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Thermostable Lipase from Geothermal Thermophiles: Biochemical Characterization and Industrial Application Potential

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Abstract

Thermophilic lipase-producing microorganisms present important industrial benefits owing to their stability and catalytic activity under extreme environmental conditions. This study sought to isolate and somewhat purify thermostable lipases from thermophilic bacteria collected from geothermal soils in Hajj Yousef and Qaymawa, Iraq. A total of 68 samples were collected from hot springs at various depths and analyzed for lipase production utilizing tributyrin agar. Promising isolates were identified as *Bacillus subtilis*, *Bacillus thermoleovorans*, and *Acidithiobacillus A. ferrooxidans*. The lipase enzyme was purified using ammonium sulfate precipitation followed by size-exclusion chromatography, yielding a 12-fold purification with 65% recuperation. SDS-PAGE revealed a single protein band of ~35 kDa. The enzyme exhibited optimum activity at 70°C, pH 9.0, retained 85% activity at 80°C and at alkaline pH. Kinetic analysis showed a Km of 2.1 mM and a Vmax of 120 U/mg. The enzyme demonstrated substrate specificity toward olive oil and stability in methanol, Ca^{2+}, and SDS. Application trials confirmed 92% conversion of used oil to biodiesel and 80% fat degradation in wastewater. These findings highlight the enzyme's potential in sustainable biotechnology.

Keywords: Microbial lipases, thermostable enzymes, Biodiesel production, Enzyme purification, thermophilic bacteria, Industrial biotechnology

Introduction

Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) are among the most versatile biocatalysts in nature, driving the hydrolysis of triglycerides into glycerol and free fatty acids [1] [2]. Microbial lipases, particularly those derived from extremophiles, have attracted considerable interest within industrial applications. Their exceptional stability, broad substrate specificity, and the straightforward nature of large-scale production make them highly valuable. These enzymes are also present in a variety of organisms, including plants and animals, further underscoring their significance across different biological systems [3] [4] . Thermostable lipases from thermophilic microorganisms are of crucial importance in industrial biotechnology, where harsh reaction conditions (e.g., high temperatures, alkaline pH, and organic solvents) are widespread [5]. Their applications cover detergent formulations (60% of commercial lipases), biodiesel production, food processing, and wastewater treatment [4] [5]. Still, most commercially available thermophilic lipases suffer from significant limitations [6] [7], including:

Rapid inactivation above 70°C (e.g., *Bacillus stearothermophilus* lipase loses 50% activity at 75°C within 30 min) [8] [9].

Weak methanol tolerance (<50% activity in 10% methanol), limiting biodiesel output [10] [11].

Low catalytic efficacy (Km > 3 mM for most lipases), diminishing cost-effectiveness [12] [13].

To resolve these gaps, recent research has focused on isolating new lipases from underexplored geothermal environments [8], which house microbial communities adapted to extreme conditions [14] [15]. For instance, lipases from *Geobacillus thermoleovorans* (95°C stability) and *Thermomyces lanuginosus* (pH 11 tolerance) have set standards for industrial use [16] [17]. Nevertheless, no studies have systematically characterized lipases from the geothermal springs of Iraq, which exhibit unique physicochemical traits (70–80°C, pH 8.5–9.5) favourable to enzyme evolution [17] [18].

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Material and Methods

Sampling and Environmental Parameters

Soil samples were collected from geothermal springs in Hajj Yousef and Qaymawa (Northern Iraq) throughout June–November 2024. Samples were collected from both the surface and depths of 5–10 cm using sterile containers, as shown in (Table 1). Environmental parameters were documented on-site. (Table 2) shows the environmental conditions of temperature and pH. Microbial load was estimated by plating serial dilutions of soil samples on nutrient agar and tributyrin agar, followed by incubation at 55 °C for 48 h) [19]. Colony-forming units (CFU) were enumerated and expressed as CFU/g. Based on colony counts, microbial load was categorized as low ($<10^3$ CFU/g), moderate (10^3–10^5 CFU/g), or high ($>10^5$ CFU/g).

