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Kinetic properties of Lipase obtained from *Pseudomonas aeruginosa* isolated from crude oil contaminated soil

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Abstract
Soil contaminated with crude oil has negatively affected some of the communities in Ekiti State region in Nigeria. There is an increased interest in microbial lipases because of their enormous potential in industrial and biotechnological applications. This study investigated the characteristics of partially-purified lipase from *Pseudomonas aeruginosa* isolated from oil-contaminated soil in Ado-Ekiti, Ekiti State. The lipase was purified by gel-filtration, having a molecular weight of 39.11 kDa, with K_m and V_{max} values of 12.50 and 28.86, respectively. The enzyme had an optimal pH of 8.0 and exhibited its maximal activity at 50°C, whereas the relatively stable temperature and pH were 40°C and 5.0, respectively. The enzyme activity was enhanced by olive oil, which served as the carbon source. Sodium chloride enhanced lipase activity, while calcium chloride acted as mild inhibitor, and iron chloride acted a strong inhibitor. The lipase from *Pseudomonas aeruginosa* possessed properties of an industrial enzyme and will be useful for biodegradation and bioremediation studies.

Introduction

Enzymes are compounds of biological origin which accelerate chemical reactions. Almost all catabolic and anabolic reactions in cells require enzymes to rapidly catalyze life-sustaining reactions (Stryer et al., 2002). In a similar manner to catalysts, enzymes lower the minimum energy required to initiate a reaction, resulting in increased reaction rates, with some enzymes even converting substrates to products a million times faster. Enzymes are generally globular proteins, acting either independently or in association with proteins (Miller et al., 2007). Lipases are glycerol ester hydrolases (EC: 3.1.1.3) which hydrolyze ester linkages of glycerides at the water–oil interface (Garlapati, Vundavilli & Banerjee, 2010). According to Ramani et al. (Ramani et al., 2010), lipases form a lipase-acyl complex by abstracting an acyl group from glycerides, which is then transferred to the acyl group on the hydroxyl ion of a water molecule. In a non-aqueous environment, these naturally-occurring hydrolytic enzymes transfer their acyl groups from carboxylic acids to nucleophiles, leading to the formation of different stereospecific esters, amides, and sugar esters (Singh et al., 2003).

It is worth noting that in the last several decades, many lipases of microbial origin have been discovered and utilized by different industries, including applications in wastewater treatment, food preservatives, fine chemicals, leather, cosmetics, pharmaceuticals, and medicine (Bora & Kalita, 2006; Sebdani et al., 2011). Generally, inducers increase the production of enzymes of industrial interest. However, for lipases, triacylglycerol, surfactants, vegetable oils, oil industry wastes or their hydrolytic products, there is a significant inducible effect when inoculated

in a culture medium for lipase production (Damaso et al., 2008).

Most of the well-studied lipases of microbial origin are inducible extracellular enzymes which are synthesized within microbial cells and then transferred to the surroundings (Tan & Yin, 2003). Of all bacteria involved in the production of lipase enzymes, *Bacillus* and *Pseudomonas* species have distinctive features which make them prospective candidates for biotechnological applications. *Pseudomonas* species are prevalent in natural habitats, and are desired for their ability to utilize different organic compounds, including haloaromatics (Van der Meer, Ravatn & Sentchilo, 2001). *Pseudomonas* can extract energy from a variety of relatively inactive compounds, making them suitable for xenobiotic waste treatment and oil spill management, treatment of fossil fuels for improved quality, and biocatalysis to synthesize various compounds (Olivera et al., 2001). Lipases produced by *Pseudomonas aeruginosa* are suitable indicators for hydrocarbon degradation testing in soil (Verma, Thakur & Bhatt, 2012).

