

Isolation, Molecular Identification, and Characterization of Extracellular Lipolytic Activity of *Bacillus* Strain from Petroleum-contaminated Soil

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ABSTRACT

Lipases are versatile industrial enzymes with broad applications in the food, pharmaceutical, detergent, and biofuel industries. Petroleum-contaminated soils are promising sources of microorganisms adapted to hydrocarbon-rich environments and may harbor bacteria capable of producing extracellular enzymes with valuable biochemical properties. This study aimed to isolate and screen lipase-producing bacteria from petroleum-contaminated soils and characterize the extracellular lipolytic activity of the most active isolate. Five soil samples were collected from petroleum product-contaminated sites in Mahan and Kerman, Iran. Bacterial isolates were screened using Rhodamine B-olive oil agar, and extracellular lipase activity was quantified using p-nitrophenyl palmitate. The effects of nitrogen and carbon sources, edible oils, pH, temperature, and incubation time on extracellular lipolytic activity were evaluated, along with thermal stability. Molecular identification was performed using 16S rRNA gene sequencing and phylogenetic analysis. Among the isolates, strain S4, obtained from gasoline-contaminated soil, exhibited the highest lipolytic activity and was selected for further characterization. Peptone was the most effective nitrogen source, while olive oil produced the highest activity among the tested carbon sources (0.098 U/mL). Castor oil further increased extracellular lipolytic activity to 0.134 U/mL. Maximum activity was observed at 30°C after 48 h of incubation and at pH 9.0 (0.154- 0.159 U/mL). The extracellular lipolytic activity retained notable activity at 52.7°C for 40 min but showed rapid inactivation at 60°C, indicating moderate thermal stability. 16S rRNA sequencing identified strain S4 as a *Bacillus* strain with 99.8% similarity to the *Bacillus cereus* group (GenBank accession PP092157). Overall, the isolated strain represents a promising source of extracellular alkaline lipolytic activity with moderate thermal stability and potentially useful biochemical characteristics for further investigation and optimization in industrial biotechnology.

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Introduction

Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) are important hydrolytic enzymes that catalyze the breakdown of triglycerides into free fatty acids and glycerol. These enzymes are characterized by their ability to act at the oil-water interface through a mechanism known as interfacial activation (McDonald *et al.*, 2015; McDonald and Tipton, 2023). In addition to hydrolysis, lipases are capable of catalyzing esterification and transesterification reactions in

non-aqueous systems, which significantly expands their industrial and biotechnological applications (Casas-Godoy *et al.*, 2012).

Due to their catalytic versatility, lipases have become one of the most widely used classes of enzymes in biotechnology. They are extensively applied in food processing, detergent formulations, pharmaceutical synthesis, cosmetics, and biodiesel production (Casas-Godoy *et al.*, 2012; Borrelli and Trono, 2015). Their high substrate specificity, regio- and enantio-selectivity, and their ability to function



under relatively mild reaction conditions make them attractive alternatives to conventional chemical catalysts (Borrelli and Trono, 2015; Sharma and Kanwar, 2014; Ali *et al.*, 2023). As a result, the search for novel lipases with desirable biochemical properties remains an active field of research.

Microorganisms represent the most important sources of industrial lipases because of their rapid growth, ease of cultivation, and ability to secrete extracellular enzymes (Ali *et al.*, 2023; Sharma and Sharma, 2018). Among microbial sources, bacteria have attracted particular attention due to their metabolic diversity and their ability to adapt to a wide range of environmental conditions (Mobarak-Qamsari *et al.*, 2011; Ilesanmi *et al.*, 2020). Environmental factors play a significant role in shaping microbial communities and can influence both enzyme production and enzymatic characteristics (Ilesanmi *et al.*, 2020; Ul ain Rana *et al.*, 2019; Ulwiyyah *et al.*, 2019).

