Research Article

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Extraction and Purification of Lipase and Dioxygenase from Pseudomonas luteola Isolated from Petroleum-Contaminated Soil

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Abstract

This study examined the recovery and preliminary biochemical characterization of extracellular lipase and catechol-cleaving dioxygenase activities from a bacterial isolate identified phenotypically as Pseudomonas luteola and obtained from petroleum-contaminated soil. Crude enzyme preparations were subjected to ammonium sulfate precipitation followed by gel filtration chromatography. For the dioxygenase preparation, specific activity increased from 15.3 U/mg in the crude extract to 588 U/mg after gel filtration, corresponding to a 38.4-fold enrichment with a final apparent yield of 15.9%. For lipase, specific activity increased from 0.00537 U/mg to 6.99 U/mg, corresponding to a 1302-fold enrichment, while the calculated apparent yield exceeded 100%, indicating increased measurable activity after purification and therefore requiring cautious interpretation. Chromatographic elution profiles showed discrete protein peaks associated with the pooled active fractions. The purified lipase displayed highest activity and stability at 30–35°C and near-neutral pH, whereas the dioxygenase preparation showed maximum activity under mesophilic temperatures and around pH 7.0. Taken together, the results indicate that petroleum-associated bacterial isolates can provide enzyme preparations that remain active under mild conditions relevant to environmental biotechnology. The present work should be viewed as a preliminary purification and characterization study; it establishes a biochemical basis for future investigations of enzyme identity, catalytic performance, and direct hydrocarbon biodegradation.

Keywords: lipase, dioxygenase, Pseudomonas luteola, petroleum-contaminated soil, purification

1. INTRODUCTION

Microbial enzymes are increasingly valued in environmental biotechnology because they operate under mild conditions, display useful substrate selectivity, and can contribute to the transformation of complex organic pollutants. Among these enzymes, lipases (EC 3.1.1.3) and ring-cleaving dioxygenases are of particular interest in petroleum-associated systems, where mixtures of fats, oils, aliphatic residues, and aromatic hydrocarbons require different catalytic functions for effective biodegradation. Bacteria recovered from hydrocarbon-impacted environments are therefore attractive sources of enzymes with potential relevance to contaminated soils and wastewater.

Lipases are serine hydrolases that catalyze the hydrolysis of long-chain triglycerides into free fatty acids and glycerol. In addition to their role in lipid metabolism, microbial lipases are widely studied for applications in bioremediation, food processing, detergents, and biofuel production because they can remain active in both aqueous and partially non-aqueous systems [1, 2]. Dioxygenases, by contrast, are oxidoreductases that incorporate molecular oxygen into aromatic substrates and often initiate ring cleavage during the microbial transformation of toxic hydrocarbons and related intermediates [3, 4]. Catechol-cleaving enzymes are especially important because they channel aromatic compounds into central metabolic pathways.

Pseudomonas species are well known for their metabolic versatility, and isolates recovered from oil-affected environments frequently express enzymes relevant to hydrocarbon transformation. However, fewer studies describe the side-by-side recovery and preliminary characterization of both lipase and catechol-cleaving dioxygenase activities from the same petroleum-associated isolate. Basic purification and stability data are needed before such enzyme systems can be evaluated more rigorously for environmental or industrial use.

Accordingly, the aim of this study was to isolate a petroleum-associated bacterial strain identified phenotypically as Pseudomonas luteola, recover extracellular lipase and dioxygenase activities from its culture supernatant, and characterize the effect of temperature and pH on these enzyme preparations after ammonium sulfate precipitation and gel filtration. The study is intended as a preliminary biochemical characterization and not as a direct quantitative demonstration of petroleum hydrocarbon removal.

2. METHODOLOGY

2.1. SAMPLE COLLECTION AND BACTERIAL ISOLATION

2.1.1 The Studied Area

The study area is located in the southeastern part of Kut city near the Tigris River, in the vicinity of the Wasit oil depot and adjacent agricultural land. The depot is one of the major petroleum-storage sites in the region. Geographically, the area lies between longitude 45° 25/ 10//–45° 53/ 15// E and latitude 32° 31/ 40//–32° 25/ 00// N.

