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Studies on Properties of Lipase Produced from *Aspergillus* sp. Isolated from Compost Soil

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Abstract

Lipase producing *Aspergillus* was isolated from soil collected from a refuse dump site located at Awka, Anambra State, Nigeria using standard microbiology and biochemical techniques. Crude extract of lipase was produced after a successful screening of the isolates using mineral broth containing p-NPP through submerged fermentation system with optimized physiologic conditions. Three steps of purification were carried out: Ammonium sulphate, dialysis and gel filtration (sephadex G-150). Crude extract was precipitated by using 70% saturation of ammonium sulphate at pH 6.0 which gave the optimum precipitation of the protein with specific activity of 260.56 U/mg. Precipitation using ammonium sulphate carried out at pH 6.5 and 8.0 gave specific activity of 217 U/mg of the protein. The precipitates were further desalted through dialysis for twelve hours and specific activity of 343.20 U/mg was recorded from the dialysate afterwards. Further purification was done by using sephadex G-150 and specific activity of 490.55 U/mg was recorded from the active pooled fractions. The purification table showed a 2.32 purification folds of lipase was gotten after gel filtration (sephadex G-150) with a lipase percentage yield of 2.00%. The specific activity of lipase increased from 211.81 to 490.55 U/mg. Characterization of β-galactosidase gave optima pH and temperature of the enzyme at 6.0 and 60˚C respectively. Kinetic constants: K_m and V_{max} values were obtained at various concentrations of p-NPP where 0.32 mM and V_{max} of 200.00 µmol/min respectively. Ca^{2+} and Co^{2+} showed greater effect on lipase activity in a concentration-dependent manner (0.03 - 0.05 M) when compared to Mn^{2+} and Fe^{2+}. The results from this study have shown that lipase produced from filamentous *Aspergillus* has a wide range of activity over physiologic conditions in regards to industrial and clinical standard operational procedures.

Keywords

Lipase, Purification, Specific Activity, Precipitation, Purification Folds

1. Introduction

Lipases, (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are ubiquitous enzymes of considerable physiological significance and industrial potential. They catalyze the hydrolysis of triacylglycerols at oil-water interface [1] to release glycerol and free fatty acids [2].

Lipases are ubiquitous in existence and can be found in plants, animals and microorganisms. There are several studies on lipase production from bacteria, fungi, yeast and the actinomyce strains [2]. Microbial lipases boast of strong advantages to their corresponding sources in terms of high doubling time of organisms, ease of separation during downstream purifications and high yield of the biocatalyst [3] [4].

Purification of lipases from their sources is an important protein chemistry technique used for separation of enzymes from their crude mixtures respectively. Lipase purification takes the basic steps of protein precipitation through common ion effect as described by [5]. Precipitation of extract using salts of different concentration is based on the principle of common ion effect. Salt redistribution in solution induces salting in of macromolecules at its different concentrations and salting at high concentrations [6]. The precipitated proteins are usually dialysed across gradient using a dialysis bag for removal of the appending salts. It is recorded the specific activity (*i.e.* lipase activity per milligram protein) of purified lipases increase in folds as the purification step progresses. Further steps in purification involve ion exchange using exchange resins and size exclusion chromatography [3].

Lipases as stated are important clinical and biotechnological bias enzyme with wide need of bio utilizations. Purification of proteins and enzyme characterizations is though a cost implicative aspect in production and application of enzymes, lipases having seen their application in both industries, clinical and other biotechnological bias institute require to undergo some level of purification [7]. These take care of elimination of various isoforms of the enzyme thereby allowing only the desired protein to be studied and utilized. Upon doing this, it will be of noteworthy that pure lipase with identified properties will allow the application of molecular technique to harness their potentials and improvement of their activity [8].

