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Lipase Production and Characterization by *Pseudomonas aeruginosa* Isolated from Oil Mill Effluents

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Abstract
Lipases have numerous biotechnological uses. This study aimed to isolate lipase-producing bacteria from oil mill effluents and to optimize, purify, and characterize the lipase produced. Fifty bacteria isolates were obtained from the various samples screened in this study. Eighteen (18) isolates showed zones of hydrolysis on tributyrin agar, while the isolate with the highest lipase activity was selected for lipase production. The selected isolate was identified as *Pseudomonas aeruginosa* based on its physiological attributes. Optimal lipase activity (3.72U/ml) was observed at a 36-hour incubation period and total protein content was 13.5mg/ml. The optimization of various production parameters was evaluated. The optimal conditions were an incubation period of 36 hours, yeast extract as nitrogen source, palm oil as carbon source, temperature of 50ºC and pH 8.0. The lipase produced was purified by ammonium sulphate precipitation, dialysis, and column chromatography. The protein determination at each purification step was highest for gel chromatographic step (4.814) and lowest for the crude enzyme (0.999). A determination of the molecular weight using Sodium dodecyl sulphate polyacrylamide gel electrophoresis SDS-PAGE analysis of the lipase showed a molecular weight of 30.68 kDa. Characterization showed the lipase was stable at a temperature of 50ºC (98.6%), pH of 7.0 (97.3%) in the presence of Mg^{2+} (107.7%). This result showed that *Pseudomonas aeruginosa* is a good producer of lipase, which can be further researched for their biotechnological applications.

Keywords: Lipase, *Pseudomonas Aeruginosa*, Dialysis, Column Chromatography, Oil Mill Effluents

Introduction
Lipases (EC 3.1.1.3) are glycerol ester hydrolases involved in the enzymatic breakdown of triglycerides into glycerol and free fatty acids typically at the oil and water interface through catalytic hydrolysis (Borkar et al., 2009). Lipases have been applied in food, dairy, detergent and pharmaceutical sectors, and in the oleo chemical industry and in medical applications (Bornscheuer et al., 2002). Lipases are typically produced by microorganisms inhabiting diverse habitats, including industrial wastes, fat and oil processing plants, dairies, oil-contaminated soils, oil seeds, decomposing food, compost heaps, coal deposits, and hot springs (Chaturvedi & Khare, 2016; Muthumari et al., 2016; Veerapagu et al., 2016). Lipases were produced naturally in several species of animals, plants, bacteria, yeasts, and fungi (Nagarajan, 2012). However, bacteria lipases have gained more attention due to their higher activities under optimum pH at neutral or alkaline condition. Microbial lipases also have shorter generation times, and genetic manipulations can be performed more easily on bacterial cells to increase the enzyme production (Nagarajan, 2012). Besides, bacterial cultures were more readily scaled up for production and purification with lower production costs (Saxena et al., 2011). The activities of lipases are highly pH dependent (any alteration in pH of the reaction mixture is likely to affect the catalytic potential of the lipases) and the metal-ions have been reported to be effective antagonists and modulators of lipases (Okino-Delgado & Fleuri, 2014). Lipases have been isolated from several genera including *Bacillus*, *Pseudomonas*, *Staphylococcus*, *Acinetobacter*, *Enterococcus*, *Geobacillus* sp (Veerapagu, Jeya & Sankaranarayananan, 2016). Hence, this study was aimed at isolating bacteria with copious lipase-production attributes from oil mill effluents; and to optimize and characterize the lipase produced.

Methodology
Sample collection
Vegetable oil effluents were obtained from vegetable oil factories in Owo, Ondo State. They were stored in falcon tubes and immediately transferred to the Department of Science Laboratory Technology laboratory for

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processing and analysis. Samples were immediately analyzed microbiologically and isolates were kept in the refrigerator at 4ºC until needed.

Bacterial isolation

Bacteria present in the samples were isolated by serial dilution using pour plate method. Dilutions of the stock up to 10^{-6} was prepared. 0.1ml of the dilutions 10^{-5} and 10^{-6} of the samples (POME and groundnut oil residues) were aseptically transferred into sterile plates, after which nutrient agar was dispensed into Petri plates, the plates were twirled to permit proper mixing of agar and distribution of inoculum. Plates were left to solidify on the bench and then incubated for 24 - 48hours at 37ºC aerobically in an incubator.