Lipases produced by *Pseudomonas* species are utilized to degrade organic pollutants through a series of reactions such as hydrolysis, esterification, alcoholysis, and aminolysis which confirms their ability to effectively clean up the environment (Karigar & Rao, 2011). The structures of these organic pollutants are completely transformed, which greatly reduces their toxicity before they are eventually converted into their inorganic components (Erdogan & Karaca, 2011). Hence, the aim of this study was to characterize and optimize lipase enzymes produced by *Pseudomonas* species isolated from soil contaminated with crude oil to exploit their use in crude oil cleanup.

Materials and methods

Collection of contaminated soil samples

Contaminated soil samples were collected randomly from crude oil contaminated soil in the oil-rich Niger Delta in the Ogoni and Ijaw communities and was transported to the laboratory for analysis.

Isolation of bacteria

Serial dilution was used to isolate bacteria, which were identified and then inoculated into mineral salt medium (MSM) amended with crude oil. The amended MSM was set up using 1–3% (v/v) of crude oil as a carbon source in a basal mineral salt medium with the following composition (g/L):

$NaNO_3$ (2.0 g/L), NaCl (0.8 g/L), KCl (0.8 g/L), $CaCl_2 \cdot 2H_2O$ (0.1 g/L), KH_2PO_4 (2.0 g/L), $Na_2HPO_4 \cdot 12H_2O$ (2.0 g/L), $MgSO_4$ (0.2 g/L), $FeSO_4 \cdot 7H_2O$ (0.001 g/L). Sub cultures were made and transferred to fresh plants stored at 4°C for further experimentation (Ajuzie, Atuanya & Enerijiofi, 2015).

Qualitative test for lipase production using tributyrin agar

The identified bacterial isolates were screened for their ability to produce lipase using agar plates containing tributyrin (1%, w/v) and agar. The formation of clear halos around the colonies on the tributyrin-containing agar plates by the test bacterial isolates indicated lipase production (Shah & Bhatt, 2011).

Lipase enzyme production (using MSM)

The composition of the production medium used in this study was: $MnSO_4$ 0.002 g/L; $FeSO_4 \cdot 7H_2O$ 0.02 g/L; KCl 0.2 g/L; $MgSO_4 \cdot 7H_2O$ 0.2 g/L; K_2HSO_4 0.9 g/L; $ZnSO_4$ 0.002 g/L; and olive oil 10 g/L. Cultures were suspended overnight in 5 ml of sterile deionized water and used as the pre-culture inoculum to obtain an initial cell density which was thereafter adjusted to 0.5 McFarland turbidity standard. The submerged bacterial cultures were inoculated in 500 ml Erlenmeyer flasks containing 100 ml of liquid medium on a rotary shaker at 150 rpm. They were grown using an incubator at 30°C for 24 h before centrifuging at 4,000 rpm for 10 min at 4°C. The cell-free culture supernatant was harvested as the lipase enzyme (Shah & Bhatt, 2011).

Enzyme assay

Lipolytic activity was determined using a differential calorimeter which was based on the ability to utilize *p*-nitrophenyl palmitate at pH 8.0 (Lotrakul & Dharmsthiti, 1997). The reaction mixture contained 180 µl of solution A (0.062 g of p-NPP in 10 ml of 2-propanol, sonicated for 2 min before use), 1620 µl of solution B (0.4% triton X-100 and 0.1% gum arabic in 50 mM Tris-HCl (pH 8.0)) and 200 µl of diluted enzyme sample. The product was incubated at 37°C for 15 min and analyzed at a wavelength of 410 nm. One unit of lipase activity was defined as one mmol of *p*-nitrophenol released per minute by 1 ml of enzyme.