2. Material and Methods

2.1. Materials

All chemicals/reagents and equipments used in the present were of analytical grade and products of the following companies: BDH chemical limited (Eng-

land), Merck (Germany), May and Baker limited (England), Riedel-DeHaen Hannaves (Germany), Hopkins and Williams Essex (England), Fluka chemical company (Germany), Kermel chemicals (China) and Lab. Tech Chemicals, Avighkar (India). The reagents which include basically: acetate, p-NPP, BSA, folin ciocateau, other buffer salts and corresponding acid and bases, tween 80, sephadex G-150, ammonium sulphate, chlorides of Ca, Co, Mg, Fe, Mn were prepared at each use; the equipments were basically: UV-VIS spectrophotometer, water bath, pH meter, vortexed machine, column units were calibrated at each use.

2.2. Methods

2.2.1. Sample Collection

Soil from refuse dump site was collected from three ranges of refuse dump sites within Awka metropolis, Anambra state, Nigeria stratifiedly as described by [9]. The collected soil was taken to the laboratory in a clean sterile sample bottle for further analysis.

2.2.2. Isolation and Identification of Strains of *Aspergillus* sp. from the Collected Soil Sample

Strains of the fungi (*Aspergillus* sp.) was isolated from the soil using standard microbiology (culturing and microscopy mounting) and biochemical (sugar fermentations, nitrogen digestions) technique as described by [10].

2.2.3. Assay Protocol for the Enzyme Activity

Lipase activity was determined by the colorimetric method according to [11] [12]. This method was based on the cleavage of p-nitrophenylpalmitate (p-NPP). 0.5 ml of lipase was pippetted using a micropipette and mixed with 1 ml of sodium acetate buffer solution of pH 5.0, the solution is rapidly mixed with 0.5 ml of 2 mM of p-NPP and incubated at 50˚C for 30 min.

Protein Determination

The total protein content of the enzyme was estimated as described by [13] using bovine serum albumin (BSA) as the standard protein. Absorbance was taken at 750 nm.

2.2.4. Enzyme Production

Submerged fermentation technique was used for lipase production as described by [4]. Production set-ups contain 100 ml of liquid media optimized for lipase production contained: 1% $(NH_4)SO_4$, 0.4% K_2HPO_4, 1% sugarcane baggase, 0.2 ml tween 80, 0.01% sodium acetate, 01% di-ammonium citrate, 0.05% $MgSO_4{\cdot}7H_2O$, 0.2% $MnSO_4{\cdot}4H_2O$ were incubated at pH 6.0 and at room temperature (37˚C) for seven [7] days. The whole setups were sterilized at 121˚C/15psi for 20 minutes using the electronic autoclave.

2.2.5. Ammonium Sulphate Precipitation Profiling

This was carried out as described by [14]. Test tubes numbering eight were used

to form the ammonium sulphate precipitation profile. Lipases were precipitated by gentle stirring at 20% - 90% saturation of solid ammonium sulphate at intervals of 10% in each test tube. Lipase activity of the precipitates and the supernatants were assayed simultaneously as described above to determine the percentage ammonium sulphate saturation that precipitated the enzyme from the solution.

1) Dialysis of the Precipitated Proteins

Dialysis of the precipitated protein across gradients was carried out as described by [14].

2) Gel Filtrations

This was carried out as described by [15]. Sephadex G-150 used as the stationary phase for the purification was gently packed in the column. The protein concentration of each fraction was determined using a spectrophotometer at wavelength of 280 nm. Lipase activity of each fraction was assayed using the spectrophotometer at wavelength of 400 nm as described above.

2.2.6. Studies on Partially Purified Enzyme

1) Effect of pH on Lipase Activity

The optimum pH for lipase activity was determined using 0.2 M sodium acetate buffer of pH ranging from 3.5 - 5.5, sodium phosphate buffer of pH 6.0 - 7.0 and Tris-HCl buffer of pH 7.5 - 9.0 at intervals of 0.5 units as described by [14]. Lipase activity was assayed as described above.

2) Effect of Temperature on Lipase Activity

This was carried out as described by [14]. Lipase activity was assayed as described above.

3) Effect of Substrate Concentration on Lipase Activity

This was carried out as described by [15]. The V_{max} and K_m values of the enzyme were determined using the double reciprocal plot. Activity of lipase was determined as described in above.