Hydrocarbon-contaminated environments are considered selective ecological niches for microorganisms capable of metabolizing hydrophobic compounds (Das and Chandran, 2011). While numerous studies have reported the isolation of lipase-producing bacteria from crude oil-contaminated soils, comparatively limited attention has been given to gasoline-contaminated sites (Mobarak-Qamsari *et al.*, 2011; Abubakar *et al.*, 2024). Gasoline differs markedly from crude oil in its composition, containing higher proportions of low-molecular-weight and volatile hydrocarbons, including monoaromatic compounds such as BTEX (Koshlaf and Ball, 2017). These characteristics may exert distinct selective pressures on indigenous microbial communities, potentially influencing both the diversity and functional properties of lipase-producing bacteria (Singh *et al.*, 2017). Therefore, investigating gasoline-contaminated soils may reveal strains with different enzymatic profiles compared to those isolated from crude oil-polluted environments.

Accordingly, the present study aimed to isolate and screen lipase-producing bacteria from petroleum hydrocarbon-contaminated soils collected from Kerman province, Iran. The isolates were initially screened for lipase activity, followed by quantitative evaluation of enzyme. Furthermore, the effects of different carbon and

nitrogen sources, various oils, pH, temperature, and incubation time on lipase production were investigated to characterize the enzymatic potential of the selected isolate. Finally, molecular identification of the most promising strain was performed based on 16S rRNA gene sequencing and phylogenetic analysis.

Materials and Methods

Sample collection

Soil samples were collected from five petroleum-product-contaminated environments (in Mahan and Kerman, Iran), including: S1) an area exposed to petroleum; S2) an oil change shop; S3) a diesel-contaminated site; S4) a gasoline (benzin)-contaminated site (S4); and S5) a mechanic workshop. These sites were selected because they represent distinct hydrocarbon contamination profiles, with gasoline-contaminated soils being less studied compared to crude oil-contaminated environments (Abubakar *et al.*, 2024). One gram of each sample was suspended in 9 mL sterile distilled water, incubated at 37°C for 3 h with shaking (180 rpm), and serially diluted up to 10⁻⁶ for bacterial isolation.

Isolation of lipase-producing bacteria

Diluted samples (100 µL) were spread onto LB agar and incubated at 37°C for 48 h. Ten colonies from each plate were inoculated into LB broth and incubated under the same conditions. Lipolytic activity was screened on Rhodamine B-olive oil agar (Merck) as previously described (Ilesanmi *et al.*, 2020; Ul ain Rana *et al.*, 2019). This lipid substrate was selected because it is a natural triglyceride commonly used for detecting true lipase activity, whereas substrates such as Tween 80 and tributyrin are more suitable for esterases (Jaeger and Eggert, 2002; Srinivas Kumar *et al.*, 2012). Colonies producing an orange fluorescent halo under UV light were considered lipase-positive. Positive colonies were transferred to a screening medium (peptone, yeast extract, olive oil; pH 7.0) and incubated at 37°C, 180 rpm for 48 h. Cultures producing the highest fluorescence were selected for further analyses and preserved at -80°C in 20% glycerol.

Lipase activity assay

Extracellular lipolytic activity was quantified using p-nitrophenyl palmitate (p-NPP) (Merck) as

the substrate, following established protocols (Ullain Rana *et al.*, 2019; Mostafa *et al.*, 2013; Liu *et al.*, 2007). Briefly, the reaction mixture contained 100 μ L of cell-free supernatant, 800 μ L of Tris-HCl buffer (50 mM, pH 7.0), and 100 μ L p-NPP solution (10mM in isopropanol). The mixture was incubated at 37°C for 40 min, and the reaction was stopped by adding 250 μ L of 0.5 M Na₂CO₃. The absorbance was measured at 410 nm using a spectrophotometer (UV-1800, Shimadzu, Japan). A standard curve of p-nitrophenol (20-130 μ M) was prepared under identical conditions. One unit (U) of lipase activity was defined as the amount of enzyme that released 1 μ Mol of p-nitrophenol per minute under the assay conditions. To ensure comparability between samples and eliminate the influence of variable bacterial growth, all cultures were adjusted to an optical density of OD₆₀₀ = 1.0 prior to the enzyme assay. Therefore, volumetric enzyme activity is reported as U/mL. Uninoculated culture media and enzyme-free reaction mixtures were processed under identical conditions as negative controls, and their absorbance values were subtracted from all sample readings prior to activity calculations.