Protein determination

The Lowry method was used to determine the protein concentration (Shah & Bhatt, 2011) Reagents A (2% $NaCO_3$ in 0.1 N NaOH) and B (0.5%

$CuSO_4 \cdot 5H_2O$ in 1% Na tartrate). Reagents C (100 ml of Reagent A + 2 ml of reagent B) and E were formed by a 1:2 dilution using John's reagent water. Graded concentrations of bovine serum albumin (BSA) were prepared in tubes. Aliquots (0.3 ml) of each concentration were measured into test tubes, and 3 ml of reagent C was poured into them, vortexed, and left for 10 min. Thereafter, 0.3 ml of reagent E was added, vortexed, and left to stand for 30 min. The optical density (OD) was read at 600 nm. The graph of OD versus concentration of BSA was obtained and used as a standard BSA curve. Optical density values were also obtained for the samples, and the protein concentrations were extrapolated from the standard curve multiplied by the dilution factor. All readings were stated accordingly.

Partial purification of lipase of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa was grown in NB for 20 hrs at 30°C. The cell-free supernatant, prepared by centrifugation (6000 rpm, 20 min), was passed through a 0.45 μm pore size membrane, and then ammonium sulfate was added to achieve a 30% saturation. The suspension was further centrifuged (6000 rpm, 20 min, 4°C), and ammonium sulfate was added to the supernatant to reach 80% saturation. The precipitates (0–30% and 30–80%) were collected by centrifugation and separately dissolved to a minimal concentration of 20 mM. Tris buffer (pH 7.0) at 4°C and the solution were dialyzed against the same buffer to remove residual ammonium sulfate. The lipase activity in each fraction was spectrophotometrically analyzed using olive oil as the substrate (Kukreja & Bera, 2005).

Ammonium sulfate precipitation

Crude lipase enzyme was precipitated by adding ammonium sulfate until it reached a saturation of 60%. The mixture was centrifuged at 10,000 g for 15 min at 4°C. Thereafter, the precipitates were re-suspended in 50 mM Tris-HCl (pH 8.0) and dialyzed against the same buffer overnight at 4°C with three buffer changes. The enzyme solution was concentrated using 4 M sucrose solution to produce an enzyme free of metal ions and salts.

Gel filtration chromatography

The concentrated lipase solution was poured into a Sephadex G-100 column with diameter of 1.5 cm and a length of 75 cm. The solution was pre-equilibrated with 50 mM Tris-HCl at pH 8.0. Samples (5 ml) were collected at a flow rate of 20 ml/h at 27°C. The protein content of the collected fractions was analyzed at 280 nm by measuring the OD and thereafter assaying them for lipase activity. The fractions exhibiting lipase activity were harvested and concentrated for further analysis (Shah & Bhatt, 2011).

Characterization of the partially-purified lipase

Effect of temperature on lipase activity and stability

The optimal operating temperature range of the enzyme was determined to be from 30 to 80°C at a pH of 7.4. The enzyme stability was studied by incubating it in 50 mM Tris-HCl (pH 8.0) at different temperatures for 2 h, followed by activity estimation at 37°C (Mussarat et al., 2008).

Effect of pH on lipase activity and stability

The lipase enzyme samples were incubated using *p*-nitrophenyl acetate as the substrate and were prepared in various buffers, which included: sodium acetate (pH 3–5), sodium phosphate (pH 6–7), and Tris-HCl (pH 9) (Mussarat et al., 2008).

Effect of metal ions on lipase activity

To examine the effect of metal ions on lipase activity, enzymes were incubated with 10 mM solution of different metal salts for 15 min. Thereafter, the enzyme activity was monitored by examining the release of *p*-nitrophenol (Kunamneni, Poluri & Davuluri, 2003).

Effects of substrates on lipase activity

Palm, groundnut, and soya bean oils were used to determine the substrate specificity of the bacterial lipase.

