a temperature of 37°C. After the incubation period, the isolates were purified and tested for their ability to produce lipase enzyme by using specialized media such as(Spirt Blue Agar and Rhodamine B Oilve Oil Agar). Then, they were stained with Gram and examined. Bacterial isolates were identified on the basis of their morphological characteristics. Then, the purified isolates were transferred to Nutrient Agar slant

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https://doi.org/ 10.21070/ijhsm.v3i1.386
for preservation and later use. 2020.

3. Identification of lipase-producing bacteria

Genomic DNA was extracted from bacterial isolates containing lipase enzyme by sequencing the *16srRNA* gene[8] . Genomic DNA was extracted using a Geneaid kit (a Taiwanese manufacturer), and DNA extraction was performed according to the manufacturer's instructions. A NanoDrop device was used to determine the concentration of the extracted DNA . Genomic DNA was stored at -20°C until needed. The 16srRNA gene was amplified from the genomic DNA by polymerase chain reaction (PCR) using forward and reverse primers for *16srRNA*[9] .Table 2

PCR was performed in a thermal cycler according to the program shown in the table 4.

The PCR products were loaded into the appropriate wells of a 1.0% TAE (w/v) agarose gel stained with 3 µl ethidium bromide. The agarose gel was run at 80 V for 40 minutes. The DNA within the agarose gel was visualized using a UV transilluniotor and imaged using a digital camera[10].

Table2: Primer Sequence used in the study

Primer sequence (5-3)	**Bp**		**gene**
5'AGAGTTTGATCCTGGCTCAG3'	F	1492	*16SrRNA*
5'GGTTACCTTGTTACGACTT3'	R		

Table3: Mixture used in the study

Component	**Volume**
GoTaq Green Mix (promega)	12.5µL
FWD	1µL
REV	1µL
Nuclease-free water	5.5µL
gDNA	5µL
Total	25µL

Table4: PCR Program

Stage	**Step**	**Temperature and time**	**Number of cycles**
1	Pre-denaturation	95 C, for 5 minutes	1 cycle
2	Denaturation	95 C, for 30 seconds	35 cycles
	Annealing	55 C, for 45 seconds	
	Extension	72 C, for 30 seconds	
3	Final extension	72 C, for 7 minutes	1 cycle

4. Detection of Lipase Activity

A medium was used to evaluate the lipase production of bacterial isolates . 15.32 g of spirit blue agar medium was dissolved in 1000 ml of distilled water and then autoclaved at 121°C for 15 minutes. After sterilization and the medium reaching approximately 50°C, olive oil was added as a carbon source and mixed well. The medium was poured into Petri dishes and allowed to solidify. A quantity of each pure culture was placed separately on the plates and incubated at 37°C for 48 hours[11] . After incubation, a clear hydrolytic zone around the colonies indicated lipase activity. Positive cultures exhibiting the highest hydrolytic zone were selected for further testing .

5. Secondary assay for lipase activity

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2020. Rodamine B Olive Oil medium was used to detect lipase-producing .8 g of Nutrient Broth, 4 g of NaCl, and 20 g of Agar were dissolved in the medium. The pH was adjusted to 7 and the medium was autoclaved at 121°C for 15 min[12] . After reaching 50°C add 31.25ml of Olive Oil[13] and, 500 µl of filter-sterilized Rhodamine B solution (1.0% w/v distilled water) was added with continuous stirring to homogenize the medium. The medium was then poured into Petri dishes and allowed to solidify. The dishes were inoculated with purified bacterial colonies, and incubated at 37°C for 48 h. After incubation, the formation of orange fluorescent halos around the bacterial colonies upon exposure to UV light indicated lipase activity[14]. The bacterial isolates with high activity were selected for further testing.

Result and Discussion

1. Sample collection and Bacterial isolation

The number of samples in which growth occurred is seven out of ten samples.

2. Microscopic and morphological diagnosis of bacterial isolates from soil

The results of Gram staining after purification of the isolates showed that most of the bacterial isolates were Gram-positive and bacilli and medium-length rod-shaped in chains, or that they bacilli a single or double rod-shaped shape. Initially, morphological characteristics were relied upon to diagnose bacteria, which included the texture, smell, shape, colour and size of the colonies growing on culture media, and biochemical test.

3. Identification of lipase-producing bacterial strains

The extraction results showed the presence of DNA strands on the agarose gel. Figure 1

The *16srRNA* gene sequences of the five bacterial isolates were compared with the NCBI database. The strains showed similarity to various genes, including *Bacillius velezensis* (1410 bp) with 99.5% identity*, Bacillius subtilis* (1406 bp) with 99.72% identity, and *Bacillius tequilensis* (1432 bp) with 99.82% identity.
Bacillius licheniformis (1429 bp) with 98.88% identity, and *Bacillius cereus* (714 bp) with 99.16% identity. Table 6

Figure 1: shows agarose gel electrophoresis for amplification of target bacteria using PCR.