Physicochemical factors on lipase production

To characterize the enzymatic profile of the selected isolate, the effects of different nitrogen sources (peptone, tryptone, yeast extract), carbon sources (olive oil, starch, glucose), various edible oils, pH (5- 9), temperature (30- 50°C), and incubation time (24- 96 h) were evaluated. All assays were performed in the screening medium, and enzyme activity was determined as described above (Chouhan *et al.*, 2020; Gürkök 2021; Demirkan *et al.*, 2021). Thermal stability was assessed at 52.7°C (the temperature at which the enzyme showed maximum stability) and 60°C (a temperature above the stability optimum, selected to determine the inactivation point). The enzyme extract was incubated in a water bath at these temperatures, and residual activity was measured at 10-min intervals (Demirkan *et al.*, 2021).

Amplification of 16S rRNA gene

Genomic DNA was extracted using a commercial bacterial DNA extraction kit (Yekta Tajhiz Azma, Iran) following the manufacturer's instructions. The 16S rRNA gene was amplified using universal primers and a standard PCR master mix.

Cycling conditions and primer sequences .The primers and PCR reaction components used for amplification are described as follows. The forward primer (8F) sequence was 5'-AGAGTTTGATCCTGGCTCAG-3', and the reverse primer (1541R) sequence was 5'-AAGGAGGTGATCCAGCCGCA-3'. The PCR reaction mixture had a final volume of 50 μ L and consisted of 25 μ L of 2 $\times$ Master Mix, 1.5 μ L of forward primer (final concentration: 0.5 μ M), 1.5 μ L of reverse primer (final concentration: 0.5 μ M), 5 μ L of DNA template (0.2 ng/ μ L), and 17 μ L of double-distilled water. The final concentrations of MgCl₂, dNTPs, and Taq DNA polymerase were 1.5 mM, 0.2 mM, and 0.1 U/ μ L, respectively. PCR products were analyzed on a 0.8% agarose gel, and purified amplicons were submitted for Sanger sequencing.

Sequence, phylogenetic tree, and statistical analyses

Sequences were trimmed and analyzed using BLASTn against the NCBI database. Phylogenetic analysis was performed in MEGA XI using CLUSTAL W for alignment. The Neighbor-Joining method with the Kimura-2-parameter model was applied, and branch support was evaluated by 1,000 bootstrap replicates (Kimura, 1980; Felsenstein, 1985). Gap-containing positions were removed using complete deletion. All experiments were performed in independent triplicates, and the results are presented as mean $\pm$ standard deviation (SD). Statistical analysis of the data was carried out using one-way analysis of variance (ANOVA) followed by Tukey's test to compare the means. A p-value of <0.05 was considered statistically significant.

Results

Isolation and primary screening

Initial screening of all five petroleum-contaminated soil samples (S1-S5) on Rhodamine B-olive oil agar revealed varying degrees of lipolytic activity. *Bacillus* sp. strain S4, obtained from gasoline-contaminated soil, exhibited the highest abundance and the most intense orange fluorescent halos under UV light, indicating superior lipase production. Consequently, S4 was selected for further enzymatic characterization and molecular identification (Fig. 1).

