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Production, Purification and Biochemical Characterization of Lipase from *Pseudomonas aeruginosa*

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Abstract: Lipases are enzymes that have numerous applications in many industries like leather, soaps and detergents, pharmaceuticals, biofuel, food, textile, etc. Many organisms like plants, fungi, bacteria are known to produce lipase. The present study was aimed at isolating lipase producing microorganisms from different water samples which are rich in lipid content like oil mills. As oil mill samples are rich in lipid and fatty acid content it makes a very good source to find microorganisms capable of degrading lipids by producing lipase enzymes. In the present study the isolates were identified by morphological, biochemical and molecular characterization. Purification results of lipase exposed prominent specific activity of 248.4 U/mg, purification fold of 75.50, and 47.7% yield. The purified lipase demonstrated outstanding activity and stability in a temperature range of 25-45 °C and pH (5–10), revealing optimal activity at 35 °C and pH 7. The molecular weight of the enzyme was estimated to be 63 kDa. Compared to control, the lipase activity was promoted in the presence of calcium chloride.

Keywords: Lipase enzyme, lipase assay, purification, enzyme stability.

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Introduction

Lipase is one of the enzymes that catalyst the hydrolysis of oils and fats (triglycerol lipase, EC 3.1.1.3). An important characteristic of lipases is their ability not only to hydrolyze the ester bonds, trans-esterify triglycerides and resolve racemic mixture, but also to synthesize ester bonds in non-aqueous media (1). The enzyme lipase is found in the secretions of the pancreas and is responsible for the process of fat digestion and also found in animals, plants, and microorganisms (2), especially those that originated from bacteria are more stable than others. Bacterial lipases are commercially more important, mainly because of the ease of their cultivation and optimization to obtain higher yield (3). Industrial demand for new sources of lipases with different catalytic characteristics stimulated the isolation and selection of new strains. Lipase producing microorganisms have been found in different sources such as agro-industrial waste, vegetable oil processing factories, dairy plants and soil contaminated with oils (4). Furthermore, the versatile biochemical characteristics of microbial lipases, including catalytic activity in harsh surroundings, vast substrate specificity, stability in different organic solvents, and enantioselectivity bestow them the potential implementation in various industries, such as food biotechnology, pulp and paper, detergents, cosmetics, biodiesel, and pharmaceuticals (5). Generally, bacterial lipases are glycoproteins, but some extracellular bacterial lipases are lipoproteins (6). The production of extracellular lipases from bacteria is greatly influenced by medium composition besides physicochemical

factors such as temperature, pH and dissolved oxygen, the major factor for the expression of lipase activity has always been reported as the carbon source since lipase are inducible enzymes (**7**). These enzymes are generally produced in the presence of a lipid such as oil or any other inducer, such as triacylglycerols, fatty acids, hydrolysable esters, Tweens, glycerol and bile salts. However, nitrogen sources and essential micronutrients should also be carefully considered for growth and production optimization (8). From the industrial point of view, lipase enzymes are considered very important, due to their greater production potential on a large scale (9). They are mostly used in the detergent, food and pharmaceutical industries (10). Considering the important of lipase enzyme, the present study was aimed to production, purification and characterization the novel lipase from pseudomonas aeruginosa isolate from contaminated water, a study of the stability of the enzyme at different temperature and pH, in addition to the effect of metal ions on the enzyme activity.

Materials and methods

Sample collection and isolation of *Pseudomonas aeruginosa*

A total of thirty liquid samples (Oil contaminated water) were collected from various places in Dora Oil Refinery in Baghdad. The entire collected samples were instantly transported to the lab. To isolate bacterial strains, after a serial of dilutions, 1 mL of each water sample was directly cultivated on plates of nutrient agar medium with pH 9 and then maintained in a static incubator for 72 h at 37 °C. The grown colonies were purified, and the morphological features of each bacterial isolate were inspected (11).

