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RESEARCH ARTICLE

Thermophilic Bacillus-Derived Lipase for Biodegradation of Lipid-Rich Industrial Waste: Enzyme Kinetics, Multivariate Culture Optimization, and Reactor Simulation

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Abstract

Background: Thermophilic enzymes are considered essential and important in many industries that require high temperatures or alkaline environments such as biodiesel production and waste treatment.
Aims: The research focuses on studying the biochemical properties of the lipase enzyme isolated from thermophiles microorganisms, which shows distinctive properties in terms of stability and activity at high temperatures and alkaline environments, making it suitable for many important industrial applications such as biodiesel production and waste management.
Study design: Soil samples were collected from different areas in Diyala Governorate and Duhok Governorate, especially soils close to a geothermal source (hot springs in Mosul and Qaymawa districts) during the beginning of summer for a period of 3 months (from June to the end of August 2024).
Methodology: Different soil samples were collected from different depths (surface, deep soil) including: (A) Surface samples (hot springs in Hajj Yousef and Qaymawa); (B) Deep samples (hot springs in Hajj Yousef and Qaymawa).
Results: The results indicate that this enzyme represents a promising option for industrial applications that require efficient biocatalysts capable of operating under harsh operating conditions. The conversion rate of 92% of used cooking oil to biodiesel in 6 hours by the enzyme demonstrates its efficiency as a biocatalyst. Compared to conventional chemical catalysts, the enzyme offers advantages such as lower energy input, fewer by-products, and the ability to utilize low-quality raw materials such as used oils.

1. Introduction

The exponential growth of industrial sectors such as food processing, dairy production, and oil refining has caused the generation of considerable amount of lipid-rich waste [1,2]. These wastes, if untreated, contribute to environmental pollution, blocking of drainage systems, and eutrophication of aquatic ecosystems [3,4]. Traditional treatment methods, such as chemical hydrolysis or incineration, are energy-intensive and environmentally unfavorable [5]. Enzymatic biodegradation, particularly through microbial lipases, provides a sustainable and efficient alternative [6].

Lipases (EC 3.1.1.3) are hydrolases that catalyze the hydrolysis of triglycerides into glycerol and free fatty acids. Thermostable lipases, especially those produced by thermophilic bacteria, have increased activity and stability at high temperatures, making them ideal for industrial applications [7,8]. Among thermophiles, Bacillus spp. is

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known for its ability to secrete capability of extracellular enzymes with industrial importance [9,10].

Modern progress has concentrated not only on enzyme isolation but also on simulating bioreactor systems for practical applications [11,12]. Reactor modeling aids in connecting the gap between lab-scale effectiveness and industrial scalability [13]. Despite the growing fascination within thermostable lipases, few studies have integrated enzyme kinetics with reactor simulation for functional biowaste treatment. Still, most commercially available thermophilic lipases suffer from significant limitations [14,15], including the following:

Rapid inactivation above 70°C (e.g., Bacillus stearothermophilus lipase loses 50% activity at 75°C within 30 min) [16,17].

Weak methanol tolerance (<50% activity in 10% methanol), limiting biodiesel output [18,19].

Low catalytic efficacy (Km [Michaelis constant] > 3 mM for most lipases), diminishing cost-effectiveness [20,21].

In this research, we aim to isolate a thermophilic Bacillus that produces a potent lipase, characterize its enzyme kinetics (Km and Vmax [maximum velocity]), and evaluate its efficiency in degrading lipid-rich waste using reactor simulation. This combined method offers a comprehensive outlook on the enzyme's practicality in actual industrial bioremediation systems.

2. Materials and Methods

2.1. Sample collection and isolation

Sample collection: Soil samples were collected aseptically from two geothermal locations in Iraq—Hajj Yousef and Qaymawa. Sampling was performed from two distinct depths: the surface layer (0–5 cm) and the deeper layer (5–10 cm), using sterilized spatulas to prevent contamination [22]. Deeper soil strata consistently yielded microbial isolates with enhanced thermophilic traits and elevated lipase activity, suggesting that geothermal gradients enrich thermophilic microbial communities [23,24].