Screening for lipolytic bacteria

Screening was done using Tributyrin agar; a differential media (Oxoid). The molten agar was aseptically dispensed into sterile Petri dishes and left to solidify. Fresh eighteen hours old culture organisms were then streaked in a straight line onto tributyrin agar plates. The tributyrin oil forms an opaque suspension in the agar. Production of clear halo zones around the organism is evidence of lipase presence in the medium. Bacteria having more clearance zones were then selected and subcultured for further study.

Characterization of isolate

The isolated strain was characterized using physiological attributes such as cellular morphology, Gram Staining and biochemical test (Norris & Ribbons, 1971).

Lipase production media

Culture medium containing the basal medium K_2HPO_4 (900 mg/L), KCl (200 mg/L), $MgSO_4.7H_2O$ (200 mg/L), NH_4NO_3 (1000 mg/L), $ZnSO_4$ (2 mg/L), $MnSO_4$ (2mg/L), $FeSO_4.7H_2O$ (2 mg/L), and Olive oil (10 ml/L) in a 1000 millilitre Erlenmeyer flask (Jia et al., 2015). Sterilization of the basal medium was done using an autoclave at 121ºC at 1atmospheric pressure for fifteen minutes. It was allowed to cool and the inoculum was seeded. Culture media was incubated in with agitation at 28ºC for 36 h at 150 rpm during which 5 mL of the mixture at 6 h intervals were taken for 36 h. The fermenting mix was spun at 10,000 rpm for 15 minutes at 4ºC. The progressive growth of cells was estimated using absorbance at 600 nm. The supernatant obtained after centrifugation was stored at 4ºC until needed for the standard assay.

Lipase Optimization

Differing pH values (3, 4, 5, 6, 7, 8, and 9); nitrogen compounds, including ammonium chloride, ammonium nitrate, sodium nitrate, potassium nitrate, and yeast extract; carbon sources (groundnut oil, goya oil, soybean, olive oils, and palm oil), and different temperatures (30 to 90 °C) were utilized to obtain optimal lipase production conditions (Câmara Ribas et al., 2019; Javed et al., 2018a). This was done in a univariate model as described by Ribas et al. (2019).

Lipase activity assay

Lipase activity assay was evaluated with p-nitrophenyl palmitate (p-NPP) used as substrate at pH 8.0 (Lotrakul and Dharmsthiti, 1997). The assay mix comprised of -solution A with volume of 180 microliters (62 mg of p-NPP in 10 ml of 2-propanol, sonicated for 2 minutes before use), while the volume of solution B was 1620 microliters (0.4% triton X-100 and 0.1% gum arabic in 50 mM Tris-HCl, pH 8.0) and 200 microliters of the thinned out lipase. Lipase was observed using a UV-spectrophotometer with wavelength (410 nm) after incubating the reaction mix for fifteen minutes at 37°C. At this state, the molar extinction coefficient of p-nitrophenol (p-NP) released from p-NPP was 15000 M^{-1}. One unit of lipase activity was defined as 1 mmol of p-nitrophenol (p-NP) released per minute by 1 ml of enzyme (Wakil & Osesusi, 2017; Femi-Ola et al., 2018).

Protein Determination

Protein concentration was determined using the Lowry method (Lowry et al., 1951).

Purification method used

Enzyme purification was done using the method described by (Wakil & Osesusi, 2017).

Lipase Molecular weight (MW)

The protein (MW) was evaluated using SDS-PAGE, based on the protocol described by Laemmli (1970) on a vertical slab 10% (w/v) polyacrylamide gel, at 200v a constant voltage of 80V, for 5hour using phosporylase B (103.14 kDa), bovine serum albumin (81.35 kDa), ovalbumin (47.04 kDa), soybean trypsin inhibitor (27.26 kDa) and lysozyme (17.67) were used as standard protein molecular weight markers ((Javed et al., 2018a; Laemmli, 1970).