Results

Lipase purification

The crude culture supernatant was purified by gel filtration on a DEAE Sephadex G – 100 column (1.5 × 75 cm). The elution profile from gel filtration (Figure 1) showed that almost all lipase activity was found in one major protein peak. The enzyme purification results are shown in Table 1. In the first step, crude extracted enzyme was concentrated 1 fold. In the subsequent ammonium precipitation, it was further concentrated by 1.77 fold, with a 44.58% recovered activity. This specific lipase activity was 13.55 μmol/min/mg with a 14.38% purity and 19.91% yield. The table shows the specific activities

Table 1. Summary of Purification Steps

Step	Volume (ml)	Lipase activity (µmol/min/ml)	Protein conc. (mg/ml)	Total activity (µmol/min/ml)	Total protein (mg)	Specific yield (µmol/min/mg)	Yield (%)	Fold
Crude extract	100	29.70	31.51	2970.37	3151.51	0.94	100	1
NH_4 content	40	33.11	19.75	1324.44	790.30	1.67	44.58	1.77
Gel Filtration	12.5	47.33	3.49	591.66	43.63	13.55	19.91	14.38

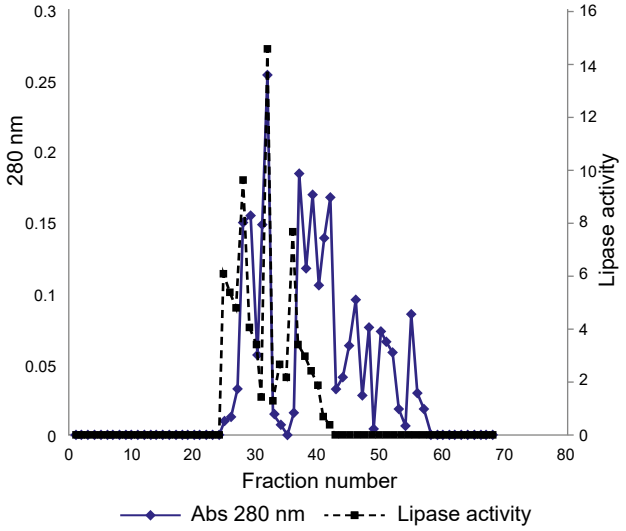

Figure 1. Ion exchange chromatography elution profile of lipase produced by *Pseudomonas aeruginosa*

for crude extract, ammonium sulfate and gel filtrations to be 0.94 µmol/min/mg, 1.67 µmol/min/mg, and 13.55 µmol/min/mg, respectively. The purification (fold) for crude extract, ammonium sulfate, and gel filtration were 1.0%, 1.77%, and 14.38%, respectively, indicating that with each purification step, the purification increased while the percent yield decreased.

Effect of temperature on lipase activity

Lipase production activity increased at temperatures between 30 and 50°C, while it declined at temperatures below 60°C. However, the optimum activity was recorded at 50°C (Figure 2).

Effect of pH on lipase activity

The optimum lipase activity was observed at a pH value of 8.0 (Figure 3). Activity was observed from slightly acidic to slightly alkaline pH values, and the minimum activity was observed at pH 3.0. Slightly changing the pH of the medium adversely affected the growth of the bacterium.

Effect of pH on lipase stability

Figure 4 shows the percentage residual activity of the enzyme at pH values from 5.0 to 8.0 against

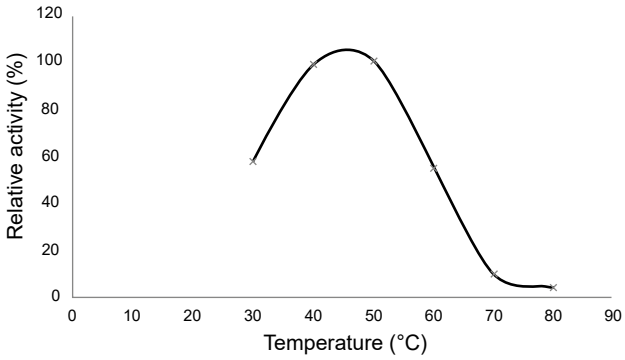

Figure 2. Effect of temperature on the activity of partially-purified lipase from *Pseudomonas aeruginosa*