4) Effects of Divalent Metal Ions on the Activity of Lipase

This was carried out as described by [16]. Chloride salts of different divalent metals: Calcium (Ca), Manganese (Mn), Magnesium (Mg) and Cobalt (Co) of concentration 30 - 50 mM respectively were incubated with the enzyme at their optimal pH and temperature; activity of the enzyme at each treatment with the metal salts was determined as described above.

3. Results

Plate one below shows the pure mycelia growth of *Aspergillus* sp on a Potato dextrose agar prepared plates respectively. From the plates it was visible their variant filamentous colours.

Ammonium sulphate precipitation of the crude lipase from the solution showed peak precipitation of the enzyme with 70% ammonium sulphate saturation (**Figure 1**).

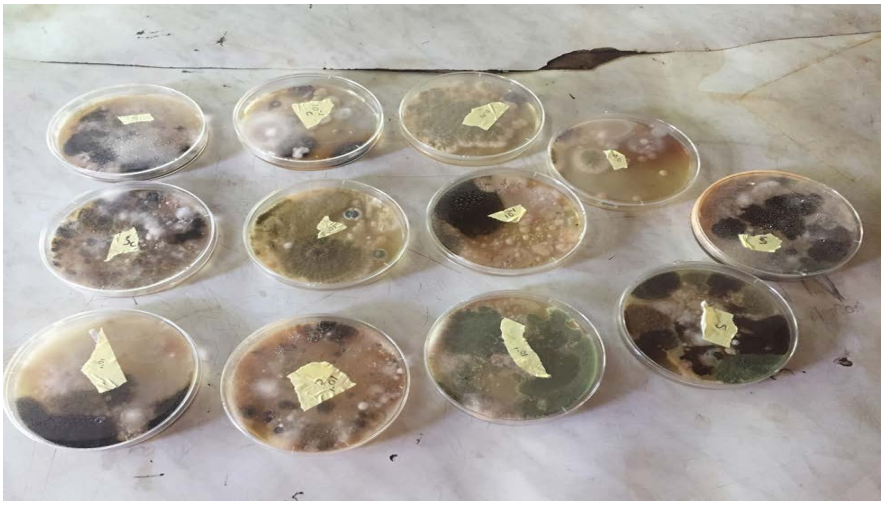

Figure 1. Strains of *Aspergillus* sp. on PDA plate.

Chromatogram of Lipase

Studies on gel elution chromatogram of the proteins using sephadex G-150 of bed height 75 and column volume of 235.65 cm^3; Figure 4, one distinct peak from the chromatogram of lipase from strains of *Aspergillus*; activity of protein was observed from tubes number 18 - 28 before a drop in activity of the enzyme. Protein concentrations of the enzyme showed single peak from the elution profile from tubes number 22 - 40. Void volume (Vo) of the elution profile was recorded from tube number 0 - 17.

Purification Table of the Protein

Crude protein from strains of *Aspergillus* was purified upto 2.32 folds after gel filtration (sephadex G-150) with percentage yield of 2.00%. The specific activity of lipase increased from 211.81 to 490.55 U/mg after gel filtration (Table 1).

Characterization of Lipase from *Aspergillus* sp.

4. Discussion

Enzymatic properties of a biocatalyst are integral parameter to be considered in the choice and application of enzymes in various fields of biotechnological bias usages. Stable enzymes with wide stability over physiologic conditions are most prime choice catalyst by protein chemists. Lipases, (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are ubiquitous enzymes of considerable physiological significance and industrial potential [1].

Soil from refuse dump site within Awka, Anambra state, Nigeria showed high heterotrophic microbial diversity and activity. Among the microbes isolated fungi kingdom was optimal in diversity. Strains of *Aspergillus* showed greater microbial presence among all other microbes on the plated potato dextrose agar media. Basic morphological and Biochemical screening were used to identify the isolate as an *Aspergillus* sp (Figure 1 and Figure 2). Strains of *Aspergillus* isolated from the refuse dump soil showed the ability to produce lipase when screened with chromogenic substrates (p-NPP).