2.1.2 Sampling

Soil samples were collected from different locations within the study area at a depth of approximately 10 cm using a soil auger. Samples were labeled immediately, kept in an ice box during transport, and processed in the laboratory as soon as possible.

2.1.3 Isolation and Identification of Pseudomonas luteola

Microbial strains were isolated from the collected samples by serial dilution and plating on nutrient agar. Distinct colonies were purified by repeated streaking. The isolate used in the present study was identified presumptively as Pseudomonas luteola on the basis of colony morphology, Gram staining, oxidase, indole, methyl red, catalase, and related biochemical tests following Bergey's Manual of Systematic Bacteriology. Because no molecular identification was performed, the taxonomic assignment should be regarded as phenotypic.

Approximately 10 g of soil sample was enriched in Bushnell–Haas (BH) broth supplemented with 2% (v/v) crude oil and incubated at 30°C for 5 days under shaking conditions (150 rpm).

2.1.4 Screening of lipase producing bacterial strains

Isolated bacterial strains were screened for lipolytic activity using the tributyrin agar plate assay method. Tributyrin agar supplemented with 1.0% (v/v) olive oil was sterilized at 121°C for 15 min and poured into Petri plates. The isolates were streaked onto the medium and incubated at 37°C for 24 h. Clear halo formation around the colonies was taken as evidence of extracellular lipase activity [5].

2.1.5 Screening of Dioxygenase producing strains

To detect catechol-cleaving activity, isolates were grown in minimal medium supplemented with catechol (0.1 mM). Changes associated with catechol transformation were monitored visually and spectrophotometrically as a preliminary screen for dioxygenase activity. Under the assay conditions used in this study, the reported activity was interpreted as catechol 1,2-dioxygenase-like activity; definitive differentiation from alternative cleavage pathways would require additional product-based confirmation.

2.2. ENZYME PRODUCTION

The selected bacterial isolate was cultivated for extracellular enzyme production in liquid media containing appropriate inducers. For lipase production, the organism was grown in a basal medium containing peptone, yeast extract, mineral salts, and olive oil or Tween-80 as lipid inducers to stimulate extracellular secretion [1, 6].

For dioxygenase production, cultures were incubated in Bushnell–Haas medium supplemented with catechol or gentisate as aromatic inducers for ring-cleaving dioxygenase activity [4, 7]. Cultures were incubated at 30–35°C on a rotary shaker (150–180 rpm) for 48–72 h. Cell-free supernatants were obtained by centrifugation at 8,000$imesg$ for 15 min at 4°C and were used as the crude extracellular enzyme preparations.

2.3. LIPASE ACTIVITY ASSAY

Lipase activity was determined spectrophotometrically using p-nitrophenyl palmitate (pNPP) as substrate, according to the method of Kouker and Jaeger [5] with slight modifications. The standard reaction mixture (1.0 mL final volume) contained 800 μL of 50 mM Tris-HCl buffer (pH 8.0), 100 μL crude enzyme, and 100 μL of 10 mM pNPP (dissolved in isopropanol containing 0.5% Triton X-100). After incubation at 37 °C for 30 min, the reaction was terminated by adding 100 μL of 1 N NaOH. The release of p-nitrophenol was measured at 405 nm against a substrate blank lacking enzyme. One unit (U) of lipase activity was defined as the amount of enzyme releasing 1 μmol of p-nitrophenol per min under assay conditions.

2.4. DIOXYGENASE ACTIVITY ASSAY

Dioxygenase activity was assayed spectrophotometrically by monitoring catechol cleavage [8]. Briefly, the reaction mixture (1 mL) consisted of 50 mM phosphate buffer (pH 7.5), 100 μL crude enzyme, and 100 μM catechol as substrate. The reaction was initiated by adding the substrate, and formation of the ortho-cleavage product was monitored at 260 nm. In the present manuscript, the reported dioxygenase data therefore refer to catechol 1,2-dioxygenase-like activity under these assay conditions. Enzyme activity was expressed as the amount of product formed per minute using the appropriate molar extinction coefficient.