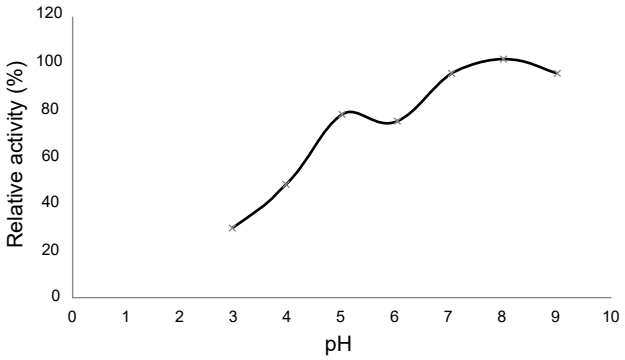

Figure 3. Effect of pH on the activity of partially-purified lipase from *Pseudomonas aeruginosa*

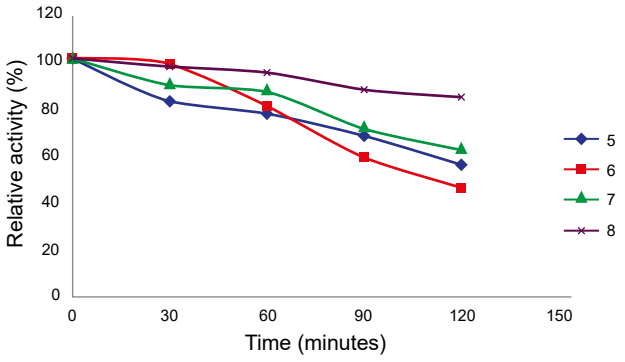

Figure 4. pH stability of partially-purified lipase of *Pseudomonas aeruginosa*

incubation time at room temperature. At all pH values examined, the enzyme was relatively stable for 2 h, and thereafter declined between pH 5–6 before stabilizing at pH 8.

Effect of temperature on lipase stability

The thermostability of the lipase activity of *Pseudomonas aeruginosa* is shown in Figure 5. Lipase production was relatively stable between 40 to 60°C before rapidly declining at 70°C.

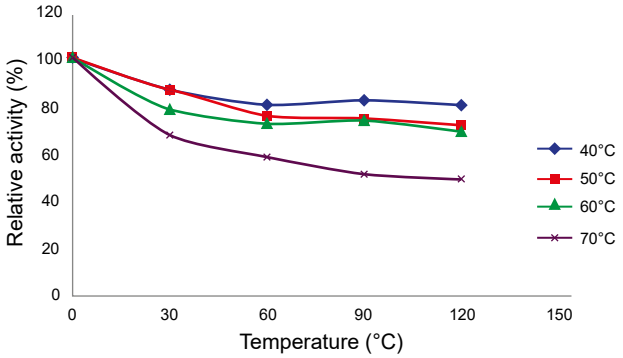

Figure 5. Thermostability of partially-purified lipase activity of *Pseudomonas aeruginosa*

Effect of metal ions on lipase activity

The effect of different metal ions activities on lipase production is recorded in Figure 6, which shows that Fe^{2+} and Zn^{2+} strongly inhibited lipase production. The maximum molar concentrations observed were 0.050 µM/mL/min, 0.025 µM/mL/min, 0.090 µM/mL/min, 0.065 µM/mL/min, 0.090 µM/mL/min, and 0.01 µM/mL/min, respectively for Zn^{2+}, Fe^{2+}, Ca^{2+}, Al^{3+}, Cu^{2+}, K^{+}, and Na^{+}, respectively. Monovalent ions like Na^{+} and K^{+} slightly inhibited lipase activity (Figure 6).