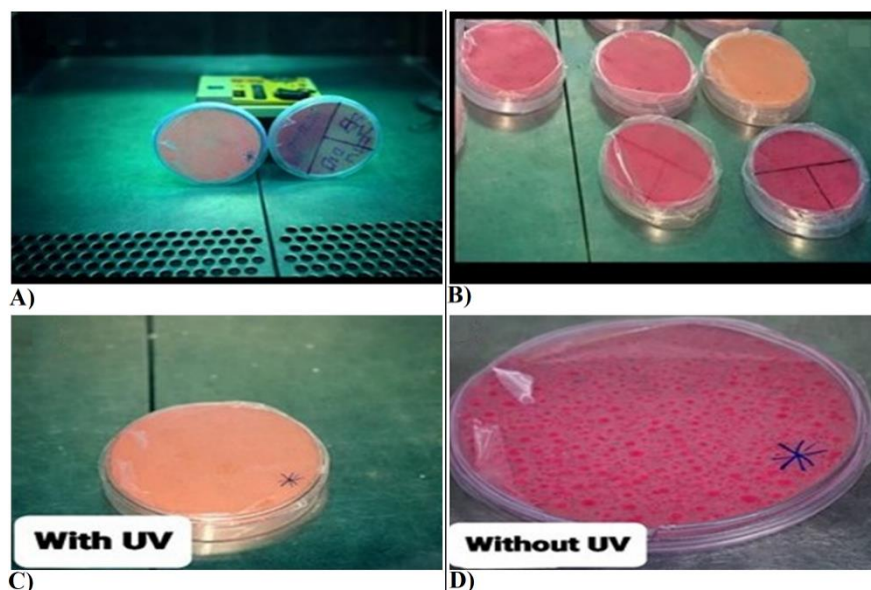


Fig. 1. Detection of lipase-producing bacteria: A) Negative control (right) and positive control (left); B and D) Detection of lipase production by *Bacillus* sp. strain S4 under UV illumination, showing the formation of an orange halo; C) *Bacillus* sp. strain S4 without UV illumination.

Optimization of physicochemical factors for lipase activity

The influence of nutritional and environmental factors on the extracellular lipolytic activity of *Bacillus* sp. strain S4 was evaluated (Fig. 2). Among the tested nitrogen sources, peptone resulted in the highest lipase activity ($p < 0.05$), while among the carbon sources, olive oil significantly enhanced enzyme activity compared with starch and glucose ($p < 0.05$), indicating the potential role of lipidic substrates as effective inducers of lipase production. Evaluation of different oils further showed that castor oil produced the highest lipase activity compared with olive and frying oils ($p < 0.05$). The extracellular lipolytic activity of the culture supernatant showed an alkaline profile, with maximum activity at pH 9.0, whereas no detectable activity was observed under acidic conditions (pH 5.0- 6.0). Temperature also significantly affected extracellular lipolytic activity, with the highest activity recorded at 30°C. Furthermore, lipase production increased with incubation time, peaking after 48 h, followed by a decline at longer incubation periods. Overall, these findings indicate that nitrogen and carbon

sources, oil type, pH, temperature, and incubation time substantially influence lipase production and activity in *Bacillus* sp. strain S4 (Fig. 2).

Thermal stability

The thermal stability of the extracellular lipolytic activity was evaluated at 52.7°C and 60°C. At 52.7°C, the activity retained a substantial portion of its initial value for up to 40 min before gradually declining. In contrast, exposure to 60°C led to rapid inactivation, with activity dropping to near zero within 40 min (Fig. 3).

Molecular identification and phylogenetic analysis

Genomic DNA extraction and subsequent PCR amplification yielded a distinct band of approximately 1500 bp, corresponding to the 16S rRNA gene (Fig. 4). BLASTn analysis confirmed that the isolate belongs to the *Bacillus* genus, showing 99.8% sequence similarity with the *Bacillus cereus* group. The sequence was deposited in the NCBI database (Accession Number: PP092157). Phylogenetic tree construction using the Neighbor-Joining method positioned the isolate in a well-supported clade with *B. cereus* strain PRW-65 (Fig. 4).