2.5. PROTEIN QUANTIFICATION

Protein concentration in the enzyme preparations was determined by the Bradford method using bovine serum albumin (BSA) as the standard. Standard solutions (0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL) were prepared from a BSA stock solution. Coomassie brilliant blue G-250 reagent was prepared in ethanol, phosphoric acid, and distilled water, filtered, and stored in a dark bottle. For the assay, 2.5 mL of dye reagent was mixed with either the standard or the test sample and left to stand for 2 min at room temperature before absorbance was measured at 595 nm. The blank consisted of 0.45 mL of 0.05 M phosphate buffer (pH 7.0) and 2.5 mL of dye reagent. Protein concentration was estimated from the BSA calibration curve. Purification parameters were calculated directly from measured activity and protein values for the representative preparations shown in Tables 1 and 2.

3. RESULTS

3.1. PURIFICATION OF LIPASE PRODUCED BY PSEUDOMONAS LUTEOLA

The lipase preparation was processed through three successive steps: crude extract, ammonium sulphate precipitation (60%), and gel filtration chromatography. As shown in Table 1, the crude extract exhibited an enzyme activity of 0.00164 U/mL and a specific activity of 0.00537 U/mg, indicating that measurable lipase activity was initially distributed within a protein-rich mixture. Subsequent purification steps progressively enriched the active fractions recovered from the culture supernatant.

After ammonium sulphate precipitation, the enzyme activity increased to 0.00483 U/mL and the specific activity to 0.0298 U/mg, corresponding to a 5.5-fold enrichment. The calculated total activity also increased to 0.724 U. Because this value exceeded that of the crude extract, the result is interpreted cautiously as an apparent increase in measurable

activity that may reflect concentration of the active fraction and removal of interfering constituents rather than true enzyme generation.

Table 1. *Show purification steps of lipase enzyme*

Purification step	Volume (ml)	Enzyme activity (U/ml)	Protein Concentration (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification (Folds)	Yield (%)
Crude enzyme	200	0.00164	0.305	0.00537	0.328	1	100
Ammonium Sulphate precipitation 60%	150	0.00483	0.162	0.0298	0.724	5.5	220
Gel chromatography	3	0.0629	0.09	6.99	1.887	1302	575

Following gel filtration, the specific activity rose to 6.99 U/mg, corresponding to a 1302-fold enrichment relative to the crude extract. Total activity reached 1.887 U, yielding an apparent recovery of 575%. Recoveries above 100% are best interpreted as evidence that the final fraction displayed greater measurable activity than the starting extract, possibly because inhibitors or interfering compounds were removed during purification [6]. In the absence of replicate purification runs and electrophoretic purity analysis, the final fraction is more appropriately described as markedly enriched rather than definitively purified to homogeneity.

3.2. CHROMATOGRAPHIC PROFILE OF LIPASE

The gel chromatography profile (Figure 1) showed two main regions. The wash fractions (1–30) contained low protein absorbance at 280 nm with only minor enzyme activity and therefore mainly represented unbound proteins and impurities. In contrast, the elution fractions (35–50) displayed a distinct peak, with maximum absorbance around fraction 40 corresponding to the highest lipase activity. This pattern indicates that the active lipase was concentrated within a defined elution window.

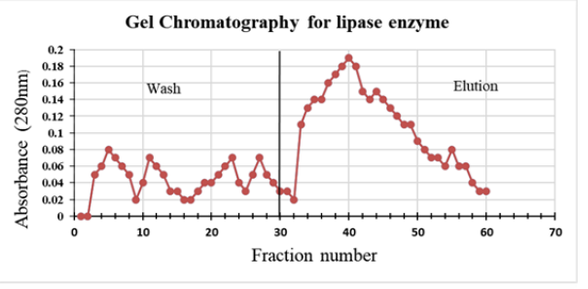

Figure 1. *Show Gel chromatography for lipase enzyme*

The agreement between Table 1 and Figure 1 indicates that the highest lipase activity was concentrated within the main elution peak, consistent with effective pooling of the active chromatographic fractions.

3.3. STATISTICAL ANALYSIS

Because the purification values presented in Table 1 were obtained from a representative preparation rather than from true biological replicates, inferential statistical testing was not applied in the revised analysis. The increases in specific activity across purification steps are therefore interpreted descriptively from the measured values reported in the table.