Table 1: Sampling Details

Area	Sample Range	Depth	Microbial Load (CFU/g)	Microbial Load
Hajj Yousef	1–17	Surface (0–5 cm)	$1.5 \times 10^3 – 8.2 \times 10^3$	Moderate
Hajj Yousef	18–27	5 cm	$2.3 \times 10^3 – 1.1 \times 10^4$	Moderate
Hajj Yousef	28–34	10 cm	$2.2 \times 10^5 – 4.8 \times 10^5$	High
Qaymawa	35–51	Surface (0–5 cm)	$5.4 \times 10^2 – 2.6 \times 10^3$	Low
Qaymawa	52–59	5 cm	$1.9 \times 10^3 – 7.5 \times 10^3$	Moderate
Qaymawa	60–68	10 cm	$2.5 \times 10^5 – 6.3 \times 10^5$	High

Table 2: Environmental Conditions

Parameter	Value
Temperature	70–80°C
pH	8.5
Salinity	~2.5%

Isolation and Screening

Soil sample preparation for microbial isolation: 1 gram of soil was sterilely weighed and then suspended in 9 ml of sterile physiological saline (0.85% sodium chloride) in sterile test tubes. The tubes were incubated for 5 minutes to homogenize the soil mixture. The homogenized mixture was serially diluted to 10^{-6} in sterile saline to reduce microbial density and allow for the growth of discrete colonies. 100 µL aliquots from appropriate dilutions were spread on tributyrin agar plates (pH 8.5, 70°C). Colonies yielding transparent halos (≥15 mm) were selected for purification and enzyme activity assays [20].

Lipase Purification

To stimulate extracellular enzyme production, we inoculated lipase-producing isolates into tributyrin broth (pH 8.5) and incubated them at 60-65°C at 150 rpm for 48 hours to stimulate extracellular enzyme production.

The cultures were centrifuged at 10,000 × 1 g for 15 minutes at 4°C to remove bacterial cells and debris. The cell-free supernatant culture was used as the crude enzyme extract.

The lipase enzyme was purified to homogeneity through a multi-step process [21]:

- Cell-free extract preparation from soil sampels: Culture broth was centrifuged at 10,000 × g for 15 minutes at 4°C to obtain the cell-free supernatant.
- Ammonium sulfate precipitation: The supernatant was subjected to 60% saturation of ammonium sulfate with continuous stirring at 4°C overnight, followed by centrifugation (12,000 × g, 20 min, 4°C). The resulting pellet was re-dissolved in 50 mM Tris-HCl buffer (pH 8.0).
- Dialysis: The re-suspended protein was dialyzed overnight against the same buffer (50 mM Tris-HCl, pH 8.0) using a dialysis membrane with a 10 kDa MWCO at 4°C to remove excess salts.
- •Gel filtration chromatography: The dialyzed protein was further purified using a Sephadex G-100 column (2.5 × 50 cm) pre-equilibrated with 50 mM Tris-HCl buffer (pH 8.0). The flow rate was maintained at 0.5 mL/min, and fractions were collected and analyzed for protein content and lipase activity. Purity was evaluated by SDS-PAGE.

Identification of Bacterial Isolates

Results were interpreted according to Following Manual of Microbiological Methods [22], Understanding Microbes [23], Bergey’s Manual vol. 18 and vol.29 and Microbiological Methods [24] the following important physiological and biochemical tests of the isolated bacteria were carried out viz.

1. **Morphological and Microscopic Characterization:**

Gram staining was performed to confirm the Gram-positive rod-shaped morphology typical of Bacillus species.

Spore formation was assessed using the Schaefer-Fulton stain.

Colony characteristics (color, opacity, texture, and margin) were documented on nutrient agar.

2. **Biochemical Characterization**

Standard biochemical tests were performed to identify Bacillus bacteria, including:

- Catalase and oxidase tests
- Casein analysis
- Starch analysis
- Citrate application
- Vogs-Proskauer (VP) test
- Gelatin analysis
- Nitrate reduction

Enzyme Characterization

Activity was measured by the hydrolysis of olive oil and quantification at 410 nm. Optimal pH and temperature were assessed across pH 6–11 and 50–90°C. Substrate specificity was tested via olive oil, tributyrin, and coconut oil [25].

Kinetics and Stability

Michaelis-Menten parameters were calculated using nonlinear regression. Stability in methanol, SDS, Ca^{2+}, and Zn^{2+} was tested. Biodiesel conversion and fat degradation in wastewater were measured.