Table 1. Purification profile of lipase from strains of *Aspergillus* sp.

Lipase from *Aspergillus* sp.	Volume (ml)	Protein (Mg)	Total protein	Activity µmol/min	Total activity U/ml	Specific activity U/mg	Purification folds	Percentage yield
Crude enzyme	1000	0.52	520	110.14	110,140	211.81	1	100
Ammonium sulphate precipitation	200	0.398	79.6	86.49	17,298	217.31	1.03	15.71
Dialysis	80	0.233	18.64	79.96	6396.8	343.20	1.62	5.80
Gel filtration (G-100)	40	0.110	4.56	53.96	2098.4	490.55	2.32	2.00

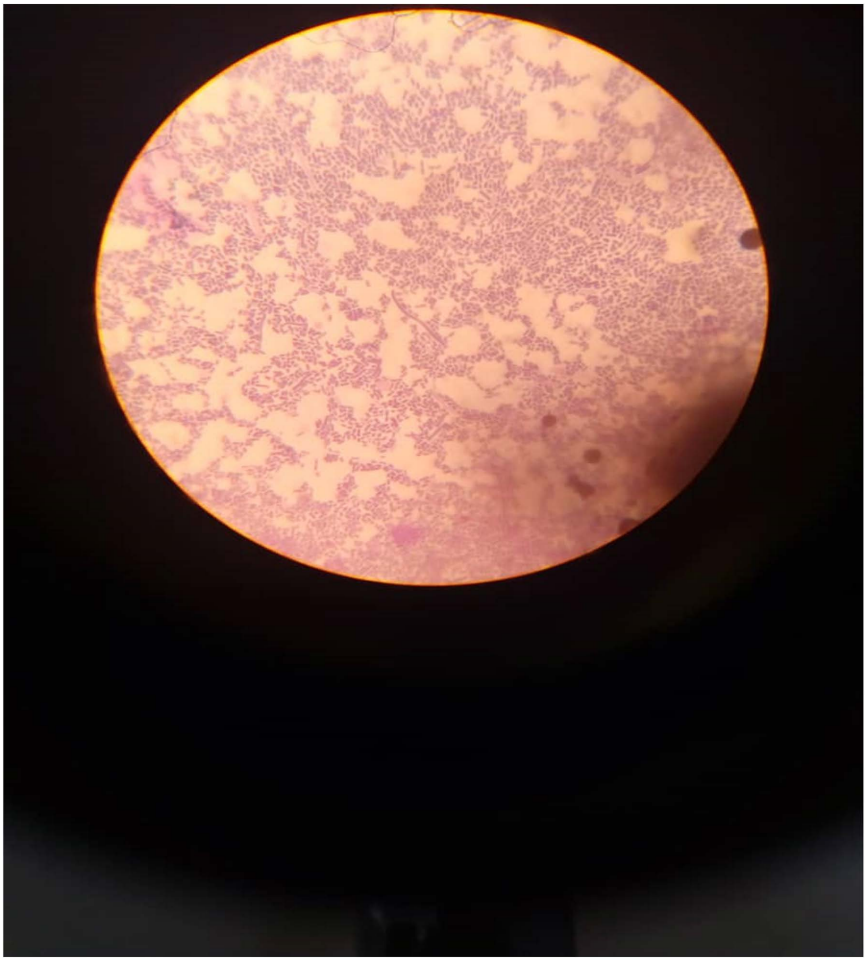

Figure 2. Micrograph of the identified *Aspergillus* strain under the objectives of ×100.

In this present study, at pH 6.0, 70% ammonium sulphate saturation was found suitable to precipitate protein with highest lipase activity (217.31 U/mg) (**Figure 3**). [15] reported an optimal precipitation of lipases from microbial sources at 60% saturation of ammonium or calcium sulpahte and at pH 7.0.

The precipitated protein was de-salted for 12 hours using 2 mm dialysis bag in an ice pack container. After the process, 20 ml of desalted protein was recovered after initial 10 ml was introduced into the bag with increased specific activity of 343.20 U/mg.