3.4. PURIFICATION OF DIOXYGENASE PRODUCED BY PSEUDOMONAS LUTEOLA

A three-step purification scheme was applied: crude extract, ammonium sulfate precipitation, and gel filtration (Table 2).

Table 2. *Purification steps for dioxygenase Enzyme*

Purification step	Volume (ml)	Enzyme activity (U/ml)	Protein Concentration (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification (Folds)	Yield (%)
Crude enzyme	100	9.2	0.6	15.3	920	1	100
Ammonium Sulphate precipitation 60%	80	2.22	0.04	55.5	178	3.6	19.3
Gel chromatography	5	29.4	0.05	588	147	38.4	15.9

The crude extract contained a high total activity (920 U) but a relatively low specific activity (15.3 U/mg), consistent with the presence of substantial non-specific protein. Ammonium sulfate precipitation (60%) increased specific activity to 55.5 U/mg (3.6-fold enrichment) while reducing total activity to 178 U, corresponding to 19.3% apparent recovery. Gel filtration yielded the highest specific activity, 588 U/mg, equivalent to a 38.4-fold enrichment relative to the crude extract, with a final total activity of 147 U and an apparent recovery of 15.9%. Collectively, these values indicate progressive enrichment of the dioxygenase-active fraction, with gel filtration acting as the principal polishing step.

3.5. PURIFICATION OF DIOXYGENASE

Figure 2 shows the gel filtration chromatography profile of the dioxygenase preparation monitored at 280 nm, the wavelength commonly used to follow protein elution through absorbance by aromatic amino acids.

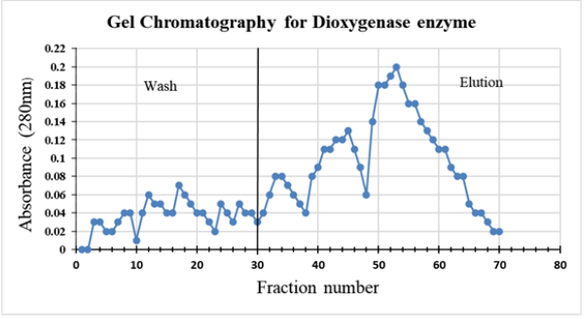

Figure 2. *Show purification of Dioxygenase enzyme produced by Pseudomonas luteola*

Gel filtration of the dioxygenase preparation showed a clear separation between the washing and elution phases (Figure 2). The wash fractions (1–30) displayed very low absorbance at 280 nm (≤0.06), indicating limited protein content, whereas a distinct peak appeared during the elution phase with maximum absorbance (0.20) between fractions 52 and 58. After fraction 60, absorbance declined toward baseline by fraction 70. This elution pattern supports pooling of the central peak fractions for subsequent characterization, although purity cannot be inferred from absorbance data alone.

3.6. STATISTICAL ANALYSIS

As with the lipase purification data, the values in Table 2 are reported descriptively because they represent a single purification workflow rather than a replicate data set. The observed fold increases (3.6× and 38.4×) therefore document substantial enrichment of the active fraction, but they are not presented here as inferentially tested statistical differences.

3.7. EFFECT OF TEMPERATURE ON ACTIVITY AND STABILITY OF LIPASE

Figure 3 shows that the purified lipase retained its highest activity around 30–35°C, with residual activity close to 100% in this range. A gradual decline was observed above 40°C: at 45°C the enzyme retained approximately 60% of its initial activity, at 55°C about 35%, and at 65°C nearly 20%. The data indicate that the lipase preparation is most stable under mesophilic conditions and becomes progressively inactivated as temperature increases.

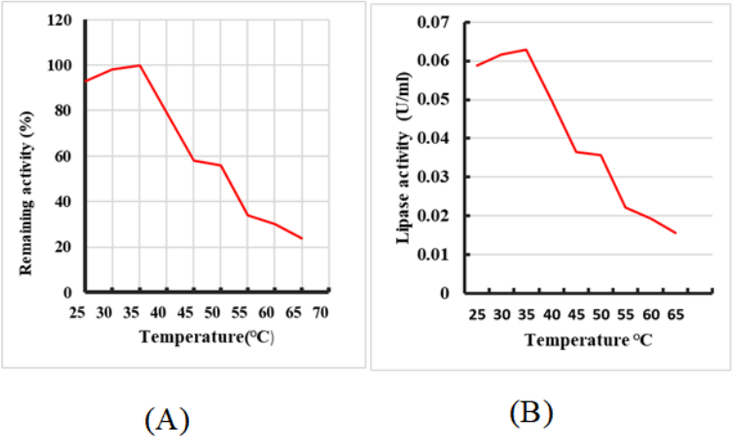

Figure 3. *Effect of Temperature on the activity (A) and Stability (B) of purified lipase produced by Pseudomonas luteola*