Lipase Characterization

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Enzyme stability at varied temperature (30 to 80°C) was determined. The pH was maintained using Sodium Acetate (pH 3-5), Sodium Phosphate (pH 6-7) and Tris-HCl (pH 9) buffers according to the protocol of Mussarat *et al*. (2008). Potential influence of metal ions and Ethylene diaminetetraacetic acid EDTA on the stability of lipase was assessed, followed by lipase assay (Kunamnein, Poluri & Davuluri, 2003).

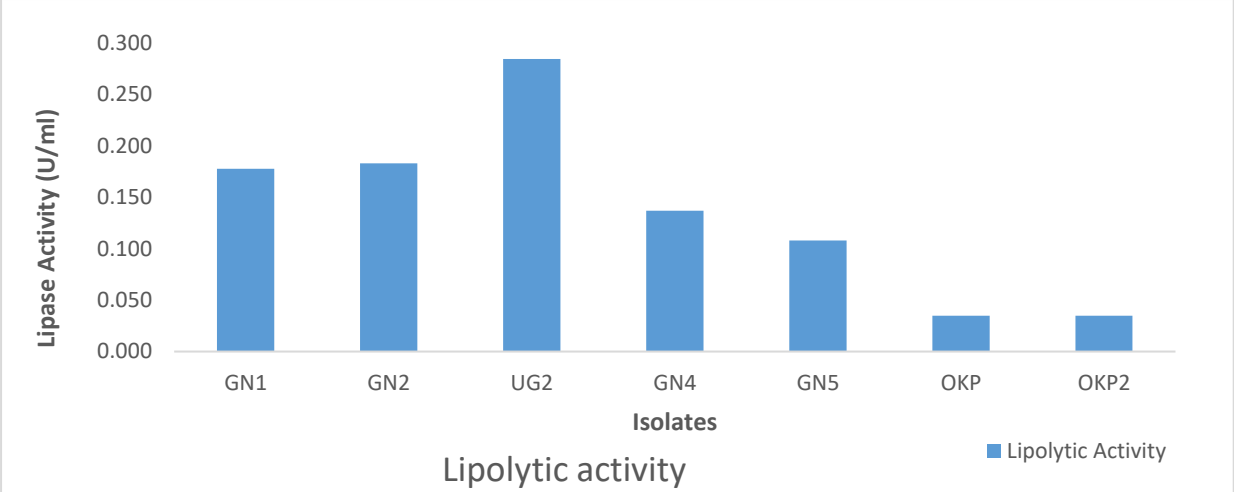
Results

Oil mill effluents are potential substrates for the production of lipase, and other enzymes. In this study, the lipase activity screening was carried out using Tributyrin agar (Cardenas et al., 2001). A total of fifty bacteria isolates were observed from the various sample sites. EW for wastes, SP and OKP for palm oil wastes, GN and UG for groundnut oil residues. Out of the fifty isolates, eighteen showed positive from the qualitative lipase screening carried out. The isolates with observable lipase hydrolysis (shown in Figure 1) were selected and quantitatively screened, with Isolate UG2 giving the best lipase yield (0.28u/ml). Isolate UG2 was identified by assessing the physiological attributes (morphological and biochemical reactions). The isolate UG2 was identified to be *Pseudomonas aeruginosa.* It showed a negative Gram reaction with circular shape. It was chocolate, shiny, with a raised elevation. It utilized catalase, urease, and citrate, with a positive reaction for oxidase utilization. It also fermented sugars such as mannose, fructose, glucose, lactose, and mannitol. *Pseudomonas aeruginosa* is an organism with prolific lipase production capabilities as highlighted by Hasanuzzaman, it is a ubiquitous organism capable of utilizing an assortment of simple to complex organic and inorganic sources (Hasanuzzaman et al., 2004).

Lipase optimization

Optimization of the production parameters showed that growth rate did not significantly affect lipase production. From figure 2, lipase production increased remarkably with increasing time of incubation. Highest lipase activity (3.72U/ml) occured at 36 hours of incubation and growth (optical density) was 1.13. This differed slightly from previous findings which reported highest production of lipase at 48hrs for *Pseudomonas putida* (Fatima et al., 2014; Javed et al., 2018b).