Screening of lipase producing

Isolated bacterial strains were screened for their lipolytic activity on the basis of the tributyrin agar plate assay method (TBA). The tributyrin agar media contain with 1.0% (v/v) olive oil were prepared and sterilized at 121 °C for 15 min, and then the sterilized media was poured into petri plate. Isolated strains were streaked on the tributyrin agar plate and it was incubated at 37 °C for 24 h to observe hydrolysis zone (12).

Enzyme production media

Screened positive bacterial strains were cultivated in lipase producing media for enzyme production. Lipase producing media consist of 2% yeast extract, 2 g $NaNO_3$, 0.5 g KCl, 0.14g KH_2PO_4, 1.2 g K_2HPo_4, 0.1 g $CaSO_4$, 1% olive oil in 100 mL distilled water in a 250 mL conical flask as submerged fermentation method. Inoculated flasks were incubated at 37 °C for 24–48 h (13).

Lipase assay

After 24 h of inoculation, the fermentation broth was taken in falcon tubes. These falcon tubes were centrifuged for 10 minutes at 5000 Xg and the supernatant was then used as the crude enzyme. This crude enzyme added (2 mL) to 2 mL of olive oil with 8 mL of emulsifier polyphenol alcohol, was added ammonium chloride at concentration of 0.3 M, and was then tested for its enzyme activity by titrating it against 0.05 M NaOH. The amount of NaOH used the amount of acid present in the solution, which was directly proportional to the amount of lipase produced (14). Acid value was calculated by the formula:

µmol fatty acid (U) = (mL NaOH for sample – mL NaOH for blank)×N1000 /M
Where:
U = µmol of fatty acid released/mL
N = the normality of the NaOH titrant used (0.05 in this case)
M = Total volume of reaction mixture used
One lipase unit has been defined as the amount of the enzyme that releases one µmol fatty acid per ml under standard assay conditions (U = µmol of fatty acid released/mL) (15). Based on the Bradford method the protein concentration was determined using the standard curve for bovine serum albumin, and the qualitative effectiveness is calculated using the law Specific activity= Activity/protein conc.

Lipase purification

Bacterial culture has grown in mineral salt medium (MSM) was centrifuged at 8000 Xg for 20 min at 4 °C in a refrigerated centrifuge. Cell free supernatant was saturated with (0-70%) ammonium sulfate with continuous stirring at 4 °C followed by centrifugation at 14,000 rpm for 20 minutes. Ammonium sulfate fraction was dialyzed against 50 mM Tris-Chloride buffer (pH 8.0) for 6 hours at 4 °C in a Dialysis tube. The concentrated enzyme after dialysis was loaded onto ion exchange column. The enzyme was eluted from the column at a flow rate of 1 mL/min. Enzyme fractions (5 mL each) were collected and the protein content was measured spectrophotomerically at 280 nm. Then the concentrated sample obtained from the ion exchange is applied to the column Sephadex G-1500, the absorbance of each fraction was measured at 280 nm The enzyme activity was also determined in each fraction and the protein concentration was determined Lipase assay was performed using fractions containing highest protein content (16).

Effect of temperature, pH, minerals and inhibitors on activity and stability of lipase enzyme

Effect of temperature on the activity and stability of purified lipase enzyme
To determine the optimum temperature for the lipase, the enzyme assay was carried out at temperatures range (25 – 50 ^{o}C) and constant pH 7. Thermal stability of the lipase was investigated by pre-incubating the lipase at different temperatures (25, 30, 35, 40, and 45 °C) for predetermined time intervals. Then, the enzymatic reaction was conducted under standard conditions and the residual activity of lipase was estimated on the basis of the activity of enzyme without incubation was considered 100% (17).

Effect of pH on activity and stability of lipase: The optimal pH value for the purified lipase was ascertained by performing the lipolytic reaction in different buffers within a pH range of 5-10. To study the enzyme stability in the various pH values, the lipase was pre-incubated in different pH values in the range of 5–10 at 25 °C for 1 h. Subsequently, lipase activity was evaluated following standard assay conditions and the residual activity was then assessed in relation to control that defined as 100% (18).