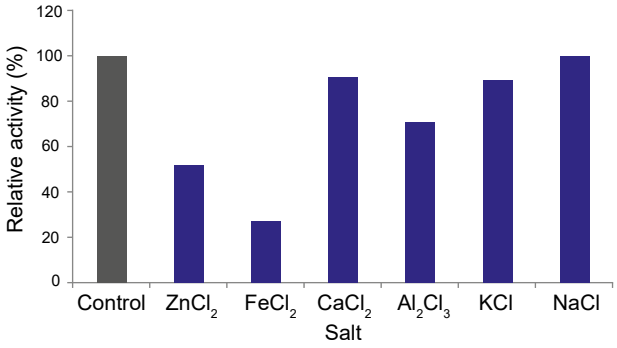

Figure 6. Effect of salts on the activity of partially-purified lipase from *Pseudomonas aeruginosa*

Effects of substrates on lipase activity

The effect of palm oil, olive oil, groundnut oil, and soya bean oil as lipase production substrates is shown in Figure 7. Olive oil gave the best result, possibly due to a reduction in the enzyme activity at higher concentrations of other substrates and molasses because the viscosity of the medium was maintained using the olive oil substrate.

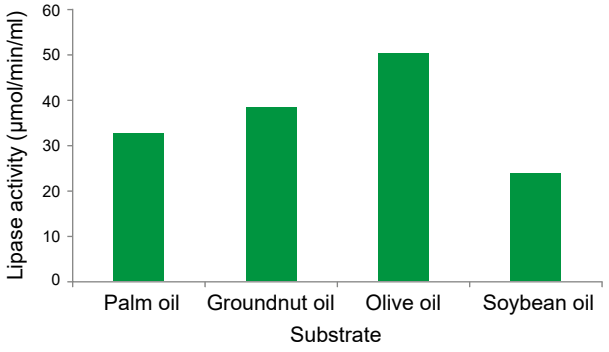

Figure 7. Effect of substrates on the activity of partially-purified lipase from *Pseudomonas aeruginosa*

SDS PAGE Electrophoresis

The SDS PAGE electrophoresis results in Figure 9 show the occurrence of a single protein band which was spotted between protein standards C and D. The estimated molecular weight of the purified lipase was 39.11 kDa.

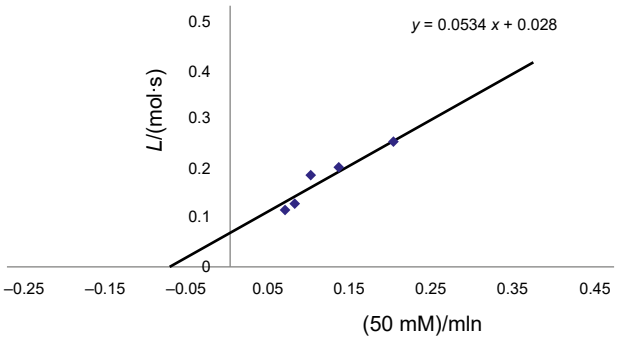

Figure 8. Lineweaver-Burk plot for the determination of Km of partially-purified lipase from Pseudomonas aeruginosa

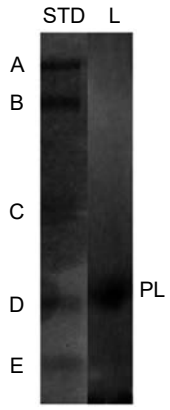

Figure 9. Electrophoretogram of partially-purified lipase from Pseudomonas aeruginosa. Standard molecular weights consisted of proteins A–E (103.14–27.26 kDa). Molecular weight of partially-purified lipase (PL) = 39.11 kDa

Discussion

The lipase activity increased with its purity, highlighting the importance of purity to achieve optimum activity. The optimum activity was observed at 50°C, and decreased as the temperature further increased. At 70°C, the residual activity declined to 9.56%, possibly due to the production of large amounts of metabolic heat. However, as the temperature increased, microbial growth and enzyme formation were inhibited (Bhatti et al., 2007). The enzyme was relatively stable at high temperatures compared with lipase obtained from *Pseudomonas aeruginosa* EF2 at 50°C (Gilbert, Cornish & Jones, 1991). Also, Gupta et al. (Gupta, Rathi & Bradoo, 2003) previously reported that extremely high temperatures lead to the deamination of enzyme proteins resulting from the breakdown of peptide bonds and the interchange and destruction of disulfide bonds. However, resistance to thermal denaturation of enzymes is regarded as one of the most important criteria for industrially useful enzymes (Sarowar et al., 2012).