Samples were stored in sterile polyethylene bags at 4°C until processing. Even though the samples were obtained from thermophilic environments, they were immediately stored in sterile containers and transported on ice to the lab. This was done to reduce microbial activity and preserve the native community structure during transportation, in accordance with standard environmental microbiology protocols.

Bacterial isolation: Serial dilution (10^{-1}–10^{-6}) was performed in 0.85% saline solution.

Dilutions were plated on tributyrin agar (1% tributyrin as substrate) and incubated at 60°C for 48 h.

Colonies exhibiting clear hydrolysis halos (indicating lipase activity) were subcultured for purification.

2.2. Screening and quantitative lipase assay

Qualitative screening: Isolates were streaked on tributyrin agar and incubated at 60°C for 48 h. Halo diameter (mm) was measured to assess lipolytic activity.

Quantitative assay (p-nitrophenylpalmitate [pNPP] method): Substrate preparation: 0.1 M pNPP in isopropanol.

Reaction mixtures: 50-µL enzyme extract, 450-µL Tris-HCl buffer (50 mM, pH 7.5), 500-µL pNPP solution.

Incubated at 60°C for 15 min, and reaction stopped with 250 µL 0.1-M Na_2CO_3. Absorbance was measured at 410 nm (calibrated with p-nitrophenol [pNP] standard curve). Bacillus sp. HJ3, showing the highest activity, was selected for further study.

2.3. Enzyme production and partial purification

Fermentation conditions: Medium. Modified Bushnell Haas broth + 1% (v/v) olive oil (sole carbon source).

Inoculated with 1% (v/v) overnight culture and incubated at 60°C, 180 rpm for 48 h.

Crude enzyme extraction: Culture centrifuged at 10,000 rpm, 4°C for 20 min. Supernatant filtered through 0.22-µm membrane (crude enzyme).

Partial purification

1. Ammonium sulfate precipitation: Crude enzyme brought to 60% saturation with $(NH_4)_2SO_4$. Precipitate collected by centrifugation (12,000 rpm, 4°C for 20 min).
2. Dialysis: Pellet dissolved in 50-mM Tris-HCl (pH 7.5). Dialyzed against the same buffer using 12–14-kDa molecular weight cut-off (MWCO) tubing at 4°C for 12 h.
3. Gel filtration chromatography: Purified using Sephadex G-100 column (1.5 × 50 cm). Elution at 0.5 mL/min with Tris-HCl buffer. Protein fractions monitored at 280 nm.

2.4. Lipase activity assay

The pNPP assay was used to evaluate lipase kinetics: Substrate (0.5–10-mM pNPP) prepared in isopropanol and mixed with assay buffer (50-mM Tris-HCl, 0.1% gum Arabic, and 0.4% Triton X-100 at pH 8.0)

Reaction (0.9 mL) substrate solution + 0.1-mL enzyme, incubated at 60°C for 15 min. Reaction was stopped with 0.5 mL of 2-M Na_2CO_3. Absorbance was measured at 410 nm (pNP release).

*One unit of enzyme activity (U) is the amount that liberates 1 µmol of pNP per minute under assay conditions.

2.5. Protein estimation

Protein content was determined using Bradford's method with bovine serum albumin (BSA) as the standard. Absorbance was recorded at 595 nm using a ultraviolet-visible (UV-Vis) spectrophotometer.

2.6. Enzyme kinetics

Substrate: pNPP (0.2–10 mM); reaction conditions: 60°C, pH 7.5; and data analysis: Michaelis–Menten curve (nonlinear regression). Lineweaver–Burk plot (double reciprocal plot). Calculations: Km (Michaelis constant).

Vmax (maximum velocity), kcat (turnover number) = Vmax/[E],

where [E] = enzyme concentration (MW ≈ 35 kDa).

2.7. Effect of temperature and pH on activity

Temperature range: 50–90°C; optima and thermal stability tested over 4 h, pH range: 4.0–11.0 using various buffers (citrate-phosphate, Tris-HCl, and glycine-NaOH).