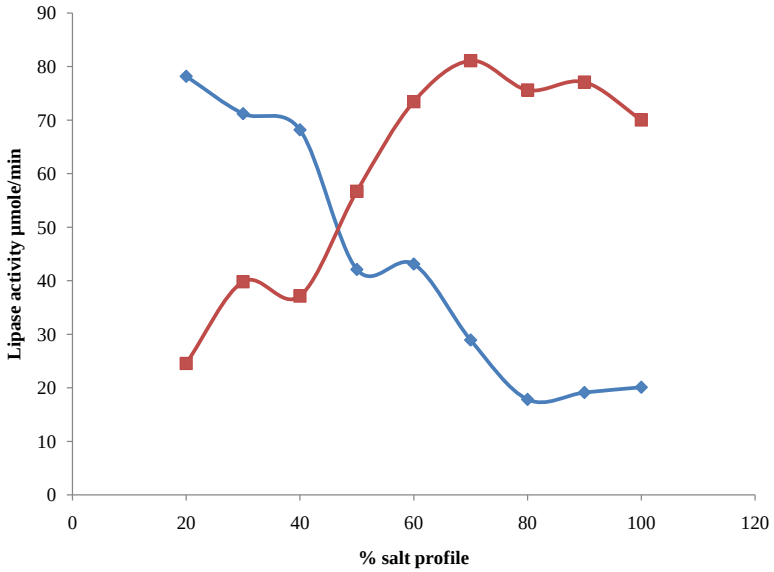

Figure 3. Ammonium sulphate precipitation profile of lipase from strains of *Aspergillus* sp.

Sephadex G-150 was used for further purification of the protein to various molecular sizes and weight. Elution was done with 0.1 M of sodium phosphate buffer at pH 6.0. One distinct peak was seen from the chromatograms respectively on tubes number 18 - 29 and 22 - 39 before a drop-in activity (**Figure 4**). Void volume (Vo) of the elution profile was recorded from tube number 0 - 17 and 0 - 21 respectively which approximately represent one-third of the column volume.

As reported by [14], they stated that there appears to be a relationship between dialysis of enzymes usually after ammonium sulpahte precipitation and the presence of isoenzymes, they went further to state that when dialysis is replaced by gel filtration in enzyme purification, isoenzymes were lost out. Multiple peaks of the enzyme activity could be attributed to ionic scrambling encouraged by dialysis and this leads to formation of aggregates with incorrect ionic bond pairs.

The specific activity of the lipase increased from 211.81 to 490.55 U/mg, respectively after gel filtration **Table 1**). The specific activity of the enzyme increased from 175.78 to 604.20 U/mg after gel filtration (**Table 1**). [15] reported specific activity of 568.61 U/mg and purification folds of 21.2 after gel filtration of *β*-galactosidase produced from *Lactobacillus acidophilus* isolated from fermented ragi.

Optimum pH for lipase produced from *Aspergillus* sp. was 6.0 (**Figure 5**). [17] reported optimum pH of 8.0 for isolated from bacteria strains isolated from a local palm oil processing site while [15] reported an optimum pH of 7.0 for lipase produced from coconut seeds under different production conditions.

Optimum temperature for lipase produced from strains *of Aspergillus* is 60˚C (**Figure 6**). Reports from [5] stated an optimum temperature of 50˚C for lipase produced from seeds of *Jatropha cucas*.