Lipase was active in the pH range of 7.0–9.0 and showed an optimum relative activity (100%) at pH 8.0. Lipases have been shown to retain over 65% of their activity at pH 8.0 (Gupta, Gupta & Rathi, 2004). Notable activity was observed from slightly acidic to slightly alkaline pH values, which agrees with a previous report (Amin, Bhatti & Perveen, 2008) which showed that most lipases produced by *Pseudomonas* sp. have an optimum pH near pH 7.0–9.5 because this pH range supports optimum lipase production activity. Each microorganism grows and acts within a unique optimum pH range (Bhatti & Nawaz, 2009). A comprehensive review of some bacterial lipases carried out by Gupta et al. reported that in many case studies, optimum lipase activity was observed at pH values greater than 7 (Gupta, Shai & Gupta, 2014). Bacterial lipases display optimum performance at a neutral or alkaline pH, with the exception of one produced by *Pseudomonas fluorescens* which performs the best at an acidic pH of 4.8. In general, bacterial lipases are stable from a pH range of 4.0–11.0, but they are most stable at pH 7.0–8.0 (Sidhu et al., 1998).

Lipase activity was stable from 40–60°C, but declined at 70°C. At pH 7.0–8.0, lipase activity was stable, but began to decline below a pH of 7.0. Yuzo et al. (Yuzo, Kojima & Sakaya, 2003) reported that most bacterial lipases possess an optimal pH stability in the range of 6.0–8.0, and are unstable at pH values above 8.0. Additionally, lipase stability decreased sharply after 2 h of incubation at higher temperatures. Various metals, such as the divalent ions Fe^{2+} and Zn^{2+}, strongly inhibited the enzyme activity (Figure 6), which is consistent with earlier reports (Sarowar et al., 2012; Ojo & Ajele, 2011). In enzymatic reactions, metallic co-factors are important because their presence or absence regulates catalytic activities. Some lipases produced by *Pseudomonas aeruginosa* are Ca^{2+} dependent (Borkar et al., 2009). In certain *Pseudomonas aeruginosa* lipases, Ca^{2+} may be involved in the correct positioning of the histidine residue of the catalytic triad (Jinwal et al., 2003; Khattabi et al., 2003). Yamamoto and Fujiwara (Yamamoto & Fujiwara, 1988) also reported that Zn^{2+} inhibits lipolytic activity, which is also exhibited by other *Pseudomonas* lipases that may have arisen from the direct interaction of metals with the catalytic site.

The substrate specificity of lipase is important for analytical and industrial applications. The profiles of the different substrates on *Pseudomonas aeruginosa* showed a greater preference for the olive oil substrate. Similar hydrolytic activity in groundnut oil had been previously reported (Dahiya et al., 2010). Similarly, the purified lipase from *Pseudomonas* sp. efficiently hydrolyzed various oils and fats such as palm oil, soybean oil, and olive oil (Jinwal et al., 2003). Also, Fodiloglu and Erkmen (Fodiloglu & Erkmen, 1999) reported that when combined with other nitrogen sources, olive oil enhanced lipase production. Lipase migrated as a single protein band on SDS-PAGE with an estimated molecular weight of 39.11 kDa. This is higher than that reported in earlier studies where lipase was isolated from *Pseudomonas aeruginosa* MB5001 with a molecular weight of 29 kDa (Chartrain et al., 1993). The difference in the molecular weight as determined by gel filtration may be due to interactions between the enzyme and gel, which may have resulted in a reduced mobility, leading to an underestimation of its molecular weight.

Conclusions

In this study, *Pseudomonas aeruginosa* was shown to be suitable for the production of lipase enzyme, especially at an alkaline pH of 8.0 using olive oil as the substrate. However, the production of this versatile enzyme should scaled-up and purified because it is urgently needed in numerous industries and biotechnological applications.

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