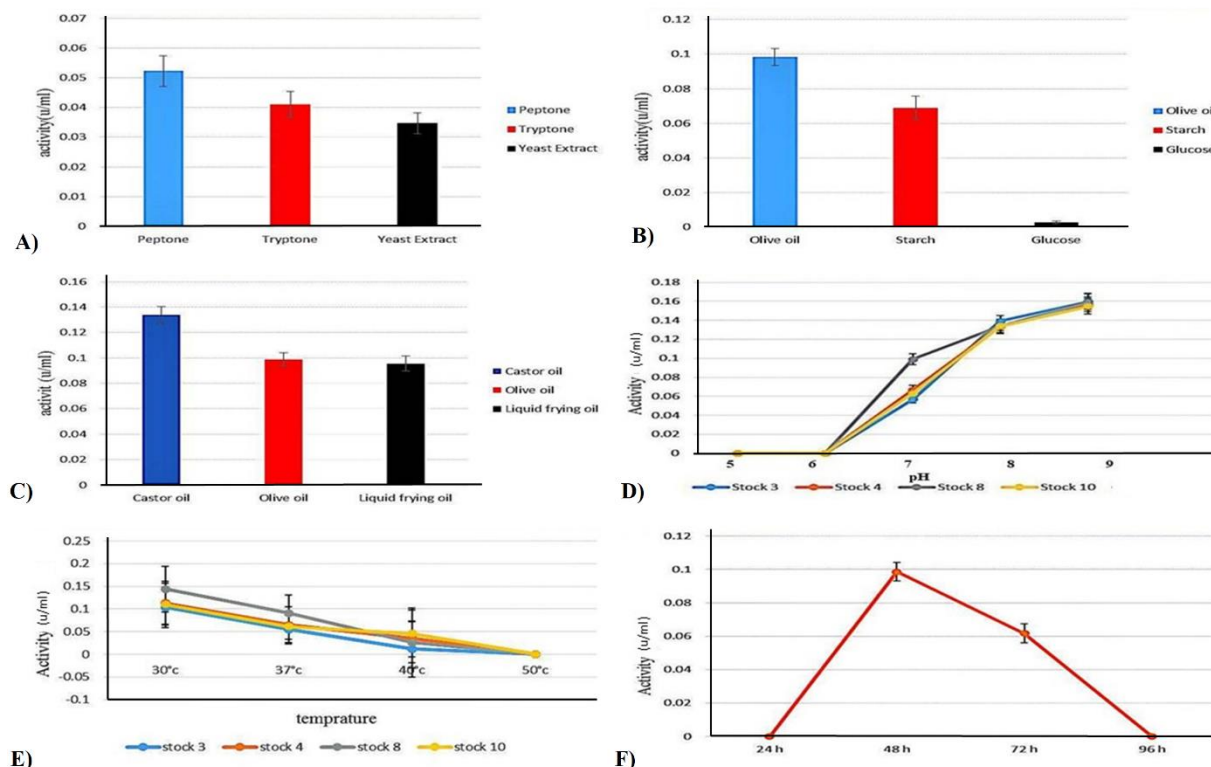


Fig. 2. Effects of different nutritional and environmental factors on the enzyme activity of *Bacillus* sp. strain S4: A) Effect of different nitrogen sources (peptone, tryptone, and yeast extract); B) Effect of different carbon sources (oil, starch, and glucose); C) Effect of different oil sources (castor, olive, and frying oils); D) Effect of pH; E) Effect of incubation temperature (30, 37, 40, and 50°C); F) Effect of incubation time on enzyme activity. Data are presented as mean $\pm$ SD (n= 3).