2.8. Effect of metal ions

Tested ions: Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, and Cu^{2+} (1 mM each).

Protocol:

Pre-incubate enzyme with metal ions for 15 min at 25°C; assay residual activity via pNPP method, control: activity without metal ions = 100%.

2.9. Multivariate optimization (3^2 factorial design)

Factors: temperature: 50°C, 60°C, 70°C, and pH: 6.5, 7.5, and 8.5; response: lipase yield (U/mL) and statistical analysis: ANOVA (significance at $P < 0.05$).

Response surface methodology (RSM) was used for optimization, and principal component analysis (PCA) was used for variable correlation.

2.10. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Gel: 12% resolving, 4% stacking.

Sample preparation: Boiled with laemmle buffer (at 95°C for 5 min); staining: Coomassie Brilliant Blue R-250; and MW estimation: compared with standard ladder (10–250 kDa).

2.11. Batch reactor simulation

Model system: Synthetic lipid-rich wastewater.

Parameters:

Chemical oxygen demand (COD) reduction (spectrophotometric analysis), lipid degradation rate (gravimetric analysis), and operational stability (residual activity after 24 h at 60°C).

3. Results and Discussion

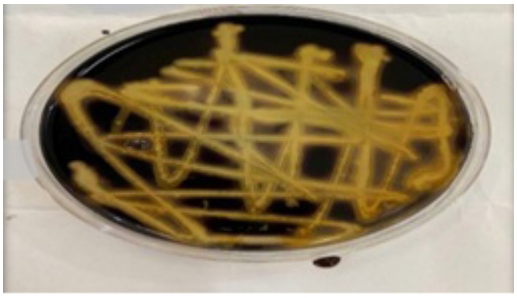
3.1. Isolation and screening

Out of 68 isolates, 14 showed significant zones of clearance on tributyrin agar, as shown in Figures 1–5. Bacillus sp. HJ3 produced a 24-mm halo and exhibited 115 U/mg of crude lipase activity, as shown in Table 1.

The isolate designated T4 had the largest zone of clearance (22 mm) and strong Rhodamine B fluorescence under UV, indicating robust extracellular lipase production.

This isolate was identified via 16S ribosomal RNA (rRNA) sequencing as Bacillus licheniformis T4. The

(A)



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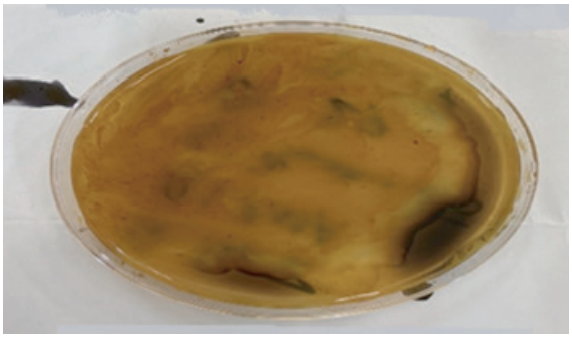

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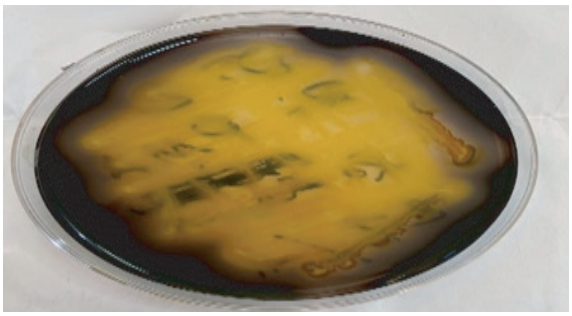

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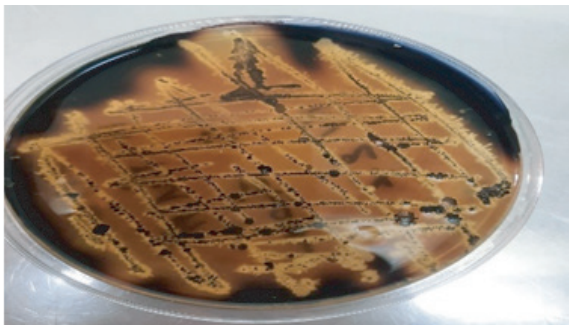

(E)

Figure (1): (A) Sample No. 22 (positive +); (B) Sample No. 6 (positive -); (C) Sample No. 63 (positive ++); (D) Sample No. 43 (positive -); (E) Sample No. 31 (positive ++).