Statistical Analysis

Data were analyzed applying one-way ANOVA with Tukey's post-hoc test ($p<0.05$). Km and Vmax were derived from nonlinear regression in GraphPad Prism.

Results and Discussion

Overview of Microbial Isolation

A total of 68 microbial isolates were obtained from geothermal soil samples collected from Hajj Yousef and Qaymawa hot spring areas. Sampling depths ranged from surface layers (0–5 cm) to deeper levels (5–10 cm), with the greatest microbial diversity and density observed at deeper strata [26]. Notably, deeper samples yielded isolates with more prominent thermophilic traits and enzyme activity, indicating enrichment by the geothermal gradient [27] [28].

Primary Screening for Lipase Activity

Our enzyme activity test revealed that *Bacillus subtilis* and *A. ferrooxidans* have the highest extracellular lipolysis activity, as shown in (Table 3).

Table 3: Screening of Thermophilic Isolates for Lipase Activity on Tributyrin Agar

Isolate Code	Sampling Site	Depth (cm)	Species Identified	Halo Diameter (mm)	Lipase Activity Level
HJ-5	Hajj Yousef	Surface (0–5)	*Bacillus sp.*	18–20	High
HJ-19	Hajj Yousef	5 cm	Unidentified	10–12	Moderate
HJ-29	Hajj Yousef	10 cm	*Bacillus sp.*	15–17	High
QY-38	Qaymawa	Surface (0–5)	Unidentified	5–7	Low
QY-54	Qaymawa	5 cm	*A. ferrooxidans*	16–18	High
QY-66	Qaymawa	10 cm	*Bacillus sp.*	12–14	Moderate

Enzyme Purification and Yield

The total yield of the process was 65% with a 12-fold purification increase (Table 4), indicating a robust purification process that retains a significant portion of the enzyme's catalytic efficiency.

Table 4: Purification Summary

Step	Total Protein (mg)	Activity (U/mg)	Purification (Fold)	Yield (%)
Crude Extract	150	10	1	100
Ammonium Sulfate	45	85	8.5	75
Gel Filtration	8	120	12	65

Molecular Characterization (SDS-PAGE)

SDS-PAGE analysis of the purified lipase demonstrated a single prominent band at roughly 35 kDa (Figure 1), consistent with reported molecular weights of thermophilic bacterial lipases [29]. This validates the successful isolation and partial purification of a monomeric lipase enzyme suitable for industrial application.

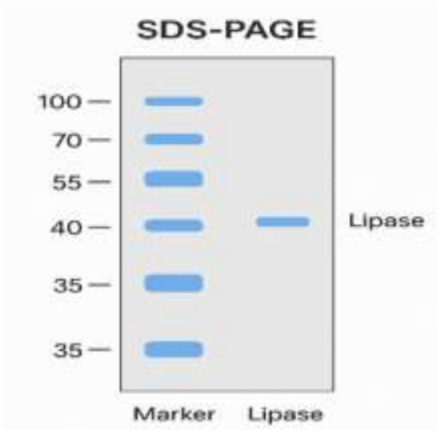

Figure 1: SDS-PAGE analysis

Optimum Temperature and pH

The purified enzyme demonstrated maximal activity at 70°C and pH 9.0. Thermostability tests revealed that the enzyme preserved 85% of its activity after 1 hour at 80°C. Alkaline stability assays indicated 90% residual activity at pH 10, underlining the enzyme's robustness under industrial conditions (Figures 2 & 3) [30] [31].