3.8. EFFECT OF PH ON ACTIVITY AND STABILITY OF LIPASE ENZYME PRODUCED BY PSEUDOMONAS LUTEOLA

pH is an important determinant of enzyme activity and stability because it influences ionization of catalytic residues and overall protein conformation [9].

The effect of pH on the stability of purified lipase was evaluated between pH 5 and 9. The enzyme showed the highest stability and retained almost 100% of its initial activity at pH 6–7, indicating a neutral to slightly acidic optimum. At pH

8, the enzyme maintained approximately 80% residual activity, whereas a sharper decrease was observed at pH 9, where residual activity dropped to nearly 40%. At pH 5, the enzyme retained only about 55% of its original activity. These findings suggest that the purified lipase is best suited to near-neutral conditions and is progressively inactivated under more acidic or alkaline environments (Figure 4).

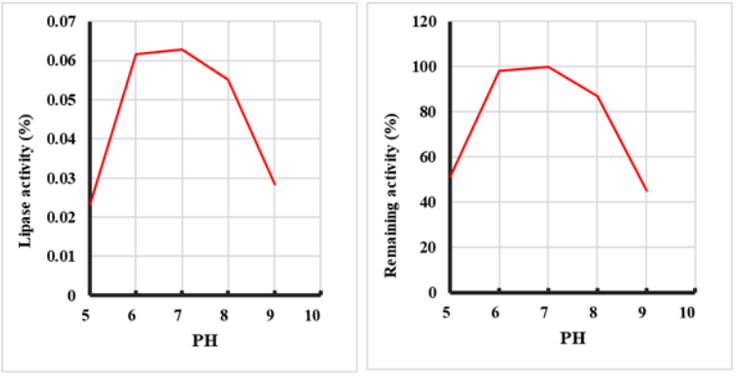

Figure 4. *Effect of PH on Activity (A) and Stability (B) of purified lipase produced by pseudomonas luteola*

3.9. EFFECT OF TEMPERATURE ON ACTIVITY AND STABILITY OF CATECHOL 1,2-DIOXYGENASE

The effect of temperature on the activity and stability of the purified catechol 1,2-dioxygenase preparation produced by Pseudomonas luteola was evaluated over the range 25–65°C. Maximum activity was observed at 35°C (29 U/mL), while moderate activity was maintained between 25 and 30°C (25–28 U/mL). Activity declined progressively above 40°C, dropping to 20 U/mL at 45°C, 15 U/mL at 55°C, and 12 U/mL at 65°C.

The thermal stability profile showed the same general trend. The enzyme retained nearly 100% of its activity at 35°C, about 55–60% at 45–50°C, and only 20–30% at 60–65°C. These results indicate that the dioxygenase-active preparation is best suited to mesophilic conditions and undergoes marked thermal inactivation above 45°C (Figure 5).

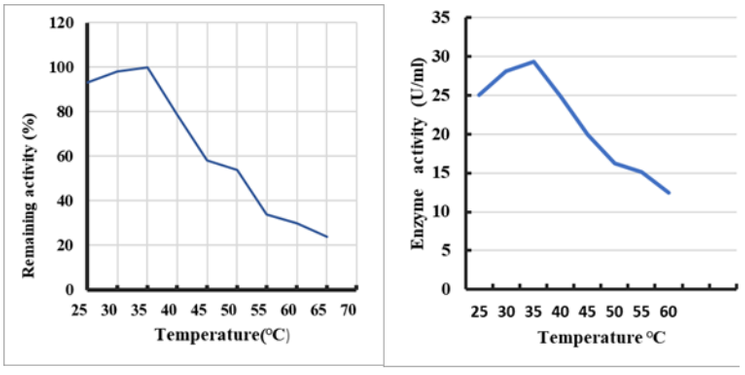

Figure 5. *Effect of temperature on activity (A) and Stability (B) of purified catechol 1,2 dioxygenase produced by pseudomonas luteola*