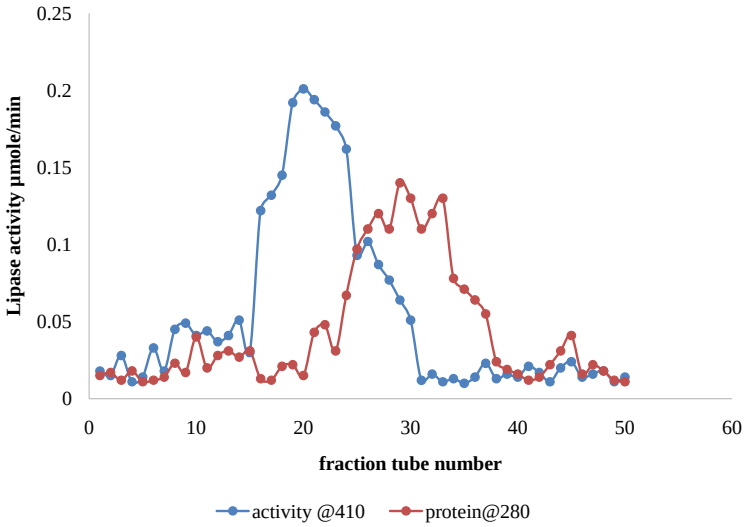

Figure 4. Chromatogram of lipase produced from *Aspergillus* on sephadex G-100.

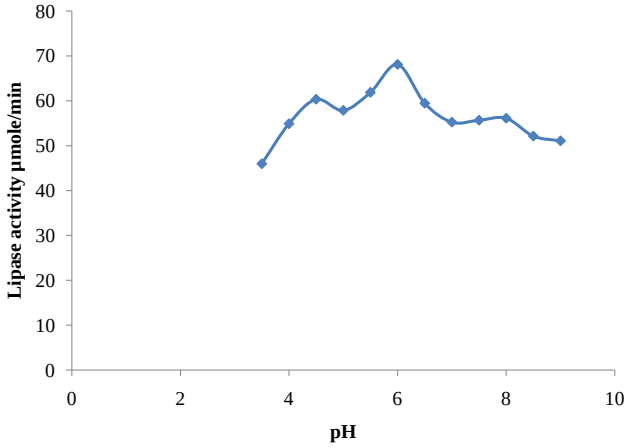

Figure 5. Effect of pH on lipase activity.

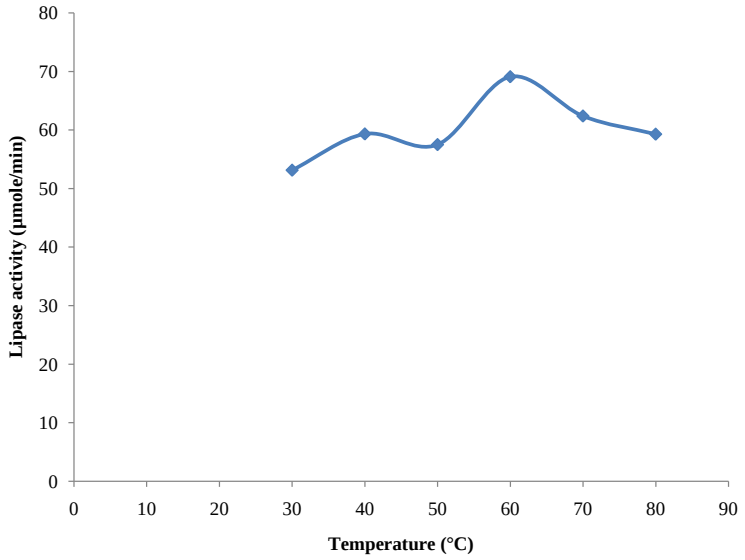

Figure 6. Effect of temperature on lipase activity.

Substrate specificity determination of lipase showed that p-NPP gave better kinetic properties of the enzyme. Kinetic constants (K_m and V_{max}) of lipase determined during the study showed K_m of 0.32 (**Figure 7**). Velocity maximal (V_{max}) which shows the catalytic efficiency (rate of turnover) of lipase during catalysis shows V_{max} of 200 (**Figure 8**). [8] reported K_m and V_{max} of 1.94 mM and 255 µmole/min respectively for lipase activity at various concentrations of p-NPP.