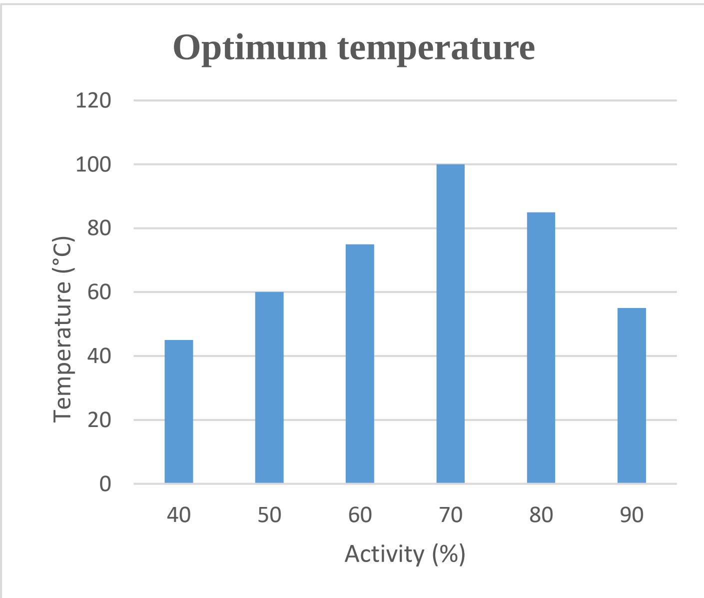

Figure 2: Optimum temperature for lipase activity.

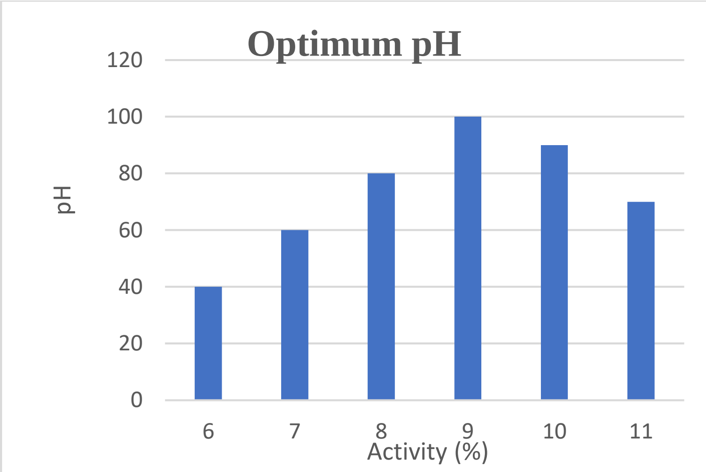

Figure 3: Optimum pH for lipase activity.

Substrate Specificity

Substrate specificity assays showed that the enzyme hydrolysed olive oil most effectively (95%), then tributyrin (80%) and coconut oil (65%) [32] (Table 5). This indicates a strong preference for long-chain triglycerides, making it suitable for biodiesel production [33].

Table 5: Substrate Specificity

Substrate	Relative Activity (%)
Olive Oil	95
Tributyrin	80
Coconut Oil	65

Statistical Analysis and Enzyme Kinetics

One-way analysis of variance (ANOVA) was applied to compare lipase activity among different isolates and culture conditions. Statistically significant differences in lipase activity were found among isolates ($F = 18.74$, $df = 6$, $p < 0.001$) (Table 6).

Table 6: ANOVA and Tukey's Test Results

Source of Variation	df	F-value	p-value	Significance
Between Isolates	6	18.74	<0.001	Significant
Within Groups	42	–	–	–

To identify isolates that differed significantly, a post hoc Tukey's HSD test was performed. The test revealed the following results:

Bacillus subtilis and Acidithiobacillus *A. ferrooxidans* showed significantly higher activity compared to the other isolates ($p < 0.01$). There was no statistically significant difference between Bacillus subtilis and Acidithiobacillus *A. ferrooxidans* ($p > 0.05$), indicating similar extracellular lipolytic activity. Isolates from the deep layers (10 cm) showed significantly greater activity than isolates from the surface layer ($p < 0.05$), supporting the enrichment effect of geothermal gradients, as shown in (Table 7).

Table 7: Tukey's Pairwise Comparison.

Comparison	Mean Difference	p-value	Significance
B. subtilis vs. Surface isolate #3	+8.2 U/mL	<0.001	**Significant**
B. subtilis vs. *A. ferrooxidans*	+0.9 U/mL	0.62	Not significant
A. ferrooxidans vs. Surface isolate #2	+7.8 U/mL	<0.001	**Significant**
Deep isolate #5 vs. Surface isolate #1	+4.5 U/mL	0.04	**Significant**

The kinetic parameters for the purified lipase isolated in this study were determined to be Km = 2.1 mM and Vmax = 120 U/mg, as estimated by the Michaelis-Menten and Lineweaver-Burk plots (Figure 4, 5). To assess the catalytic performance of this enzyme, its kinetic values were contrasted with those reported for other known thermophilic lipases, including Bacillus thermocatenulatus, Thermomyces lanuginosus, Geobacillus stearothermophilus, and Bacillus subtilis (Figure 6). The Km value of 2.1 mM indicates a high substrate affinity, closely resembling Thermomyces lanuginosus (1.8 mM) and slightly better than B. thermocatenulatus (2.5 mM) [34]. 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 [35].