Figure 1: Quantitative Assay for Lipolytic activity



Optimal lipase yield occurred with yeast extract (3.36U/ml), potassium nitrate yielded the least production (2.4U/ml) as shown in Figure 3. This differed slightly from earlier findings which reported that peptone gave the highest lipase yield by *Pseudomonas fluorescens* NS2W with peptone (KhemikaLomthaisong, 2012). Organic nitrogen sources (peptone, yeast extract) reportedly gave high lipase yields by *Bacillus* sp. and *Pseudomonas* sp (Sharma et al., 2002). Lipase activity was highest (3.32U/ml) with palm oil as carbon source. Olive oil also gave significant enzyme activity (3.17U/ml) as observed in Figure 4. Goya oil (2.9U/ml), Soybean oil (2.74U/ml) also showed potentials as carbon sources. This quite agrees with findings that reported higher lipase activity by *Bacillus* spp when palm oil was used as carbon source (Kalapatapu & Bhaskar, 2015). This highlights the attributes of lipases as being inducible enzymes that are normally synthesized when lipid substrates, including oils and so on are present (Mobarak-Qamsari et al., 2011). Carbon is a major nutrient essential for the expression of lipase activity. Optimal lipase activity was observed at temperature 50ºC (99.9%) expressed as relative activity, from which lipase activity began decreasing with no activity observed at 80ºC (Figure 5). Hence, it could be deduced that 50°C was the most suitable temperature level for this *Pseudomonas aeruginosa* lipase and

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this contrasts with reports of a cessation in lipase production at 50ºC (Veerapagu et al., 2016). It also differs from earlier findings which observed the best growth and lipase production by *Pseudomonas fluorescens* at temperature 36ºC (Shanthi, 2002). Lipase yield by *Pseudomonas aeruginosa* was highest at pH 8.0 (139%) as shown in figure 6. This agrees closely with reports that the pH range of 7 to 9 is where microbial lipases are most active (Mahmoud et al., 2015; Zhang et al., 2005).

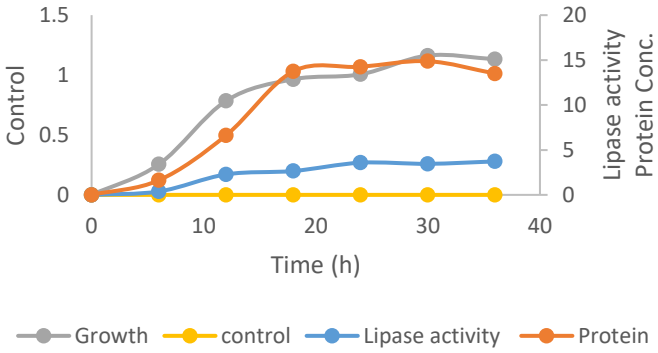

Figure 2: The Growth and production of lipase by *Pseudomonas aeruginosa*.

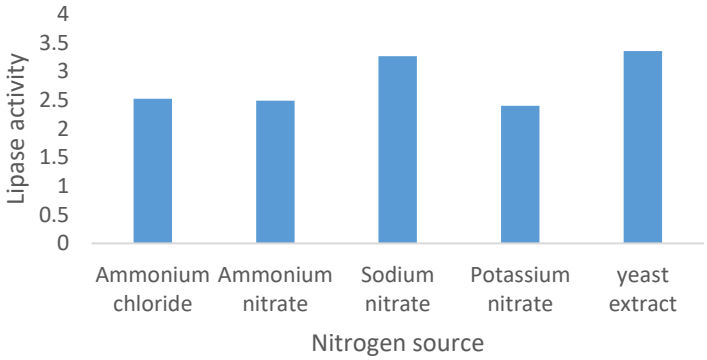

Figure 3: Effect of Different Nitrogen Sources on Lipase production by *Pseudomonas aeruginosa*.

