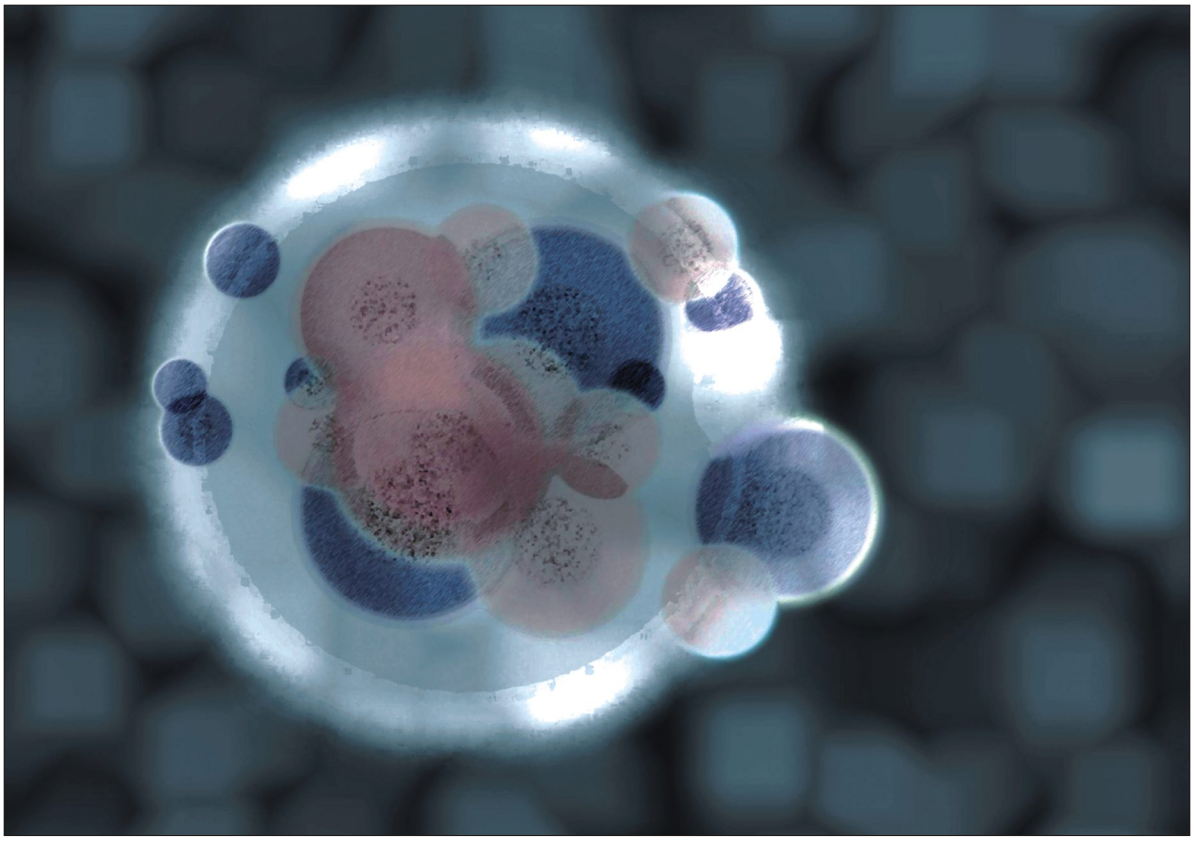

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Research Article
Purification and Kinetics of Lipase of *Pseudomonas fluorescens* from Vegetable Oil Polluted Soil

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Abstract

Background and Objective: Bacterial lipases occupy a place of prominence among biocatalysts and are used for various biotechnological processes. This study was designed to observe the pure lipases and to enable the establishment of the structure-function relationships. **Materials and Methods:** The lipase of *Pseudomonas fluorescens* was partially purified using ammonium sulphate, dialysis, column and ion-exchange chromatography. The purity and molecular weight of lipase was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis and their kinetics was studied. **Results:** The enzyme was purified 8.005-fold from *Pseudomonas fluorescens*. The molecular mass of the partially purified *Pseudomonas fluorescens* lipase was 45 KDa. Enzyme kinetics displayed Michaelis-Menten constant K_m 1.25 mg mL^{-1} and V_{max} value of 0.7 μg sec^{-1}. **Conclusion:** Hence, knowledge of the kinetics, molecular weights of purified lipases, as well as the three-dimensional structure of lipases plays an important role in designing and engineering lipases for specific purposes.