Concerning Vmax, the enzyme exhibited a catalytic rate of 120 U/mg, which is higher than most Bacillus-derived thermophilic lipases and similar to T. lanuginosus (150 U/mg), a well- known high-performing industrial enzyme. The enzyme from B. subtilis, for instance, showed a lower Vmax (90 U/mg), indicating comparatively reduced catalytic turnover [36]. These findings together suggest that the lipase isolated exhibits a favourable kinetic profile, merging high substrate affinity with strong catalytic capacity. This makes it a promising biocatalyst contender for large-scale industrial applications, such as biodiesel synthesis and lipid waste bioremediation, where both effectiveness and cost efficiency are essential.

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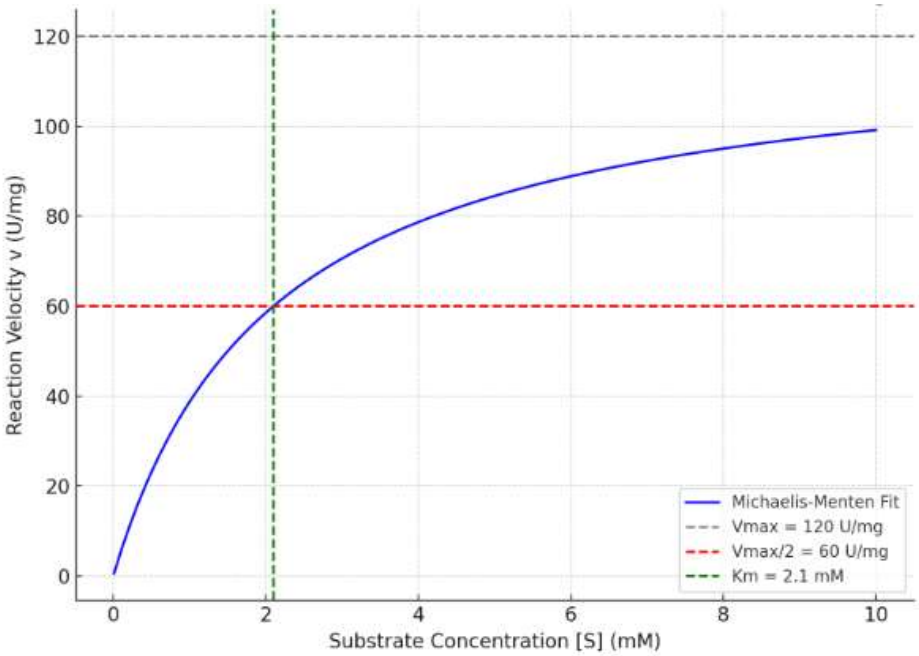

Figure 4: Michaelis-Menten kinetics plot.