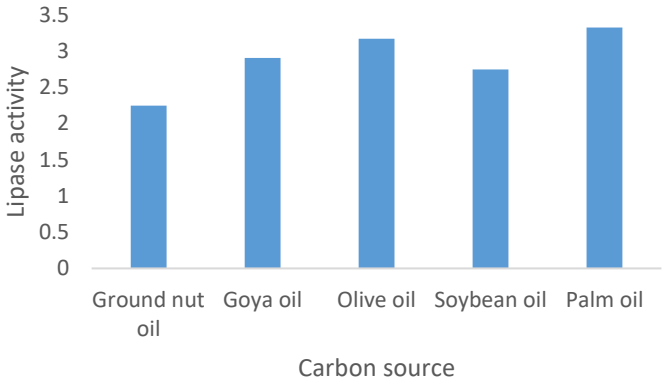

Figure 4: The Effect of Different Carbon Sources on Lipase Production by *Pseudomonas aeruginosa*.

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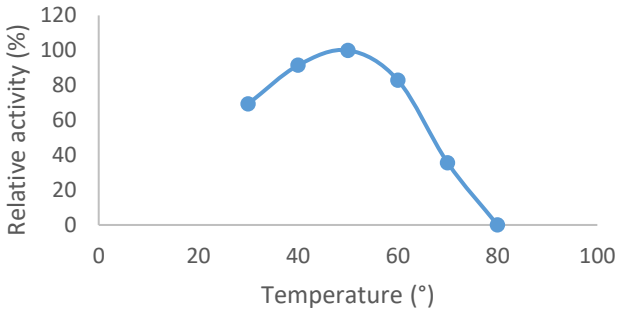

Figure 5: The Effect of Temperature on Lipase Production by *Pseudomonas aeruginosa.*

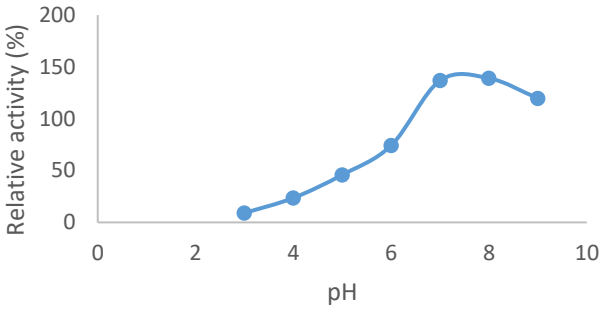

Figure 6: The Effect of pH on the production of lipase by *Pseudomonas aeruginosa.*

The purification rundown of the produced lipase is presented in Table 4, including the protein content and the total lipase activity. Purification of the enzyme by Sephacel S-200 (figure 7) was done in fractions and purification by chromatographic analysis using Sephadex G-100 (figure 8) was also done in fractions. Using SDS-PAGE analysis, the molecular weight of the purified lipase was found to be 30.68 kDa (figure 9). This contrasts slightly with reports of lipase of 33.9kD produced by *Pseudomonas aeruginosa* CS-2 (Peng et al., 2010). Temperature had the best impact on lipase stability (100%) at 50^{o}C whereas lipase activity decreased at 70^{o}C and was almost inactivated at 80^{o}C (Figure 10). This temperature influence on lipase stability also showed the highest activity (98.6%) at 50^{o}C for 30 hours (Figure 11), this shows that *Pseudomonas aeruginosa* is relatively thermostable. Higher enzyme activity was observed at pH 6.0 – 8.0, with optimum at 8.0. Upon purification lipase also showed a relative activity 74.9% at pH 9.0 whereas lipase activity dropped at acidic condition 5.0 and at pH 3.0 the enzyme was inactivated as shown by loss of activity. In this study, *P. aeruginosa* lipase demonstrated stability at varying pH, retaining 50% in residual activity between pH (5.0 – 9.0) as depicted in Figure 12. The highest lipase stability occurred at pH 7 with a residual activity of 97.3%. At pH 5.0, the least amount of residual activity (70.1%) was detected.

Among the metal ions tested, an enhancement in the enzyme activity occurred in the presence of Mg^{2+} with 107.7% relative activity in comparison to the control (Figure 13). High activity was also showed with Al^{3+} (99.3%), Na^{2+} (98.3%) and Mn^{2+} (89.9%). In contrast, lipase activity dropped with K^{+} (55.3%) and then Hg^{2+} (2.6%). There was complete lipase inactivation when Pb^{2+} was tested. This slightly differs with earlier reports of high lipase activity by different degree when Ca^{+2} and Mg^{+2} ions were tested (Benattouche & Bouzian, 2012). This influence of metal ions may have resulted from modifications to the catalytic attributes of the enzyme itself, as well as modifications to the solubility and behavioural patterns of the ionized fatty acids at interfaces (T. et al., 1994; Mobarak-Qamsari et al., 2011).