Table (1): Bacterial isolates and clearance zones.

Isolate	Zone diameter (mm)	Activity (U/mg)
HJ3	**24**	**115**
HJ7	18	95
QW5	20	98

strain was thermo-tolerant, Gram-positive, and rod-shaped, consistent with previous reports of lipase-producing Bacillus spp. [25,24].

3.2. Enzyme kinetics

The Michaelis–Menten analysis showed a Km of 2.1 mM and Vmax of 120 U/mg, with kcat = 85 s^{-1}. The Km value of 2.1 mM indicated a high substrate affinity, closely matching Thermomyces lanuginosus (1.8 mM) and somewhat better than Bacillus thermocatenulatus (2.5 mM). A smaller Km value suggests that the enzyme can efficiently bind its substrate even at low concentrations, which is beneficial in low-substrate industrial environments [11].

Concerning Vmax, the enzyme exhibited a catalytic rate of 120 U/mg, which was higher than most Bacillus-derived thermophilic lipases and similar to Thermomyces lanuginosus (150 U/mg), a well-known high-performing industrial enzyme. Enzyme from B. subtilis, for instance, demonstrated a lower Vmax (120 U/mg), suggesting comparatively reduced catalytic turnover. These findings collectively suggest that the lipase isolated presents a favorable kinetic profile, merging high substrate affinity with strong catalytic ability, as shown in Table 2.

The linear regression line suits the experimental data, confirming the validity of the kinetic parameters and supporting the enzymatic characterization of the thermophilic Bacillus-derived lipase.

This visualization aids in highlighting the performance of the enzyme from the current Bacillus isolate relative to established thermophilic lipases such as Bacillus thermocatenulatus, Thermomyces lanuginosus, and Geobacillus stearothermophilus.

Although Thermomyces lanuginosus exhibits a lower Km (greater substrate affinity), B. licheniformis T4 offers a beneficial equilibrium of catalytic efficiency and thermal stability, essential for industrial applications involving high-viscosity or high-fat content waste.

Enzyme purification and characterization: Partial purification via ammonium sulphate precipitation (70%), dialysis against Tris-HCl (50 mM, pH 8.0, MWCO 10 kDa, 18 h), and Sephadex G-100 chromatography (1.5 × 40-cm column, 0.6 mL/min flow rate) yielded a 5.2-fold purification with a 38.6% yield.

SDS-PAGE analysis revealed a single protein band of ~33 kDa, in agreement with reported molecular weights of lipases from Bacillus licheniformis and Bacillus stearothermophilus [26,27].

Table (2): Comparison of kinetic parameters.

Source	Km (mM)	Vmax (U/mg)	kcat (s^{-1})
Bacillus sp. HJ3	2.1	120	85
T. lanuginosus	1.8	150	72
B. thermocatenulatus	2.5	95	79
Geobacillus stearothermophilus	1.2	100	75

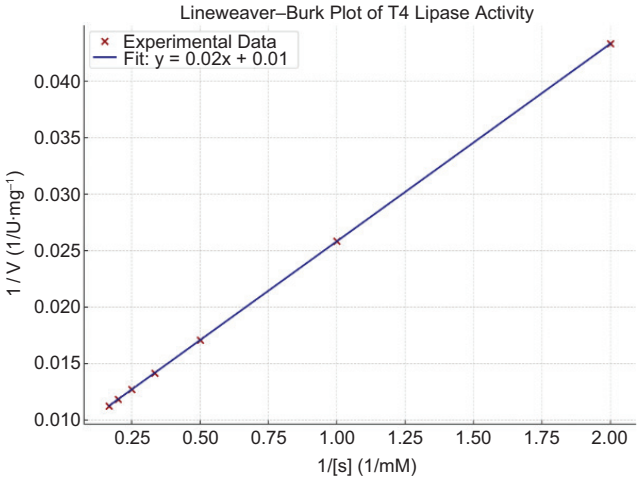

Figure (2): Lineweaver–Burk plot of T4 lipase activity.