Influence of metal ions and other substances on enzyme activity: To examine the effect of metal ions on lipase activity, the enzyme was pre-incubated with 10 mM $CaCl_2$, KCl, $CuCl_2$, $ZnCl_2$ and Ethylene-diamine-tetra-acetic (EDTA) for 1 h at 37 °C, and then the remaining activities were determined, with the untreated lipase activity considered to be 100%.

Results and discussion

Isolation and screening of lipase production from *P. aeruginosa*

Isolation of lipolytic bacterial cultures from industrial and oil-contaminated wastewater resulted in the characterization and identification of 10 cultures. Ten strains were identified by gram staining and purified by sub-culturing on agar plates.

Screening of lipase producing

Based on the clear zone production on a tributyrin agar plate, 5 bacterial strains were identified as lipase producers as shown in Figure 1. There are 3 bacterial strains, SP1, SP5, SP7 showed high intensity of clear zone, SP3 and SP8 strains showed moderate intensity of clear zone and other 5 strains (SP2, SP4, SP6, SP9, SP10) did not show any zone around the colonies. Olive oil used as the substrates for screening of lipase producing bacterial strains. Among natural oils, olive oil has been referred as one of the best inductors and substrate for lipase production. Bharathi and Rajalakshmi (19) founds the areas appeared stained with bacterial strains green spots appeared when bacteria were cultured on Tributyrin agar plates at 36 °C.

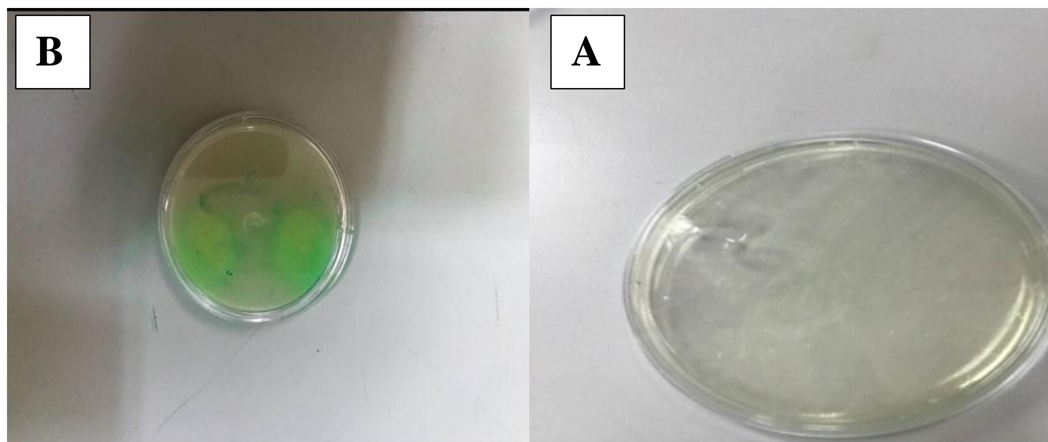

Figure (1): Screening of lipase activity in Tributyrin Agar plates. Figure A shows the shape of the productive isolates as it showed a clear area, while Figure B shows the shape of the non-productive isolates.

Lipase purification

Lipase purification was done to get a protein of interest and to remove unnecessary one. The purification process of lipase occurs in a sequential manner. The enzyme produced over 48 hours of culture was purified by ammonium sulfate precipitation for salting out the proteins. For increased enzymatic activity desalting was performed for removing the traces of salt. According to Tripathi *et al* (20), increased lipase activity depends on the concentration of ammonium sulfate. An extracellular lipase from *P.aeruginosa* was purified by ammonium sulfate precipitation and Sephadex G-150 column chromatography with a total yield of 47.7 % and 75.50 fold purification (Table 1). A low yield of the enzyme may be due to difficulty in removal of the high content of lipopolysaccharide present in *P. aeruginosa* and coupled with lipid hydrolysis (20).