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Table 4: Lipase purification profile

Step	TLA	TP	SP	Yield	Fold
Crude extract	1672.222	10412.5	0.160598	100	0.999997
Amm ppt/ Concentra	121.6467	571.825	0.212734	7.274552	1.324637
Ion exchange	130.8667	260.475	0.502415	7.825915	3.128404
Gel	125.3233	162.1125	0.773064	7.49442	4.813659

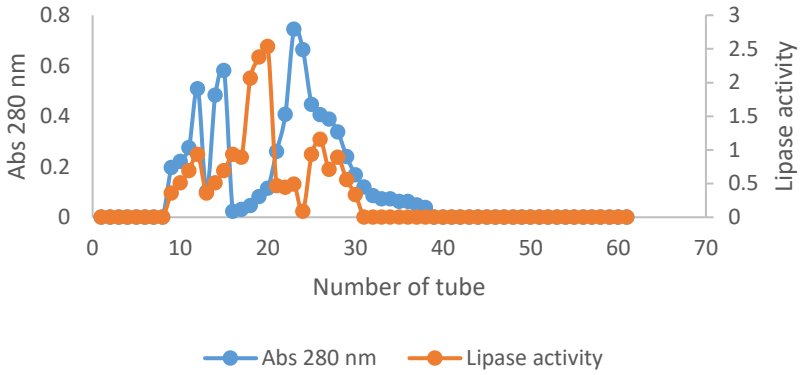

Figure 7: Purification of dialysate by Sephacel S-200

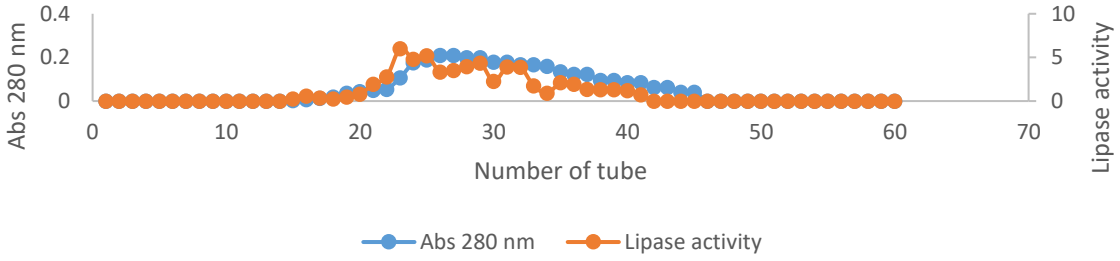

Figure 8: Purification by Sephadex G-100

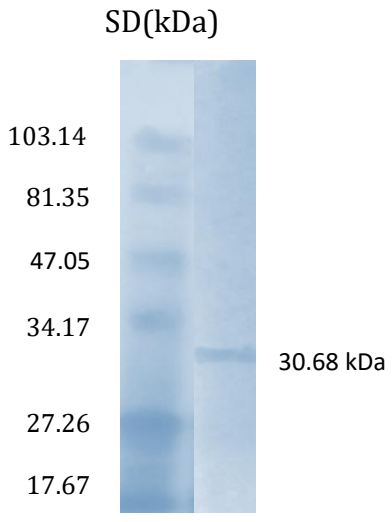

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Figure 9: SDS-PAGE of purified dialysate.

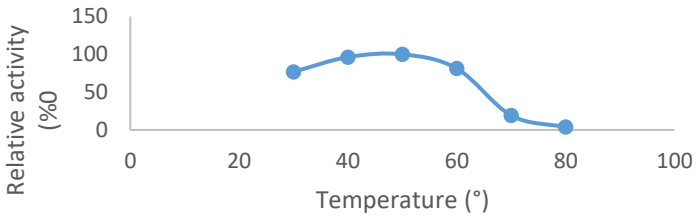

Figure 10: Influence of Temperature on Purified Lipase Activity.