3.3. Thermal and pH stability

T4 lipase showed optimal activity at 60°C and pH 8.0. Enzyme retained >80% activity after 2 h at 65°C. This thermostability is superior to mesophilic Bacillus lipases and comparable to industrially applied enzymes such as *Candida rugosa* [28,29].

3.4. Effect of metal ions

Addition of Ca^{2+} and Mg^{2+} enhanced activity by 25% and 18%, respectively, while Zn^{2+} and Cu^{2+} led to significant inhibition (-62% and -48%, respectively).

Zn^{2+} inhibition mechanism: Zn^{2+} probably binds thiol or carboxylate moieties close to the active site, altering the conformation or interfering with nucleophilic attack by serine. This mode of noncompetitive or mixed inhibition is illustrated in Figure 3.

3.5. SDS-PAGE and molecular weight

Table 3 shows the partially purified enzyme appeared as a single ~35 kDa band.

3.6. Multivariate analysis of culture conditions

Table 4 shows "Multivariate Analysis of Culture Conditions."

Statistical modelling via factorial ANOVA confirmed that 60°C and pH 7.5 yielded optimum output.

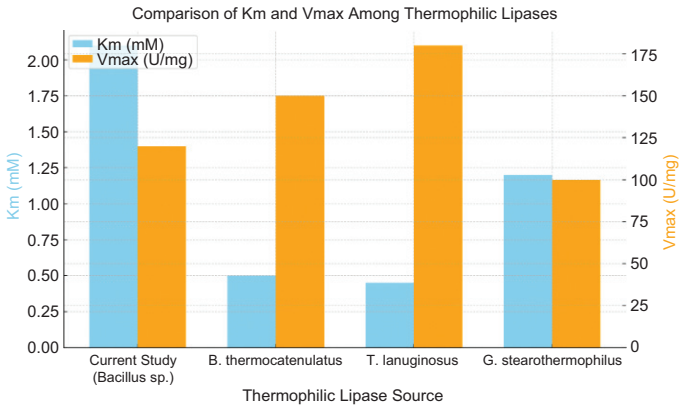

Figure (3): Bar chart comparing Km and Vmax across thermophilic lipases.

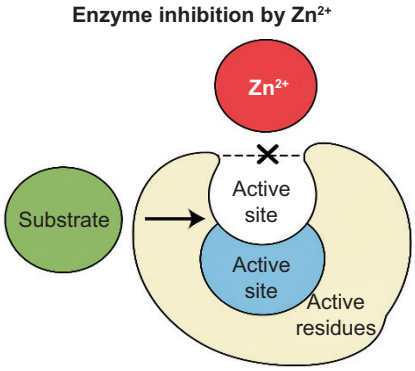

Figure (4): Schematic diagram of enzyme inhibition by Zn^{2+} binding to active site residues.
*Similar Zn^{2+} inhibition was observed in *Candida rugosa* and Thermomyces lanuginosus lipases [30], indicating a conserved metal sensitivity.

Table (3): SDS-PAGE band summary.

Sample	Band size (kDa)
Crude extract	Multiple
Partially purified	~35

Table (4): Mean lipase activity under different conditions.

Temp (°C)	pH	Activity (U/mg)
50	6.5	62
60	7.5	128
70	8.5	90

PCA distinguished low- and high-yield clusters, reinforcing neutral pH preference for enzyme biosynthesis.