3.10. EFFECT OF PH ON ACTIVITY AND STABILITY OF DIOXYGENASE

The effect of pH on the activity and stability of purified catechol 1,2-dioxygenase produced by Pseudomonas luteola was evaluated across the range pH 5.0–9.0. Maximum activity was observed at pH 7.0 and was normalized as 100% relative activity in the figure. At pH 6.0 and 8.0, the enzyme maintained moderate activity, whereas sharper declines were observed at pH 5.0 and pH 9.0. The stability assay showed that the enzyme retained approximately 70–80% of its activity at pH 6–8 but less than 60% at pH 9. Overall, the results indicate that the dioxygenase-active preparation is most active and stable under near-neutral conditions (Figure 6).

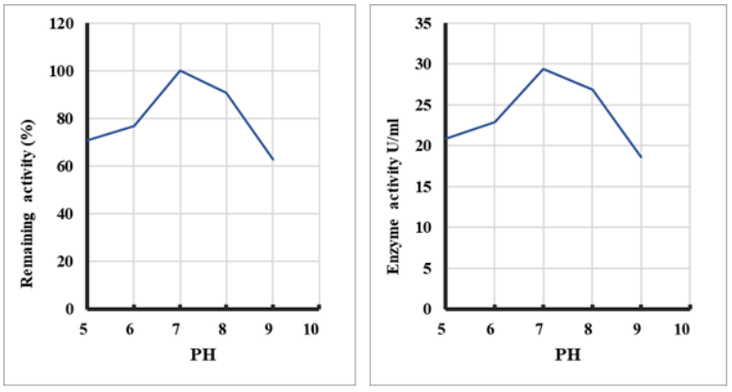

Figure 6. *Effect of pH on Activity(A) and Stability (B) of purified catechol 1,2 dioxygenase*

4. DISCUSSION

4.1. PURIFICATION OF P. LUTEOLA EXTRACELLULAR LIPASE

The lipase purification pattern is broadly consistent with previous reports showing that ammonium sulfate fractionation followed by chromatographic polishing can substantially enrich bacterial lipases [1, 2]. In the present study, the strongest increase occurred during gel filtration, indicating that this step was primarily responsible for concentrating the lipase-active fraction.

At the same time, the apparent lipase recovery above 100% should not be interpreted as literal mass recovery. Rather, it most likely reflects improved measurable activity after the removal of inhibitory or interfering constituents from the crude extract [6]. Similar behavior has been reported for microbial lipases, but the magnitude observed here remains unusually large and therefore warrants cautious interpretation.

The final specific activity of 6.99 U/mg and the 1302-fold enrichment indicate a strong purification effect relative to the starting material. However, because the present study did not include replicate purification runs, kinetic constants, or electrophoretic verification of purity, the final preparation is best described as an enriched lipase fraction rather than a fully resolved enzyme preparation. Even with this limitation, the data support the usefulness of the purification workflow as an initial recovery strategy for downstream characterization.

4.2. PURIFICATION OF PSEUDOMONAS LUTEOLA DIOXYGENASE

The overall enrichment pattern observed for the dioxygenase-active fraction is in line with previous studies of ring-cleaving dioxygenases, for which multi-step purification commonly yields moderate-to-high increases in specific activity with partial loss of total activity [8]. The 38.4-fold enrichment reported here falls within the upper part of the range commonly described for comparable preparations.

The final apparent yield of 15.9% is also reasonable for an oxygen-sensitive enzyme system, since partial activity loss during precipitation and chromatography is frequently reported for dioxygenases [10]. The gel filtration step preserved a substantial portion of the remaining activity while markedly increasing specific activity, indicating that it functioned effectively as a polishing step.

Nevertheless, the interpretation should remain conservative. The assignment to catechol 1,2-dioxygenase is operational and based on the assay conditions used here, not on direct product identification, sequence analysis, or electrophoretic confirmation. Accordingly, the data support enrichment of a catechol-cleaving dioxygenase-active preparation suitable for preliminary characterization, while more definitive biochemical identification remains necessary.