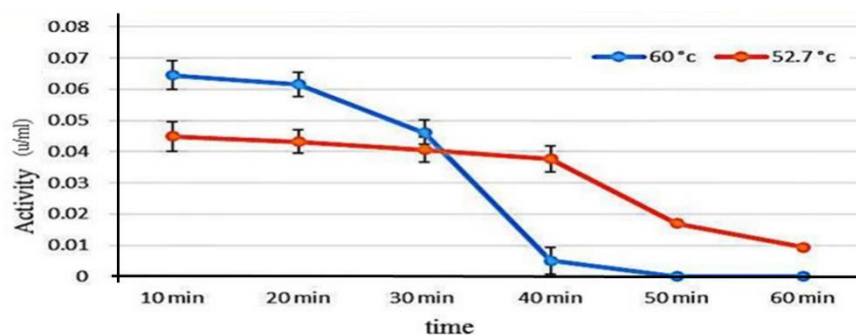


Fig. 3. Investigating the stability of *Bacillus* sp. strain S4 at two different temperatures (52.7, 60°C). Data are presented as mean $\pm$ SD (n= 3).

Discussion

The rising industrial demand for robust and application-specific lipases highlights the necessity of exploring new microbial sources, especially from unique ecological niches such as petroleum-contaminated soils. These environments are known to harbor microorganisms that have adapted to hydrophobic compounds and often display enhanced lipolytic

capabilities (Saitou and Nei, 1987). In the present study, a potent lipase-producing *Bacillus* strain was successfully isolated from gasoline-contaminated soil. Molecular identification using 16S rRNA gene sequencing and phylogenetic analysis placed the isolate within the *B. cereus* group, a taxonomic cluster well-recognized for secreting high levels of extracellular enzymes and widely utilized in industrial biotechnology (Kimura, 1980;

Felsenstein, 1985). While *Bacillus* species have been extensively isolated from heavy crude oil environments over the past decades, the specific microenvironment of gasoline-contaminated soil presents a different ecological challenge. The high volatility, distinct toxicity of light hydrocarbons (such as BTEX), and altered solvent conditions in gasoline-polluted soils exert

a unique selective pressure. Gasoline-contaminated soils may therefore select for microorganisms with different enzymatic profiles compared with crude oil-contaminated environments. However, whether the lipolytic activity of strain S4 reflects specific adaptive mechanisms requires further genomic and biochemical investigation.