Key words: Lipase, kinetics, purification, *Pseudomonas fluorescens*, Michaelis-Menten, ammonium sulphate, molecular weight

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Lipases are acyl hydrolases and water-soluble enzymes that play a key role in fat digestion by cleaving long-chain triglycerides into polar lipids. Because of an opposite polarity between the enzyme (hydrophilic) and their substrates (lipophilic), lipolytic reaction occurs at the interface between the aqueous and the oil phases[1].

Different classes of lipolytic enzymes including carboxylesterases which hydrolyze water-soluble esters and lipases which hydrolyze long-chain triacylglycerol substrates are produced by bacteria[2]. Lipases show interfacial activation, which is a sharp increase in lipase activity; this happens when the substrate starts to form an emulsion[3]. As a result of this, the kinetics of a lipase reaction may not follow the classical Michaelis-Menten model; in contrast, esterases show normal Michaelis-Menten Kinetics in aqueous solution. In most cases, bacterial lipases are able to completely hydrolyze a triacylglycerol substrate although ester bonds are more favourable[4]; however lipases are activated only when adsorbed to an oil-water interface enabled by a mobile lid domain located over the active site[5]. These bacteria genera are important lipase-producers; *Bacillus*, *Pseudomonas* and *Burkholderia*[6,7].

It is very essential to apply purification methods in order to understand enzyme functions, its relations between environment and its 3-D structures[1]; it also allows amino acid sequence. Studies shows that X-ray of pure lipases enable the establishment of the structure–function relationships, which knowledge provides a better understanding of the kinetic mechanisms of lipase action on hydrolysis, synthesis and group exchange of esters[8]. For effective lipase design and engineering for specific purposes, knowledge of the lipase three-dimensional structure plays an important role. Pre-purification steps involve concentration of culture by ultra filtration, by ammonium sulfate or cold acetone precipitation and by extraction with organic solvents.

Protein purification processing, with affinity chromatography (especially hydrophobic interaction chromatography) known to be the best method since lipases are hydrophobic and have large hydrophobic surfaces in their active sites[9]. However, ion-exchange and size-exclusion chromatography are preferred after precipitation step because of high cost of hydrophobic resins. Several investigations on purification and characterization of lipase from *Pseudomonas spp*. has been documented[8,10-16].

This study was therefore undertaken to purify, determine the molecular weight and kinetics of the lipase of *Pseudomonas fluorescens*.

MATERIALS AND METHODS

Study area: Soil samples from which the lipase was obtained were collected from a vegetable oil mill factory located: Challenge Area, of 7°21″N,3°53″E Ibadan, Oyo State, the study was carried out for a period of 2 years. This research project was conducted from April, 2012 to March, 2014.

Lipase production

Preparation of inoculum: A loop full of the pure culture of the bacteria were grown overnight in nutrient broth.

Production procedure: One milliliter from the above preparation was inoculated into 65.0 mL of sterile medium in 250 mL Erlenmeyer flasks and incubated at room temperature (27±2°C) from 24 hrs to many days until the maximum lipase production was recorded. The medium was centrifuged at 30,000 g for 15 min using Himac High-speed Refrigerated Centrifuge (Hitachi model CR21GII).The supernatant of the centrifuged culture broth was then decanted leaving the cells at the bottom. The cell-free extract acted as the crude lipase enzyme.

Lipase assay: Lipase activity was measured by a modification[17] using as substrate a 10% Olive oil-gum arabic solution emulsified by sonication for 2 min at 25 watts output observed[18]. One milliliter of cell-free fermentation broth prepared by centrifugation at 30,000 g for 15 min using Himac High-speed Refrigerated Centrifuge (Hitachi model CR21GII) was added to 5 mL of emulsion and incubated at room temperature for 1 hr with rapid stirring. Ethanol was added to stop the reaction and the free fatty acids produced were quantified by titration to pH 9.5 against 0.1 N NaOH using a radiometer titration system. Blanks with 1 mL of fermentation broth were employed in each experiment. Blanks ran with sterile or actual uninoculated broths were the same within experimental error. Samples were run in duplicate.