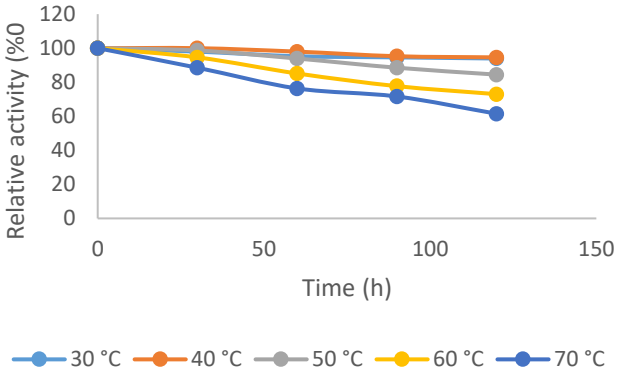

Figure 11: Influence of Temperature on Purified Lipase Activity and Stability.

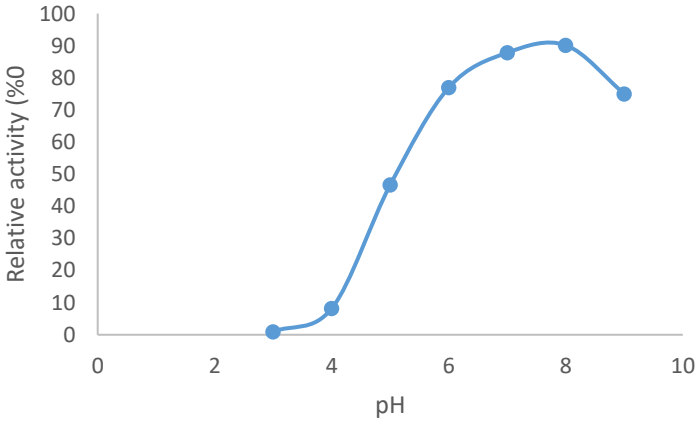

Figure 12: Influence of pH on Purified Lipase Activity

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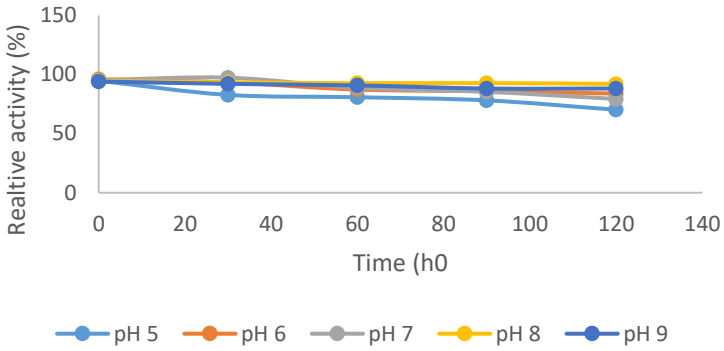

Figure 13: Influence of pH on Purified Lipase Activity and Stability.

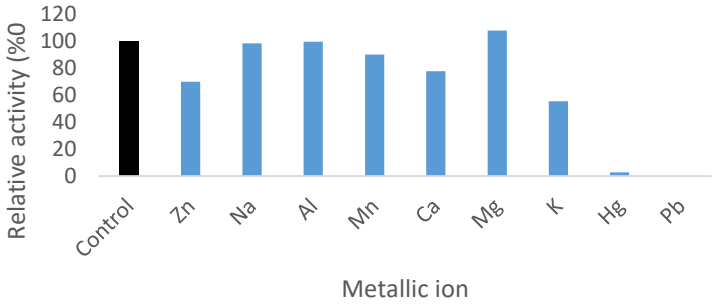

Figure 14: Influence of Metal Ions on Purified Lipase Activity

Conclusion

Pseudomonas aeruginosa was isolated from vegetable mill effluents, and optimized for lipase production. The optimal lipase activity was obtained with Yeast extract, Palm Oil, Mg^{2+}, adjusted at pH 8.0 and incubated at 50°C for 36hours. This lipase was relatively stable under a wide range of conditions. This *Pseudomonas aeruginosa* is suitable for further applications study which could include the degradation of complex hydrophobic mixtures amongst other uses.

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