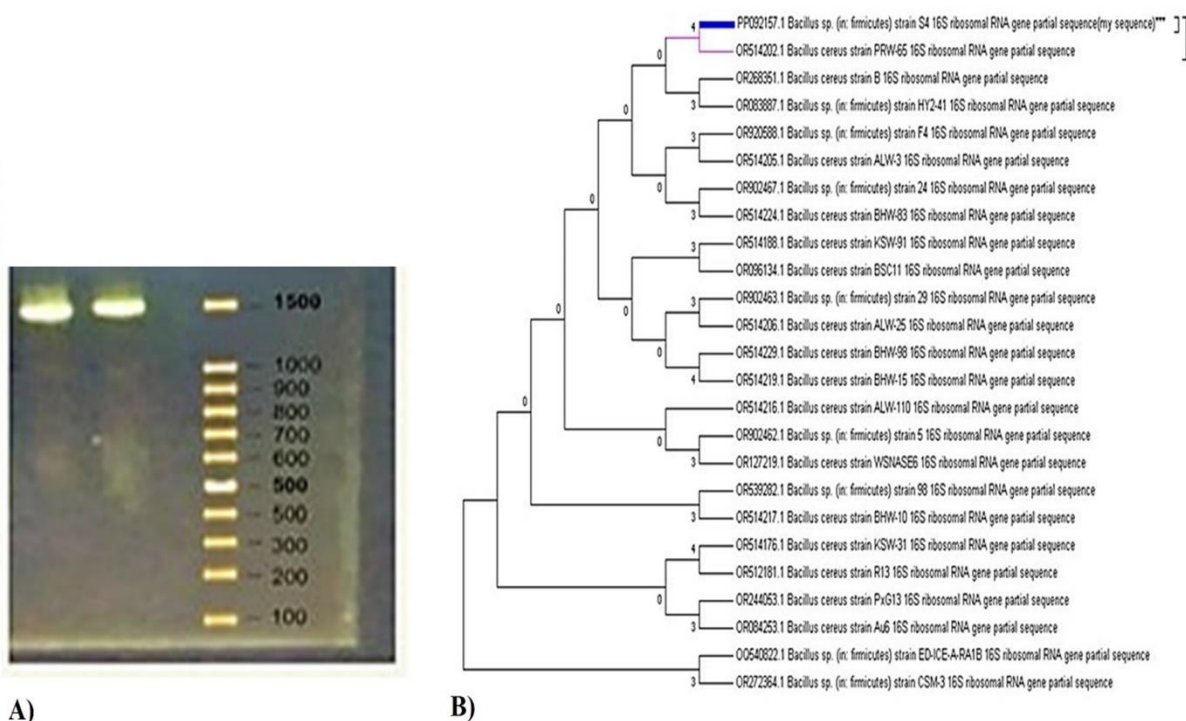


Fig. 4. Molecular identification and phylogenetic analysis of *Bacillus* sp. strain S4: A) Agarose gel electrophoresis of the PCR-amplified 16S rRNA gene of *Bacillus* sp. strain S4 in two replicates; B) phylogenetic tree of *Bacillus* sp. strain S4 and related species, constructed using the Neighbor-Joining method based on the Kimura 2-parameter model.

Among lipid inducers tested, castor oil markedly enhanced extracellular lipolytic activity compared to olive and frying oils. This strong induction may be associated with the high ricinoleic acid content of castor oil, whose hydrolysis releases intermediates that can stimulate lipase gene expression (Sharma *et al.*, 2009). Similar observations have been reported for other microorganisms grown on castor oil. However, it should be noted that the present study did not evaluate alternative lipid substrates such as soybean oil or coconut oil, which may also influence lipase production. Future studies should include a broader range of lipid sources to identify the most effective inducers. Moreover, recent studies have emphasized the industrial relevance

of unconventional lipidic substrates for example, the use of *Capparis spinosa* seed oil in biodiesel synthesis (Kattathayil *et al.*, 2024) underscoring the value of exploring microbes adapted to nontraditional lipid sources.

Regarding carbon sources, olive oil resulted in the highest lipase activity, followed by starch and glucose. This pattern aligns with classical carbon catabolite repression, in which simple sugars such as glucose suppress the expression of lipolytic genes and limit enzyme production (Akhter *et al.*, 2022). In *B. cereus* NC7401, starch and maltose supported higher lipase activity (30.6 and 40 U/mL, respectively) than other carbon sources tested, while the presence of simple sugars has been associated with reduced lipase production

due to catabolic repression (Akhter *et al.*, 2022). The moderate activity observed with starch in the present study likely reflects its slower hydrolysis and gradual release of glucose, a pattern consistent with these reports (Akhter *et al.*, 2022; Sohail, 2017). Although carbon catabolite repression is a well-documented phenomenon in *Bacillus*, direct evidence at the transcriptional level was not obtained in this study.

The nitrogen source played a critical role in enzyme production. Organic nitrogen compounds (peptone, tryptone, and yeast extract) induced significantly higher lipase activity than inorganic sources in this study. This result is consistent with reports in *B. cereus* NC7401, where tryptone was among the most effective nitrogen sources for lipase production (38.6 U/mL) (Akhter *et al.*, 2022). Similarly, in *Bacillus subtilis* PCSIRNL-39, peptone and yeast extract were identified as the best nitrogen sources for lipase production (Sohail, 2017). Peptone and yeast extract provide an abundant supply of amino acids, peptides, vitamins, and cofactors that stimulate both bacterial growth and lipase biosynthesis. However, it should be noted that the effect of nitrogen source is strain-dependent; for example, in *B. cereus* ATA179, inorganic nitrogen sources were more effective than organic ones (Demirkan *et al.*, 2021). These findings are consistent with earlier reports on enhanced lipase production in *Bacillus* species grown on complex organic nitrogen sources (Sharma *et al.*, 2009; Abro *et al.*, 2024).