A unit of lipase activity was defined as the amount of sodium hydroxide (NaOH) used in the titration to bring the reaction mixture to a pH of 9.5 min^{-1} under the defined assay conditions. Alternatively, it is considered as the release of one micromole of Free Fatty Acid (FFA) min^{-1} at room temperature.

Protein estimation: The protein concentration was determined[19]. Five milliliter of Reagent A (Reagent I Plus II) (Appendix 1) was added to 1 mL of each culture filtrate in test tubes. The mixtures were incubated at room temperature for

Appendix 1

Protein estimation (Lowry *et al.*[19])		
Reagent I		
$Na2CO_3$	2%	50 mL
NaOH	0.1 M	
Reagent II		
$CuSO_4.5H_2O$	0.5%	1 mL
Sodium potassium tartrate	1%	
Folin reagent diluted twice		

10 min. Thereafter, 0.5 mL of folin reagent (BDH) was added to the mixture and incubated at room temperature for 30 min. The optical density of each sample was taken at 670 nm using Jenway 640 (UV/VIS) spectrophotometer.

Purification of the enzymes

Ammonium sulphate purification: The crude enzyme of *Pseudomonas fluorescens* from a vegetable oil polluted soil was used for further studies. This enzyme was partially purified using ammonium sulphate within 0-100% saturation[20]. The filtrates were treated with 24.3, 28.5 and 15.7 g of ammonium sulphate to 0-40, 40-80 and 80-100% saturation respectively. The mixture for each batch of percentage saturation was stirred continuously for 15 min until the ammonium sulphate dissolved. The mixture was kept at 4°C overnight in a LG GR-131 SSF refrigerator set at 4°C after which it was centrifuged at 30,000 g for 30 min using Himac High-speed Refrigerated Centrifuge (Hitachi model CR21GII) at 4°C. The supernatant was treated to the next batch until the final batch. The precipitates for each batch were pooled together and the volume adjusted to the initial volume of the culture filtrate with the appropriate buffer (phosphate buffer).

Dialysis: The enzyme solutions were further purified by dialysis. The procedure involved introducing the previous precipitate in solution after dissolving in the appropriate buffer; phosphate buffer, into a visking dialysis tube and dialyzed extensively against the same buffer at 4°C for 18 hrs. The clarified extract obtained was then made available for the next application, column chromatographic technique.

Dissolution of sephadex beads: The preparation of the gel was carried out according to a modified method[21]. Sephadex G-75 was used for parking of the column. Ten gram of Sephadex G-75 was weighed out into a clean bowl, 50 mL of sterile distilled water was added and allowed to dissolve into paste form, 300 mL of the appropriate buffer was added and slurry was allowed to swell for 3 days at room temperature.

Loading of the column: This was carried out as using a modified method[21], the slurry was mixed together after the third day and dispensed into clean chromatography column with the use of a clean funnel. The Sephadex was allowed to compact in the column with occasional removal of the supernatant (buffer) and refilling of the column with the soaked Sephadex (the slurry) to the zero point of the column. This was done for 48 hrs after which enzyme was injected into the column.

Fractionation of the sample: The fractionation was carried out according to a previous method[21]. One milliliter of the enzyme preparation sample was carefully applied to the top of the gel and allowed to pass into the gel by running down the column. The appropriate buffer was also added without disturbing the gel surface and to the reservoir to elute the enzyme. The lipases were eluted with phosphate buffer at a flow rate of 0.5 mL min^{-1} and the fractions (10 mL) were collected in each case. The eluents from the chromatographic column were also analysed for total protein and lipase activity. The fractions that showed the highest lipase activity were pooled and assayed for protein content. The specific activity of the purified enzyme was compared with that of the crude enzyme and the purification factor was calculated.

Ion exchange chromatography (CM): The pooled and concentrated active fractions (10 mL) obtained after gel filtration were re- run on ion-exchange chromatography CM (1.5×15 cm) equilibrated with phosphate buffer[21]. The column was washed with two bed volumes of the same buffer and the enzyme was eluted with 0-0.5M NaCl-phosphate in a linear gradient the flow rate was adjusted to 0.5mL min^{-1} and fractions showing lipase activity were pooled and concentrated. The specific activity of the purified enzyme was compared with that of the initial crude enzyme and the purification factor was calculated. The active fractions were stored at 4°C until used for polyacrylamide gel electrophoresis and further enzyme characterization.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): The enzyme samples were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on vertical electrophoresis kit SE u245 series using a discontinuous gradient gel buffer system[22]. The following were prepared:

- 30% acrylamide, 0.8% Bis-acrylamide, 30 g of acrylamide and 0.8 g of N, N[1] Methylene Bis-acrylamide (BDH) were dissolved in 60 mL distilled water in 100 mL standard volumetric flask and made up to the mark. The solution was stored at 4°C
- **Stacking gel buffer:** All the components were dissolved in 500 mL distilled water in 1 L standard volumetric flask. The pH was adjusted to 6.8 with sodium hydroxide solution. The solution was then made up to mark with distilled water and stored at 4°C
- **Running gel buffer:**1.5 M Tris, 8 mM EDTA, SDS pH 8.8 in 1 L standard volumetric flask. The pH was adjusted to 8.8 with hydrochloric acid. The solution was then made up to mark with distilled water and stored at 4°C
- **1% ammonium persulphate:** One gram of Ammonium persulphate ($CNH_4)_2S_2O_8$) was dissolved in 1 mL distilled water and used immediately
- **Electrode buffer:** Glycine, SDS, Sodium salt EDTA. All the components were dissolved in 4 L distilled water with constant stirring. The solution was kept at 4°C.
- **Sample buffer:** Six milliliters glycerol mixed with 1.2 mL of sodium phosphate buffer pH 7.0, 1.5 g of sodium dodecyl sulphate (SDS), 30 mg dithiothreitol (DDT) and 10 mg bromophenol blue. Distilled water was added to make up to 20 mL. The mixture was dispensed into test tubes in aliquots of 1 mL washed and stored at 20°C until needed

Procedure: The stacking and running gels were prepared. The latter solutions were poured into the gel assembles to a level of about 4 cm below the maximum filling level. Distilled water was layered on the gel surfaces using a Pasteur pipette to ensure an even surface and also to avoid evaporation of the gel while the polymerizing gel was allowed to polymerize for 1-2 hrs.

The water on the gel was poured off and the stacking gel was poured on to the polymerized gel and a comb (1.5 mm thick) was gently inserted to obtain wells. It was allowed to polymerize for one hour before removing the comb. The gel was then clamped to the electrophoresis chamber. The upper and lower chambers were filled with electrode buffer and the bubbles formed were removed with a sterile syringe.

The protein samples were prepared by mixing sample buffer (in a dilution of 1:1 v/v) with enzyme samples. The mixtures were then placed in a dry bath fan heated for 4 min at 95°C. Equal volume of samples was applied to the bottom for the sample wells with a Hamilton syringe. Molecular weight standards (Bio rad high and low range) were then applied into wells alongside the samples.

Gel electrophoresis was conducted at 120 volts in a vertical electrophoresis apparatus for about 1.5 h or until the bromophenol blue dye migrated to the bottom of the gel.

At the end of the electrophoresis, the gel was carefully removed from the glass plates and stained by soaking in the staining solution for 20 min with gentle agitation. Excess stain was removed by immersing the gel for one h in several changes of the destaining solution each lasting for 15-20 min. The gel was then removed and allowed to dry.

Enzyme kinetics: The Michaelis constant (K_m) and the maximum reaction velocity (V_{max}) of the enzyme was obtained from line weaver-Burk plot. To obtain K_m and V_{max} of the lipase for the substrate (olive oil), 0.5, 1.0, 1.5, 2.0, 2.5% of olive oil was mixed with 1 mL of the purified lipase respectively, using the standard enzyme assay.

RESULTS AND DISCUSSION

Purification of the extracellular lipase: Lipase was precipitated to 100% saturation using ammonium sulphate. The result shows *Pseudomonas fluorescens* had maximum specific activity of 0.410 U mg^{-1} with purification fold of 1.03. The ammonium sulphate precipitated enzyme was dialyzed. Dialysis raised the specific activity of 0.73 U mg^{-1} with 1.85 purification fold as shown in Table 1.