Table (1): Partial purification steps for lipase enzyme produced by *p. aeruginosa*

Steps of purification	Volume (mL)	Enzyme activity (U/mL)	Protein concentration (mg/mL)	Specific activity (U/mg)	Total activity (U)	Purification (folds)	Yield (%)
Crude enzyme	100	60	18.2	3.29	6000	1	100
Ammonium sulfate precipitation 80%	50	78	8.4	9.28	3900	2.82	65
Ion-exchange (EDTA)	24	124	0.95	130.5	2976	39.66	49
Sephadex G150	18	159	0.64	248.4	2862	75.50	47.7

Effect of temperature on the activity and stability of purified lipase enzyme

Impact of temperature on the activity of the lipase evaluation of thermal stability of industrial enzymes is very crucial since high temperatures could cause significant damage with regards to the intermolecular bonds of enzymes, which conserve the tertiary structure and configuration of protein (21). These changes considerably influence on the catalytic activity of enzyme through impairing the affinity between enzyme and substrate, which further suppress enzyme activity. Figure 2A shows that the purified lipase activity was detectable within a broad temperature ranging from 25 to 50 °C and the maximal activity emerged at 35 °C, recording 161 U/mL. Figure 2B shows the thermal stability of enzyme with temperature, where the lipase was greatly stable after incubation for 18 h at 25, 30, 35, and 40 °C. Raza *et al* (22) reported that the highest activity of the lipase produced by the strain *Staphylococcus aureus* peaked at 52 °C.

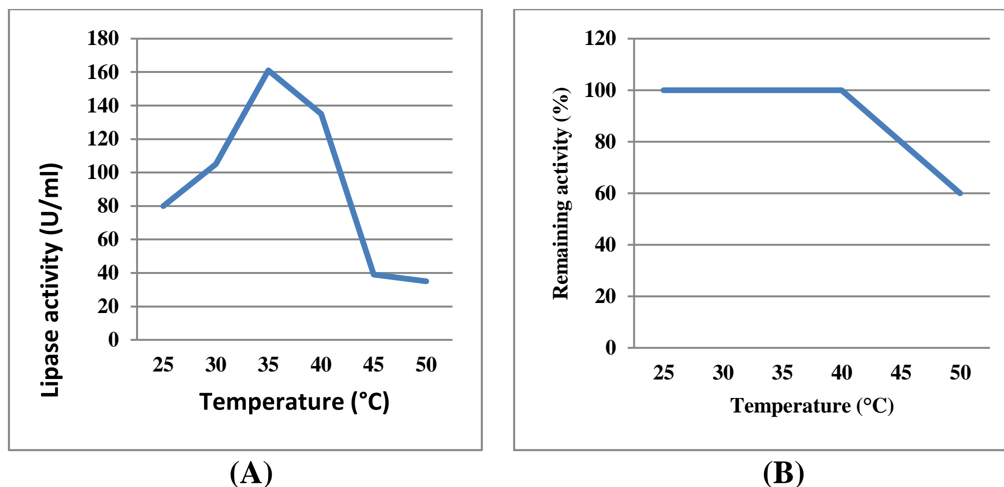

Figure (2): Effect of temperatures on activity of the purified lipase produced by *Pseudomonas aeruginosa* to estimate(A) the optimal temperature for the enzyme (B): Effect of temperatures on thermal stability of the enzyme after pre-incubation of the pure enzyme for time intervals before estimating the residual lipolytic activity.

Effect of pH on activity and stability of lipase

Enzymes are protein compounds; its activity is highly dependent on the operation of pH. From Figure 3A, it could be evidently observed the increasing in the activity of the purified lipase when the pH increases from 5 to 6, presenting maximal

activity at pH 6, and stable up to pH 7 with the activity of 160 U/mL. The activity declines slightly from pH7 to pH 8, and then decreases significantly after pH 8. In terms of stability, the lipase maintained almost all its activity post-preincubation at pH values of 5 to 10 before determination the lipolytic activity. In contrast, the lipase activity will be affected out of the range of pH (5 – 10) as revealed in Figure 3B. The optimal pH for lipase from *Pseudomonas aeruginosa* was pH (6 – 7). Moreover, lipase produced by *Penicillium cyclopium* demonstrated the highest activity at pH 10, while the stability depleted to about 60% of its original value after only 120 min of incubation in buffer of pH 10 (23). The influence of pH values on lipase activity is likely imputed to disruption or alterations of intermolecular bonds that give rise to conformational changes in functional sites of the enzyme. These modifications may augment or lessen the enzyme activity. Overall, the most striking findings to emerge from the data are the extensive stability of the lipase in relation to thermal stability and pH stability, and thus propose this studied enzyme as a promising candidate for multifarious applications.