Characterization of the extracellular lipolytic activity revealed a mesophilic and alkaline profile, which is consistent with the characteristics of many *Bacillus* lipases reported from hydrocarbon-contaminated environments. This profile suggests potential suitability for alkaline industrial applications, including detergent formulations, where activity at alkaline pH is advantageous. Although some bacterial lipases are thermophilic (Sharma *et al.*, 2009) or psychrotrophic (Kattathayil *et al.*, 2024), the moderate temperature optimum observed here may offer advantages in energy-efficient processes that do not require high-temperature operation (Guncheva and Zhiryakova, 2011).

The properties of *B. cereus* group lipases vary considerably depending on the strain and isolation source. For example, a cold-active alkaline lipase

from *B. cereus* U2 showed optimal activity at 20°C and pH 11 (He *et al.*, 2025), while an acidophilic lipase from *B. cereus* ALP E1 was optimally active at pH 5 and 40°C (Sari *et al.*, 2026). This diversity highlights the importance of characterizing individual isolates rather than assuming species-level uniformity. The strain S4 isolated in this study therefore represents a distinct member of this group with its own enzymatic profile.

A notable feature of the extracellular lipolytic activity of strain S4 is its thermal stability profile. Importantly, the temperature at which the activity showed maximum stability differed from the temperature at which it showed maximum activity. This decoupling of activity and stability optima is a well-documented phenomenon in lipases, where the temperature for maximal catalytic rate often differs from the temperature for maximal structural stability (Dasetty *et al.*, 2017). This property is advantageous for industrial operations requiring thermal tolerance, such as bioremediation of sun-heated contaminated soils or processes where mild heating is used to reduce microbial contamination (Guncheva and Zhiryakova, 2011). The thermal stability of the S4 extracellular lipolytic activity is comparable to some *Bacillus* lipases (Devi *et al.*, 2025) but lower than the thermophilic *B. cereus* ATA179 lipase, which showed optimal activity at 60°C (Irianto *et al.*, 2024). This comparison further highlights the strain-specific nature of lipase properties within the *B. cereus* group. However, thermal stability was evaluated at only two temperatures, and a broader range should be tested in future studies.

Although the effects of pH on enzyme activity were evaluated, the pH stability of the enzyme - its ability to retain activity following pre-incubation in buffers of varying pH - was not assessed due to experimental limitations. Future studies should investigate this parameter to better define application potential in environments with fluctuating pH conditions, such as wastewater treatment streams.

The enzyme production profile, reaching its peak at 48 hours, corresponds to the late exponential or early stationary phase, a stage commonly associated with the secretion of extracellular enzymes in bacilli. The subsequent reduction in activity may result from nutrient depletion, pH

drift in the culture medium, or increased endogenous protease activity. Protease-mediated degradation during prolonged fermentation is a well-documented phenomenon in *Bacillus* species and may account for the observed decline (Gupta *et al.*, 2002). Therefore, future experiments should include real-time monitoring of pH changes and direct quantification of protease activity to confirm this hypothesis.

Conclusion

The *Bacillus* sp. isolated from petroleum-contaminated soil in this study represents a promising microbial resource for industrial biotechnology, notably as a producer of extracellular lipolytic activity with a mesophilic and alkaline profile and noteworthy thermal stability. Systematic optimization of nutritional and cultural parameters established favorable conditions for enhanced extracellular lipolytic activity, providing a strong basis for future scale-up and industrial application. However, it should be emphasized that the enzyme was not purified in this study, and the observed activity represents the total extracellular lipolytic activity of the culture supernatant. Therefore, future research should focus on purifying the enzyme to homogeneity and conducting detailed kinetic and structural characterizations, as well as comprehensive assessments of its pH and solvent stability. Finally, the evaluation of its practical performance in relevant domains such as enzymatic transesterification for biodiesel synthesis and bioremediation of lipid-contaminated environments will be essential to fully establish its biotechnological potential.

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Data available

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of interests

The authors declare no conflict of interest.

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