The purification fold of the lipase increased to 3.60 after applying Sephadex G-75 column chromatography technique. A total of 30 fractions of 10 mL each were eluted at a flow rate

Table 1: Purification levels of the lipolytic extract of *Pseudomonas fluorescens*

	Total protein (mg)	Enzyme activity (U mL^{-1})	Yield (%)	Specific activity (U mg^{-1})	Purification fold
Crude enzyme	2.013	0.80	100.00	0.397	1
Ammonium Sulphate precipitated	1.2183	0.50	62.50	0.410	1.033
Dialyzed extract	0.6127	0.45	56.25	0.734	1.849
Gel filtration	0.2802	0.40	50.00	1.428	3.597
Ion-exchange (Sephadex 75)	0.0944	0.30	37.50	3.178	8.005

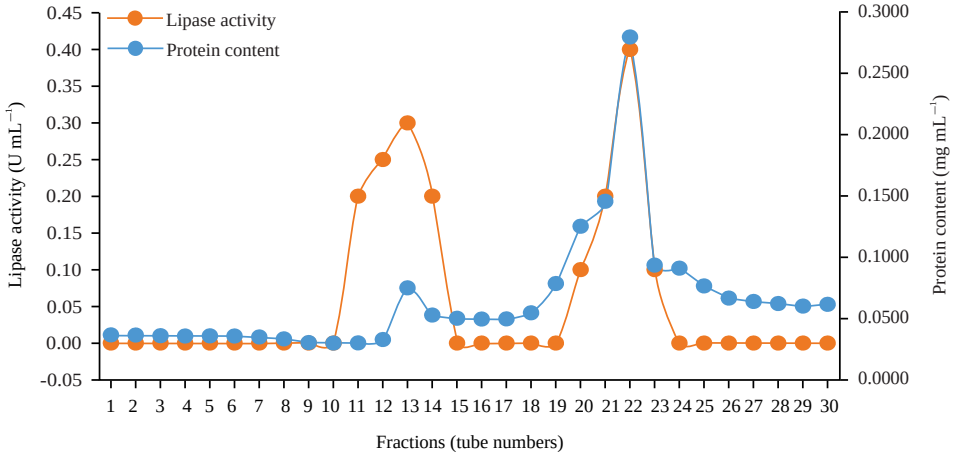

Fig. 1: Separation of proteins in concentrated extracts of *Pseudomonas fluorescens*
Dialyzed extract of the enzyme was applied to the column (1.5×15 cm) of sephadex pre-equilibrated with 0.2 M phosphate buffer, pH 7.5 at 4°C and eluted step wisely at 10 mL each into 30 fractions (in 30 tubes) using the same buffer. Flow rate: 0.5 mL min^{-1}

of 1 mL min^{-1}, each fraction of the 30 tubes elute was assayed for lipase activity (hydrolysis of fats by titrimetric method)[17] as well as the determination of the protein content of the enzyme[19] with bovine serum albumin as the standard, the result of each of the tube is as plotted in Fig. 1, the Y-axis being (lipase activity and protein content) while the X-axis represent fractions (F) of the elutes collected in tubes, the result from the assays generated separation of this *Pseudomonas fluorescens* lipase into the two major peaks F-13 and F-22, it was found that only in these two fractions with the peaks was the highest, most obvious and best lipase activity obtained (although the highest of them is F-22 with lipase activity and protein content of 0.40 and 0.2802 mg mL^{-1} respectively) as compared with other fractions where majority were 0.0 U mL^{-1} (no activity), with the exception of fractions 11, 12, 14, 20, 21 and 23, although for protein content; fractions F-19, F-20, F-21, F-23, F-24 and F-25 had higher protein content (for instance F-19 had 0.0787 mg mL^{-1}, F-20 had 0.1255 mg mL^{-1}) than that of F-13 but because their lipase activities were not as high as F-13, they were not considered for the next step of purification [F-13 (protein content = 0.0751 mg mL^{-1}, lipase activity = 0.30 U mL^{-1}) and F-22 (protein content = 0.2802 mg mL^{-1}, lipase activity = 0.40 U mL^{-1})]. These two were pooled together. The fractions pooled together was loaded onto ion exchange chromatography (CM) equilibrated with 7.5 phosphate buffer (0.5 M, pH 7.5), a total of 30 fractions of 10 mL each were also collected after purification. Fractions in each of the 30 eluted tubes containing 10 mL each (at a flow rate of 0.5 mL min^{-1}.) of the purified enzyme by ion exchange chromatography (CM) were each subjected to determination of protein content[19] and enzyme activity, which was measured by a modification[17]. In Fig. 2, this enzyme was separated into a major peak F-7 (tube 7) where both lipase activity and protein content (0.30 U mL^{-1} and 0.0944 mg mL^{-1}, respectively) was found to be the highest (meaning the activity of the enzyme is best here) of all the 30 fractions eluted. The Y-axis indicates both the protein content and lipase activity while the X-axis represents the fractions eluted in tubes, each fraction in each tube assayed gave values for both lipase activity and protein content, for instance fractions 6, 18 and 20 gave protein content and lipase activity of 0.091 mg mL^{-1}, 0.20 U mL^{-1}, 0.090 mg mL^{-1}, 0.10 U mL^{-1}, 0.089 mg mL^{-1}, 0.15 U mL^{-1}, respectively. The overall result of the fractions sampled are as shown in Table 1, for instance enzyme activity for ion-exchange chromatography is 0.30 U mL^{-1} with total protein being 0.0944 mg mL^{-1} having a specific activity of 3.178 (U mg^{-1}) with a purification fold of 8.005.Various researchers have used similar procedures/steps in the extraction and purification of lipase enzymes[13,23,24].