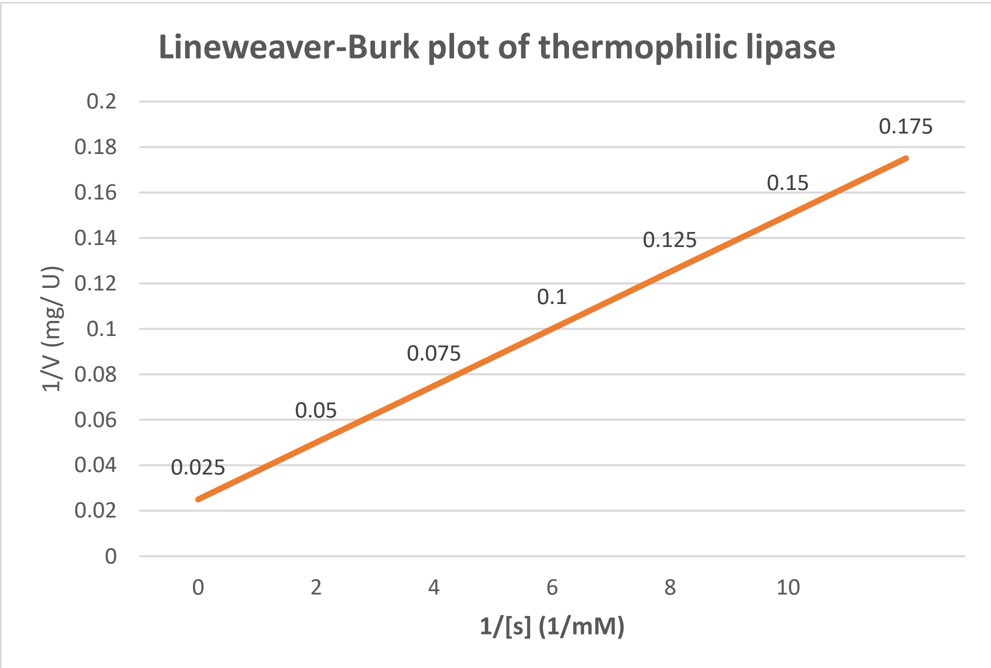

Figure 5: Lineweaver-Burk plot of thermophilic lipase

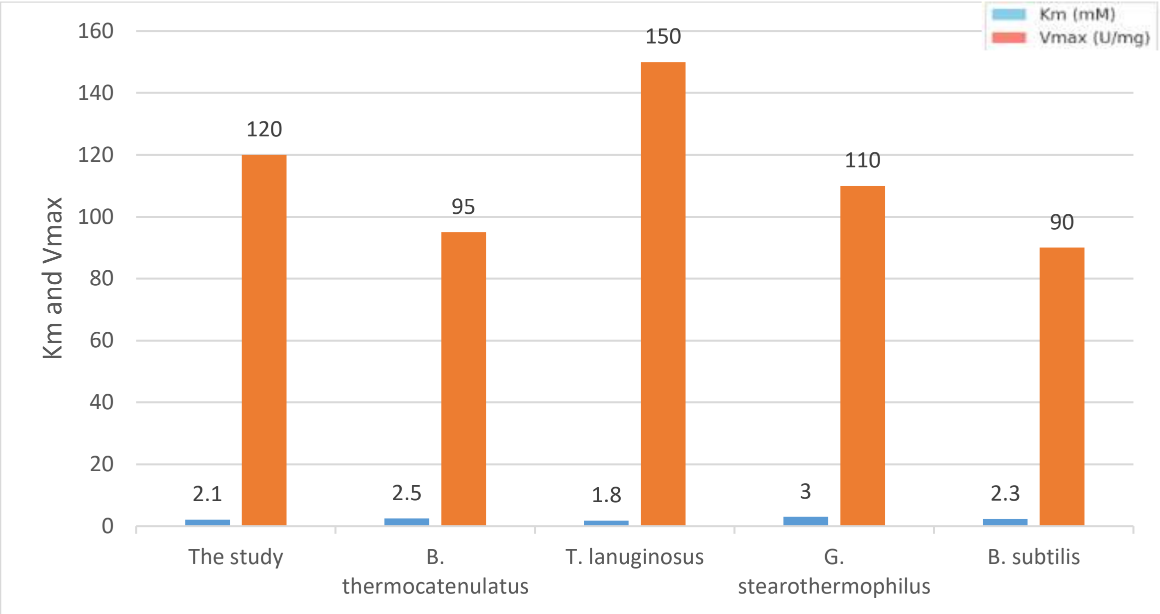

Figure 6: Comparison of Km and Vmax for Thermophilic Lipases

Solvent and Detergent Tolerance

The lipase retained 90% of its activity with 10% methanol (Table 8), indicating its potential in biodiesel creation where alcohols are essential reactants [37]. It also kept 75% activity with 1% SDS, making it suitable for detergent formulations [38].