Table 6: percentage of identity with the References sample in GenBank, the serial number of the References samples, and the serial number of bacteria regstered in GenBank

Present study sample	**Accession No.**	**genBank sample**	**Acccession No.**	**%DNA Identity**
Bacillus licheniformis	OPO361313.1	*B. licheniformis*	PV202549.1	98.88%
Bacillus velezensis	KT719749.1	*B. velezensis*	PV202546.1	99.5%
Bacillus subtilis	JN36679.1	*B. subtilis*	PV202547.1	99.72%
Bacillus tequilensis	PQ813805.1	*B. tequilensis*	PV202548.1	99.82%
Bacillus cereus	MW418333.1	*B. cereus*	PV202550.1	99.16%

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4. Screening of Lipase-Producing Bacteria

In this study, Lipase activity was detected in five bacterial isolates belonging to the *Bacillus* genus: *Bacillus subtilis* , *Bacillus cereus*, *Bacillus velezensis* , *Bacillus tequilensis* , and *Bacillus licheniformis.* the lipase-producing strains were selected from oil _contaminated soils and seawater, by studying lipase activity on plates containing Spirt Blue agar (SBA) containing olive oil as a catalyst for lipase activity. This was achieved by the formation of colonies that appeared transparent after the required incubation period of 24–48 hours at 37°C. The change in the color of the medium to a transparent color is attributed to the secretion of lipase, which decomposes the lipids present in the medium. Colonies in which the medium did not turn transparent are lipase-negative (Figure 2)

The five selected isolates were cultured using Rhodamine B Olive Oil Agar (RBOA). Lipase-producing bacterial isolates were identified by studying lipase activity on (RBOA) plates containing olive oil and Rhodamine B dye. The formation of orange fluorescent halos upon exposure to ultraviolet light after the required incubation period indicates that the isolates are lipase-positive (Figure 3). This appearance is caused by the formation of a complex between the cationic Rhodamine B and the fatty acid uranyl ion. The mechanism of this appearance may be related to the formation of excited dimers of Rhodamine B, which fluoresce at longer wavelengths than the outgoing monomer[15] .

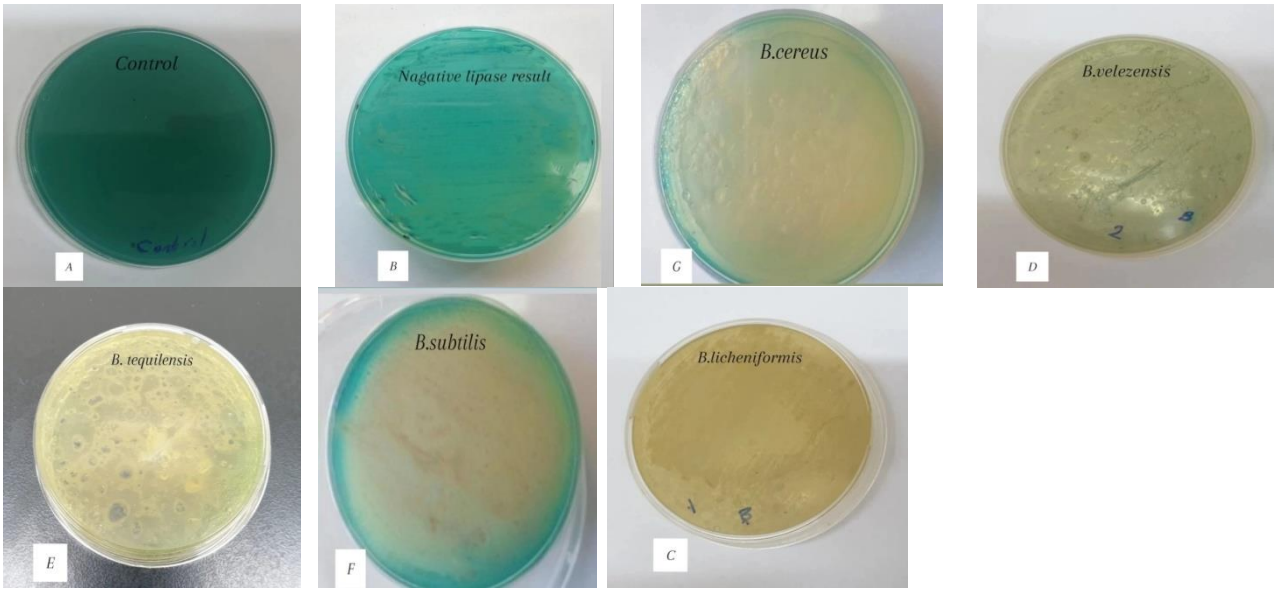
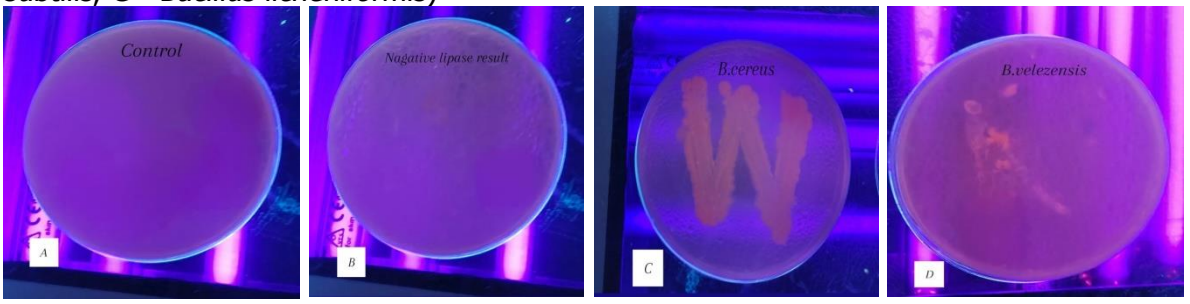