Notably, from this research ammonium sulphate method was relatively inexpensive, efficient and reliable. This is in agreement with previous study[25], they also added that the purification process is reversible and a general storage technique employed in an enzyme system. The potential advantage of the ammonium sulphate precipitation over all other techniques is the increase in stabilization of the protein[25]. A 2-3 M ammonium sulphate suspension of protein precipitates remain stable for years, thus it forms a normal packaging method for many enzymes[25]. Also, high salt concentration prevents proteolysis and bacterial activation[25].

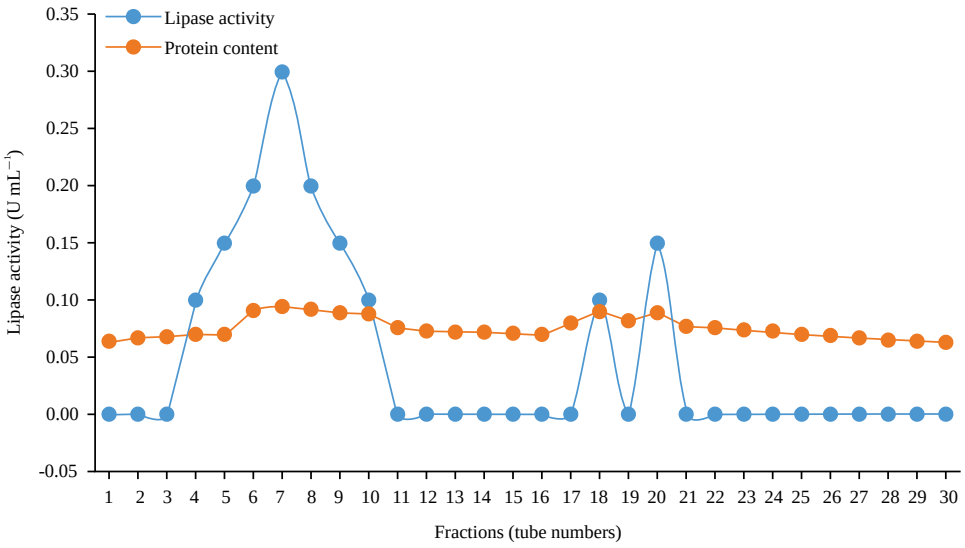

Fig. 2: Separation by Ion-exchange chromatography of high molecular weight proteins (fractions 10-24)

The pooled extract of the enzyme was applied on ion-exchange chromatography CM (1.5×15 cm) pre-equilibrated with 0.2 M phosphate buffer, pH 7.5 at 4°C and eluted step wisely at 10 mL each into 30 fractions (in 30 tubes) using 0-0.5M NaCl-phosphate buffer. Flow rate: 0.5mL min^{-1}