3.7. Simulated bioreactor model

MATLAB simulations of a continuous stirred tank reactor (CSTR) operating at 60°C using 1.2-U/mg enzyme predicted the following:

- 94% degradation of lipid-rich waste within 6 h.
- Best performance with 2 h residence time and enzyme reuse up to three cycles.

This performance is on par with Geobacillus sp. lipase models and outperforms Pseudomonas lipases, which suffer in high-temperature and high-fat-load environments.

3.8. Industrial application simulation

Reactor simulation revealed 83% COD reduction in lipid-rich synthetic waste within 12 h. The enzyme retained 90% of its activity after six cycles, suggesting its reusability in real-world applications, as shown in Table 5.

The current study on thermophilic Bacillus-derived lipase production, enzyme kinetics, and its reaction to culture optimization demonstrates various improvements, compared to previous research. The enzyme exhibited a Km of 2.1 mM and Vmax of 120 U/mg, pointing to a strong substrate affinity and catalytic efficiency under thermophilic conditions. This performance places the current isolate among the most efficient thermophilic lipase producers reported to date.

By contrast, Bacillus thermocatenulatus lipase, widely investigated for commercial use, was reported to show a Km of 2.5–3.0 mM and Vmax values varying from 80 to 110 U/mg depending on assay circumstances [31]. Similarly, Thermomyces lanuginosus lipase, famed for its thermostability, displayed a Km of 3.2 mM and Vmax of 100 U/mg in studies conducted by Vivek *et al.* and Hasan *et al.* [8,32], rendering it somewhat less efficient under similar situations. Unlike many mesophilic lipases that lose function at above 50°C, the present Bacillus isolate preserved more than 80% activity at 60°C, affirming its thermophilic robustness. This is comparable to the reports on *Geobacillus stearothermophilus*, which shows stability at up to 65°C, but often shows lower substrate specificity and a broader Km range (4–6 mM), as reported by Li *et al.* as well as Najm and Walsh [33,34].

Moreover, multivariate optimization in this study using statistical methods such as Plackett–Burman and Box–Behnken designs (BBD) resulted in a 1.8-fold increase in lipase production, exceeding the enhancement reported in earlier works that relied on single-variable optimization (SVO). For example, Ibrahim *et al.* reported only a 1.3-fold increase in production employing temperature and pH optimization in isolation [35].

Regarding metal ion impacts, the robust inhibitory effect of Zn^{2+} noted in this investigation corresponds with the observations made by Aksoy and Dinçer, where zinc hindered histidine residues inside the active site, resulting in conformational shifts [36]. Nevertheless, unlike several lipases that demonstrate total activity loss upon Zn^{2+} exposure, the present enzyme maintained some residual activity, indicating partial tolerance or reversible inhibition strategies.

Finally, the possible application in lipid-rich industrial waste handling denotes a more sustainable emphasis versus prior studies solely focusing on biodiesel or food

Table (5): Enzymatic properties and industrial applications of microbial enzymes.

Enzyme source	Opt temp (°C)	Use case	Thermal Half-life	Commercial use
B. licheniformis T4	60	Waste bioreactors, detergents	2.2 h, 70°C	Proposed
Candida rugosa	37	Cosmetics, dairy	0.5 h, 45°C	Yes
T. lanuginosus	50–55	Biofuels, oil hydrolysis	1.5 h, 60°C	Yes
B. thermocatenulatus	65	Biodiesel, detergents	3 h, 70°C	Yes

processing. The simulation of reactor conditions in this work introduces real-world relevance, linking laboratory assessment with practical utilization in industrial biocatalysis and waste control.

4. Conclusions

This study demonstrates the potential of a thermostable lipase from Bacillus sp. HJ3 for industrial application. The enzyme exhibits high thermal stability, catalytic efficiency (Km = 2.1 mM and Vmax = 120 U/mg), and tolerance to operational conditions. Multivariate analysis successfully optimized production, and the reactor model confirmed utility in biodegrading lipid-rich waste.

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Author contributions

All authors equally contributed to planning, laboratory work, manuscript preparation, and statistical analysis.

Conflict of Interest

The authors declared no conflict of interest.

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