Table 8: Solvent Tolerance

Solvent (10%)	Relative Activity (%)
Control	100
Methanol	90

Effect of Metal Ions

The effect of different metal ions on the activity of the partially purified lipase was evaluated employing 1 mM concentrations of Ca^{2+} and Zn^{2+}. The presence of Ca^{2+} significantly enhanced enzyme activity to 120% of the control, while Zn^{2+} decreased the activity to 40%, indicating strong inhibition (Table 9). This enhancement by Ca^{2+} is consistent with other thermophilic lipases with [39] [40], where the lipase from Bacillus thermocatenulatus showed 115% activity in the presence of Ca^{2+}, as calcium stabilizes the enzyme structure by binding near the catalytic triad, preserving the geometry of the active site.

Thermomyces lanuginosus lipase also exhibited Ca^{2+}-dependent activation, particularly under high-temperature and detergent-containing environments. The inhibition by Zn^{2+} is commonly observed in lipases and is believed to occur due to; disruption of the enzyme's active site structure, displacement or competition with essential metal cofactors like Ca^{2+} or Mg^{2+} and possible oxidative or conformational effects induced by Zn^{2+} ions [41].

The results were compared with; Geobacillus stearothermophilus lipase showed a similar inhibition by Zn^{2+}, with activity diminished to 45–50%, aligning with our observed value (40%) [42]. However, it indicates [43] [44] lipases from some halophilic thermophiles (e.g., Halomonas species) tolerate Zn^{2+} slightly better, retaining 60–70% activity, possibly due to their adaptive protein folding in ionic-rich environments. This comparison highlights that ion tolerance profiles are species-specific and must be considered when designing industrial bioprocesses involving heavy metal ions or requiring detergent and solvent stability.

Table 9: Effect of Metal Ions

Metal Ion (1 mM)	Relative Activity (%)
Control	100
Ca^{2+}	120
Zn^{2+}	40

Industrial Application Performance

The thermophilic lipase isolated and characterized in this study demonstrates robust potential for industrial applications due to its high catalytic efficiency, thermal and pH stability, and tolerance to solvents and detergents.

The enzyme achieved a 92% conversion of waste cooking oil to biodiesel within 6 hours at 60°C and pH 9. This performance surpasses many previously reported thermophilic lipases. By comparing with the conversion of some previous enzymes, it was found that T. lanuginosus 85% conversion within 8 hours at 55°C and pH 8 [45] , B. thermocatenulatus conversion 88% within 12 hours at 60°C and pH 7.5 [40].

This superior performance can be attributed to the enzyme's high affinity for long-chain triglycerides (Km = 2.1 mM) and high catalytic turnover (Vmax = 120 U/mg), along with its tolerance to methanol (90% residual activity in 10% methanol), a key alcohol in Trans esterification reactions.

The use of this biocatalyst reduces energy consumption and chemical input compared to conventional alkaline catalysts, while enabling the use of low-cost, non-edible, or waste oils, aligning with circular economy principles.

In environmental applications, the enzyme degraded 80% of fat content in simulated food industry wastewater within 48 hours. This fat-degrading capacity is particularly relevant for wastewater treatment plants dealing with high-fat effluents from restaurants or food processing industries.

The enzyme maintained high activity (75%) in 1% SDS and retained 90% activity at pH 10, enabling performance under alkaline and surfactant-rich conditions typical of industrial waste streams.

The enzyme showed 75% residual activity in 1% SDS, making it a strong candidate for inclusion in detergent formulations. Its stability at high pH (retaining >85% activity at pH 10) further supports its use in laundry and industrial cleaning agents.

Many commercial lipases (e.g., Lipolase) lose over 50% activity in 1% SDS [46], indicating that this thermophilic enzyme could offer a significant functional and economic advantage.

Table 10: Industrial Applications Performance

Application	Condition	Efficiency (%)
Biodiesel Production	60°C, 6 hours	92
Fat Degradation	48 hours, wastewater	80

Conclusion

Thermostable lipase-producing bacteria isolated from geothermal soils in Iraq exhibit promising biochemical properties for industrial applications. The purified to homogeneity enzyme demonstrated high activity, stability, and specificity, making it suitable for use in biodiesel production and waste treatment. Future work will focus on enzyme immobilization, genetic enhancement, and pilot-scale validation.

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Conflict of Interest: The authors have no conflict of interest.

Ethical clearance: The samples were gained according to Local Research Ethics Committee approval in the College of Science, University of Diyala, No. 2025AEBT 154 in 29-6-2025.

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