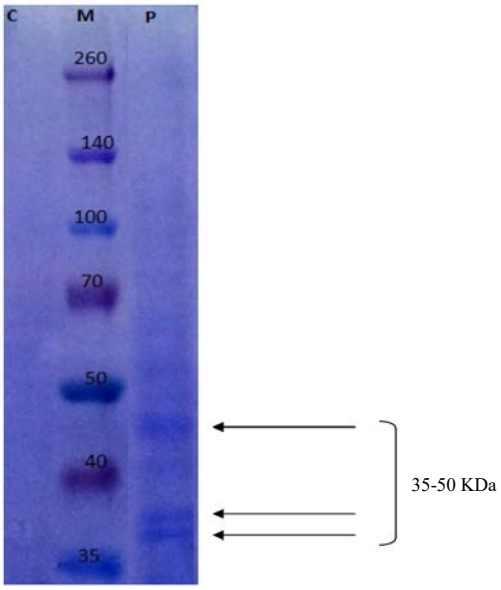

Fig. 3: SDS-PAGE of partially purified lipase

Lanes are as follows: M: Marker proteins with relative molecular masses indicated on the right, P: Lipase of *Pseudomonas fluorescens*

Enzyme precipitation was carried out by ammonium sulphate, both precipitation and dialysis showed increase in purification fold for the enzyme in this investigation. Sephadex G-75 and ion-exchange (CM) chromatography proved to be very effective for lipase activity increasing the purification folds to 3.60 and 8.01 times with specific activity reaching up to 1.43 and 3.18 U mg^{-1}, respectively in this enzyme.

These could be possible probably due to the removal of contaminants such as proteases and cellular debris that could decrease or mask the lipase activity during these purification stages. A purified and concentrated lipase preparation is essential before characterization of an enzyme, since an impure (crude) lipase preparation could pose undesirable and ambiguous results[26]. Purified a lipase observed from *Pseudomonas fluorescens* by ultra-filtration, ammonium sulphate, precipitation, DEAE-Toyopearl 650 M phenyl, Toyopearl 650 M to 6.1 fold and 42% yield. Also, similar to this study a lipase fraction from *P. fluorescens*[27] was obtained with a 10-fold increase in purification.

This study shows that partially purified lipase of *Pseudomonas fluorescens* has an estimated molecular weight range of 35-50 based on the position of the protein marker as shown in Fig. 3. Similar findings were reported[28], strain KE38 of partially purified lipase of *Pseudomonas* sp. was screened to be homogenous on SDS-PAGE gel and its molecular weight was estimated to be approximately 43 KDa based on the position of the protein marker. An extracellular lipase from *Pseudomonas aeruginosa* BN-1 has been reported to have an estimated molecular weight of 60 KDa[15]. The estimated molecular weight of lipase of *Pseudomonas fluorescens* HU 380 to be 64 KDa was also reported[29].

The kinetics, K_m and V_{max} of *Pseudomonas* were obtained from Line weaver-Burke plot of the reciprocal of substrate concentration against that of measured rate of hydrolysis. Results showed that apparent K_m and V_{max} values of the lipase to be 1.25 mg mL^{-1} and 0.702 μg s^{-1} for purified