4.3. EFFECT OF PH AND TEMPERATURE ON CATECHOL 1, 2-DIOXYGENASE ACTIVITY AND STABILITY

The pH and temperature profiles of the catechol 1,2-dioxygenase-active preparation follow the general behavior expected for many mesophilic ring-cleaving dioxygenases. Maximum performance near neutrality is consistent with the need to maintain favorable protonation states of catalytic residues and proper coordination of the metal center involved in aromatic ring cleavage. Activity loss at acidic or alkaline pH likely reflects destabilization of the active conformation and reduced substrate turnover.

This behavior is consistent with previous reports showing that many ring-cleaving dioxygenases exhibit maximal

activity between pH 6.5 and 7.5 and decline at more extreme pH values [8]. Comparable near-neutral optima have been described for catechol 1,2-dioxygenase from Acinetobacter radioresistens and for related aromatic-ring-cleaving enzymes [11, 12]. The present data therefore place the P. luteola preparation within the expected operating range for environmentally relevant dioxygenase systems.

The temperature profile, with maximum activity around 35°C and marked decline above 45°C, likewise indicates a mesophilic and thermolabile preparation. Similar trends have been summarized for other bacterial catechol dioxygenases [8] and experimentally observed in related systems [11, 12]. In practical terms, these results suggest that the enzyme preparation would be most compatible with mild aqueous processes rather than elevated-temperature applications.

4.4. EFFECT OF TEMPERATURE ON ACTIVITY AND STABILITY OF LIPASE

The temperature profile confirms that the enriched lipase fraction retains useful catalytic activity at moderate temperatures but is sensitive to heat stress above 40°C. This behavior is characteristic of many mesophilic microbial lipases, in which the non-covalent interactions that stabilize tertiary structure are progressively disrupted at elevated temperatures.

Comparable findings have been reported for bacterial lipases from hydrocarbon-associated environments, which generally perform best between 30 and 40°C and decline rapidly at higher temperatures [1, 2]. The agreement with the literature suggests that the present lipase preparation is most suitable for mild aqueous processes, including preliminary environmental applications conducted near ambient temperature. Where greater thermostability is required, further stabilization strategies such as immobilization or protein engineering would be needed [13].

4.5. EFFECT OF PH ON ACTIVITY AND STABILITY OF PURIFIED LIPASE

The pH profile indicates that the Pseudomonas luteola lipase-active fraction is most stable under slightly acidic to neutral conditions (pH 6–7), which is consistent with the behavior of many bacterial lipases that depend on appropriate ionization of catalytic residues for efficient turnover [1, 2]. Related studies on hydrocarbon-associated bacteria have likewise reported maximum lipase activity near neutral pH, with pronounced declines under more extreme conditions [14, 15].

The present results therefore support the view that this lipase preparation is better suited to near-neutral environments than to strongly acidic or strongly alkaline systems. This operating window is compatible with many aqueous biotechnological applications, but it also indicates that pH control would be important in any future scaled or field-based use.

5. CONCLUSION

The results of this study show that a petroleum-associated bacterial isolate identified phenotypically as Pseudomonas luteola produced extracellular lipase and catechol-cleaving dioxygenase activities that could be enriched through ammonium sulfate precipitation and gel filtration. In both cases, gel filtration provided the largest increase in specific activity, while the activity profiles indicated that the recovered preparations function best under mesophilic and near-neutral conditions. These findings strengthen the biochemical characterization of the isolate and provide a useful starting point for future work on enzyme recovery from hydrocarbon-impacted environments.

At the same time, the study should be interpreted within its limits. The taxonomic identification was phenotypic, the purification data were derived from representative preparations rather than replicate statistical experiments, the apparent lipase recovery above 100% reflects increased measurable activity rather than confirmed mass recovery, and direct petroleum hydrocarbon removal was not quantified. Future studies should therefore include molecular identification of the isolate, replicate purification workflows, electrophoretic and kinetic characterization of the enzymes, and direct assays of hydrocarbon degradation. Within these boundaries, the present work provides a coherent preliminary basis for more rigorous environmental and biocatalytic investigations.

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