Figure 2: Cultivation of lipase-producing bacteria on Spirit Blue agar test plates. A - Blank sample. B - Negative sample.
Positive samples: (C - *Bacillus cereus*, D - *Bacillus velezensis*, E - *Bacillus tequilensis*, F - *Bacillus subtilis*, G - *Bacillus licheniformis*)



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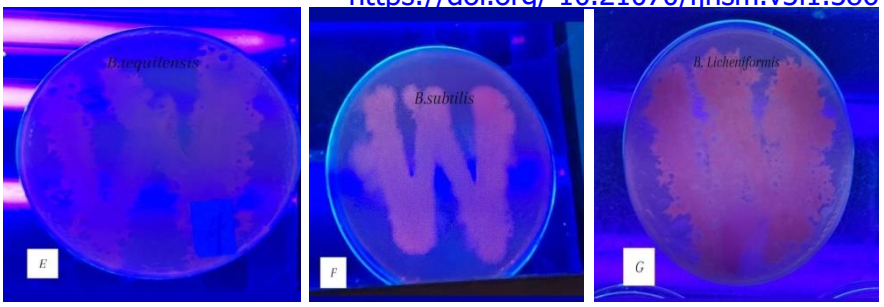

Figure 3: Cultivation of lipase-producing bacteria, on Rhodamine B Olive Oil Agar plates examined under UV light at 350 nm. A - Blank control. B - Negative Control.
Positive samples: (C - *Bacillus cereus*. D - *Bacillus velezensis*. E - *Bacillus tequilensis*. F - *Bacillus subtilis*. G - *Bacillus licheniformis*).

The study was able to increase the efficiency of *Bacillus subtilis* lipase production using cheese whey as a sustainable feed source[16]. Prior to partial purification with ammonium sulfate and the washing phase, the maximum enzyme activity value was 1314 U/ml, increasing to 6570 U/ml. The study also demonstrated that a temperature of 30°C and a 48-hour incubation period are optimal conditions for growth. These conditions are similar to those demonstrated in the current study, which showed strong activity on oil media, suggesting that the physiological requirements for optimal enzyme synthesis are potentially similar.

These findings are also in line with those of researchers[17] who employed the *Bacillus cereus* strain to generate lipases through solid-phase fermentation using industrial and agricultural waste as substrates. When soybean extract was used, the maximum observed enzyme activity was 41.2 ± 1.08 units/ml; when bagasse and wheat bran were used as substrates, the activities varied between 25 and 40 units/ml. These findings demonstrated that the strain could effectively secrete lipases from inexpensive and straightforward food sources, indicating that it may be developed for industrial or ecologically friendly production. This is especially true given that the maximal enzyme activity was attained at 40°C.

The results of researchers[18] who isolated *Bacillus licheniformis* from Indian hot springs and showed that the lipase it produces has both heat and alkaline activity its maximum activity at 60°C and pH 8.5 are likewise in keeping with these discoveries. The similarity in enzymatic behavior suggests that this species can produce stable and effective enzymes in a range of environments, increasing its potential for use in industrial applications like lipolysis and biofuel production, even though the two isolates' ideal conditions differ.

These findings also align with those of other researchers who examined the *Bacillus velezensis* EAC9 thermophilic strain that was isolated from hot compost[19]. Shea nut cake was used as a carbon source to improve the nutritional circumstances, and it showed a high lipase production capability of about 200 units/ml. This strain's lipase is an extracellular enzyme with outstanding heat stability and optimal activity at an alkaline pH of about 8, according to the study. For industrial uses and the manufacture of biofuel, these traits make it a potential strain.

Conclusions

These results highlight the importance of using two different media (Rhodamine B and Spirit Blue) for the qualitative detection of lipase activity. Rhodamine B provides accurate visual assessment of the fluorescence produced by lipase binding to the lipid substrate, while Spirit Blue allows for the detection of hydrolysis in the medium, reflecting the actual activity of the enzyme. These results indicate the high capacity of the studied *Bacillus* species to secrete extracellular lipases, enhancing their potential for industrial and environmental applications, such as lipolysis, biofuel production, and wastewater treatment.

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https://doi.org/ 10.21070/ijhsm.v3i1.386

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