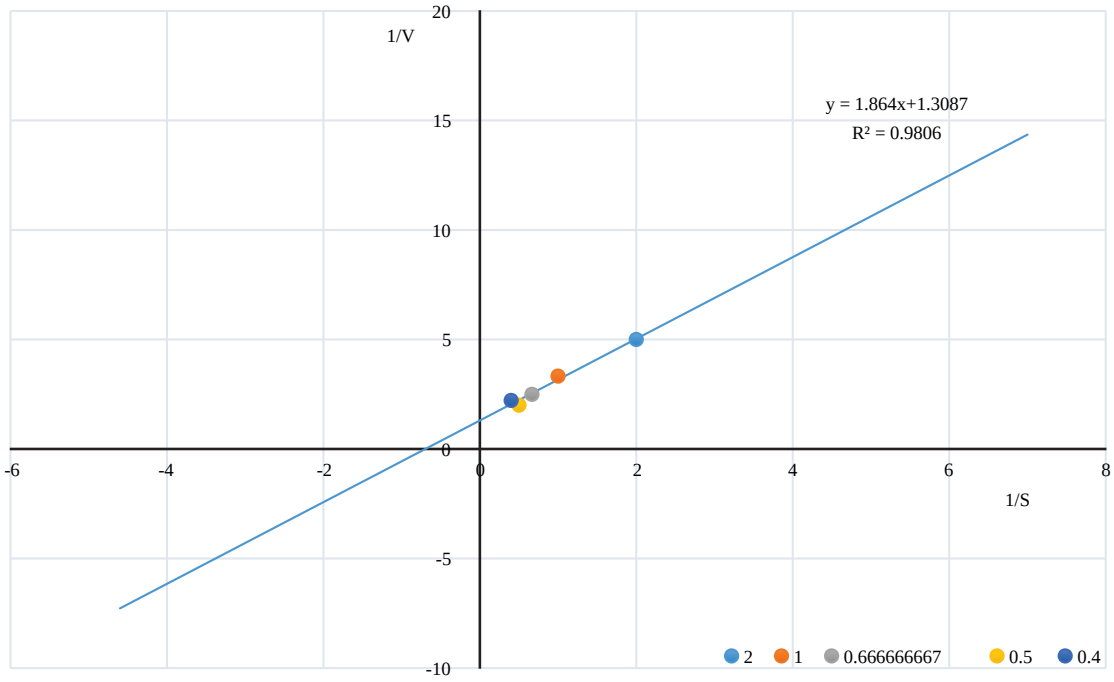

Fig. 4: Line weaver-Burke plot of the reciprocal of lipase activity of *Pseudomonas fluorescens* against that of substrate concentration

Rate of reaction (V) {reciprocal of which is on the Y axis} was examined at different substrate (olive oil) concentration (S) {reciprocal of which is on the X axis}, V was obtained thus: 0.5, 1.0, 1.5, 2.0, 2.5% of olive oil was mixed with 1 mL of the purified lipase respectively, using the standard enzyme assay. Hence, the Michaelis constant (K_m), which is the inverse of the intercept on the X-axis (1.25 mg mL^{-1}) and the maximum reaction velocity (V_{max}), being the inverse intercept on the Y-axis (0.702 µg s^{-1}) of the enzyme were obtained from the line weaver-Burk plot

Pseudomonas fluorescens, K_m value is numerically equal to the substrate concentration at which the half of the enzyme molecules are associated with substrate. It also indicates the affinity of the lipase to the olive oil substrate used, while the V_{max} was determined as the reciprocal of the intercept. The rate of reaction is directly proportional to the concentration of the olive oil. Increase in the concentration of the olive oil enhances the speed of reaction as observed in this study. The $R^2 = 0.9806$ indicates that the regression value is greater than 0.05 (i.e $p>0.05$), hence lipase activity of *Pseudomonas fluorescens* is statistically significant as shown in Fig. 4. The line weaver-Burke plots being linear indicates that hydrolysis of the triglyceride esters by the lipase followed Michaelis-Menten kinetics, in some cases, such as seen here lipases appear to obey Michaelis-Menten kinetics[30,31]. The V_{max} is the maximum rate of reaction and K_m is a measure of the affinity of an enzyme for a particular substrate, a low K_m value represents a high affinity. It has been noted that K_m values of the enzyme range widely, however for most enzymes, K_m ranges from 2-5 mM[32]. This study also corroborates with the previous work[33] who reported the Michaelis-Menten parameters K_m and V_{max} of a purified lipase of *Pseudomonas fragi* CRDA 323 to be 0.7 mg mL^{-1} and 0.97×10^{-3} U min^{-1}, respectively.

CONCLUSION

A purified and concentrated lipase preparation is essential before the characterization of an enzyme. An impure (crude) lipase preparation could pose undesirable and ambiguous results. The enzyme was purified 8.005 –fold from *Pseudomonas fluorescens*, the molecular mass of the partially purified *Pseudomonas fluorescens* lipase was 45 KDa. Enzyme kinetics displayed Michaelis-Menten constant K_m 1.25 mg mL^{-1} and V_{max} value of 0.7 µg sec^{-1}. As observed in this research, this purified enzyme had suitable affinity for the substrate used, hence there is need for the extensive characteristics of this lipase to be checked in its purified form in order to understand enzyme functions better and enhance enzyme production by applying suitable substrate as well as process parameters optimization. Also the need to produce lipase of *Pseudomonas fluorescens* with improved properties by protein engineering to further enhance usefulness of these enzyme in various industrial applications and towards fatty waste treatment.

SIGNIFICANCE STATEMENT

This study discovered that purified enzymes had suitable affinity for the substrate used, which could be beneficial in designing and engineering lipases for specific purposes. This study will help the researchers to uncover the critical areas of creation of tailor-designed enzymes that many researchers are trying to explore. Thus a new theory on protein design complemented with efficient isolation of novel lipase from the metagenome may be arrived at.

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