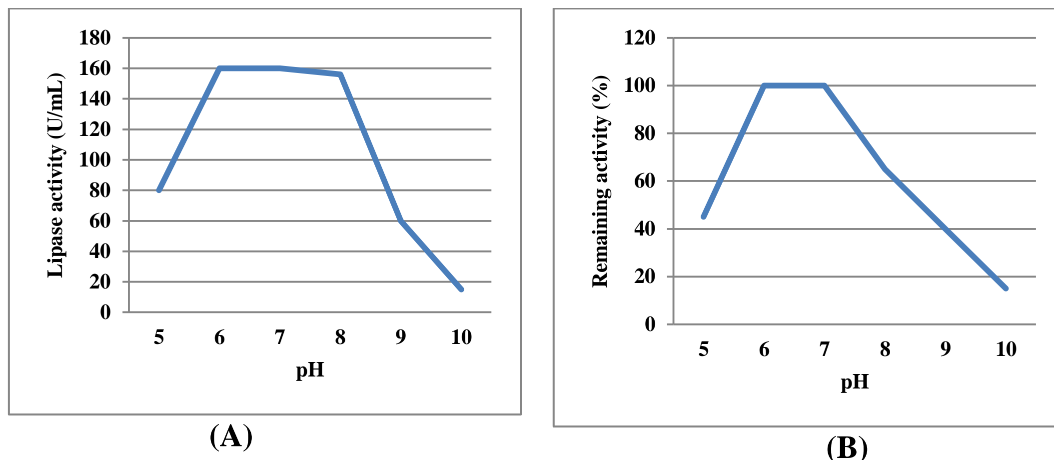

Figure (4): Effect of different pH values ranging from 5 to 10 on the activity of the purified lipase to ascertain :(A) the optimum pH and (B) stability of the enzyme.

Effect of metal ions and inhibitors on the stability of lipase enzyme

Table (1) presents the impact of various metal ions on the lipase activity at concentrations of 5 mM. The activity of lipase was significantly enhanced by $CaCl_2$ after treatment with 5 mM of calcium chloride, reaching 116% compared to the control. Several previous studies showed that the lipases demonstrated improvement in their activity in the presence of $CaCl_2$ (24). This could be ascribed to the efficacy of these metal ions to bind the lipase as cofactors, changing its conformation through producing salt bridges, which improve the stability of the enzyme (25). However, the lipase activity was decreased by $CuCl_2$ and $ZnCl_2$, besides; the concentration of 5 mM of these two metal ions strongly hindered the activity of lipase. These results are in agreement with previous studies (26). The influences of eight chemical agents on the activity of lipase produced by *Pseudomonas aeruginosa* were studied. Table 2 displays that the activity of lipase was hampered in the presence of Ethylene-diamine-tetra-acetic acid EDTA as chelating reagents, which might be attributed to the poor binding site for divalent ions in enzyme structure, and our findings concerning the impacts of metallic ions on the lipase activity uphold this exposition.

Table (1): Effect of metal ions and inhibitors on the activity of lipase enzyme.

Metal ions and inhibitors	Concentration (mM)	Remaining activity (%)
Control	---	100
$CaCl_2$	5	116
KCl	5	100
$CuCl_2$	5	83
$ZnCl_2$	5	20
EDTA	5	16

Conclusions

In this study, it is noted that the lipase purified from *Pseudomonas aeruginosa* is an extracellular enzyme, which gave the lipase stability at a temperature of 40 degrees Celsius and remained stable at pH8. It was also noted the possibility of increasing the enzyme activity when adding metal ions as $CaCl_2$ in addition to inhibiting it by adding other ions as $ZnCl_2$. The lipase enzyme also showed the potential for biodegradation as part of the waste water treatment.

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