Metal ions generally play important roles in the biological function of many enzymes (both clinical and industrial implicated ones) [16]. The various modes of metal-protein interaction include metal-, ligand-, and enzyme-bridge complexes. Metals can serve as electron donors or acceptors, Lewis acids or structural regulators (chelators) [16]. Divalent metals: Ca^{2+}, Mn^{2+}, Fe^{3+} and Co^{2+} were positive effectors to the enzyme activity relative to the control experiment (metal ions blank). Iron (Fe), Calcium (Ca^{2+}) and Cobalt ions (Co^{2+}) showed greater chelating effect to the enzyme activity when compared with the control than Mn^{2+} to the activity of the enzyme in a concentration dependent rate (0.03 - 0.05 M) (**Figure 9**).

[13] reported that differential effect of the metals on enzyme activity is attributed to spinning of electrons to the valence shells of the metals. He went further to state that paramagnetic spinned electron shell metals (especially transition metals) give more regulatory and activation effect in every enzyme/non enzymatic catalyzed reactions with respect to their ease to donate and accept (chelation power) electrons, radicals and charges during the mediated reactions. [1] reported an increase in activity of lipase produced from *Candida rugosa* in the presence of Ca^{2+} and Mn^{2+} at concentration of 50 mM respectively.

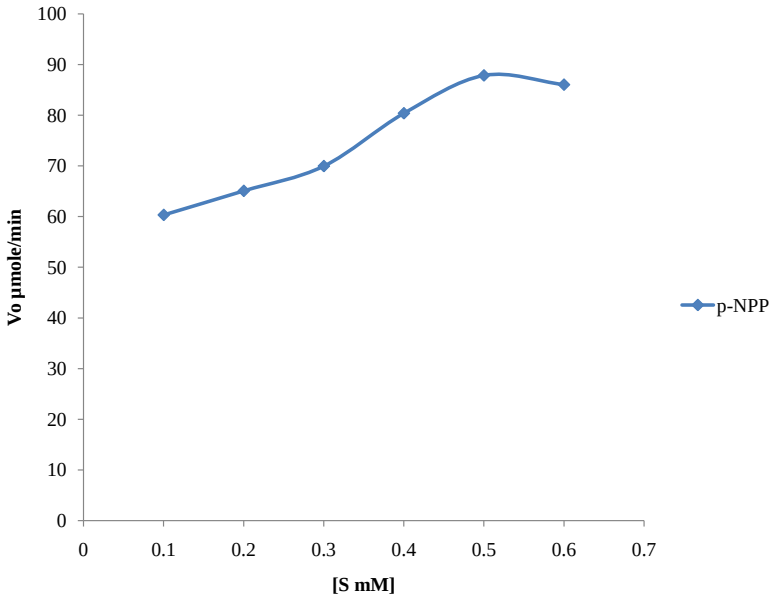

Figure 7. Michaelis-menten plot of initial catalytic activity of lipase from strains of *Aspergillus* sp.

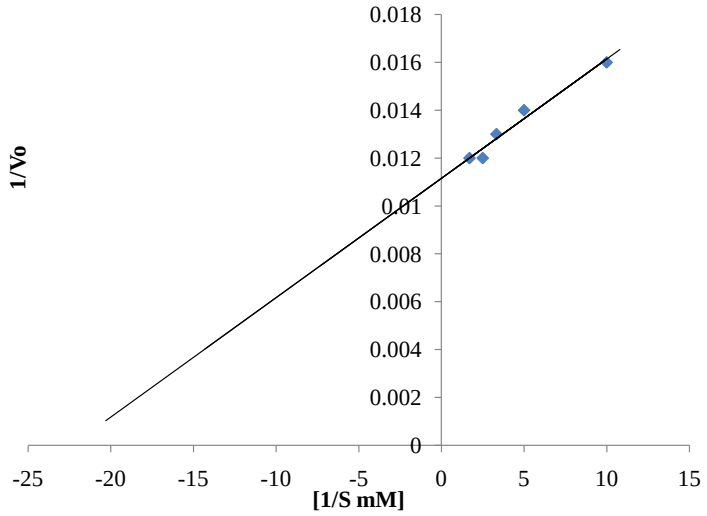

Figure 8. Linear weaver-burke plots of Lipase activity at different concentration p-NPP produced from strains of *Aspergillus* sp.

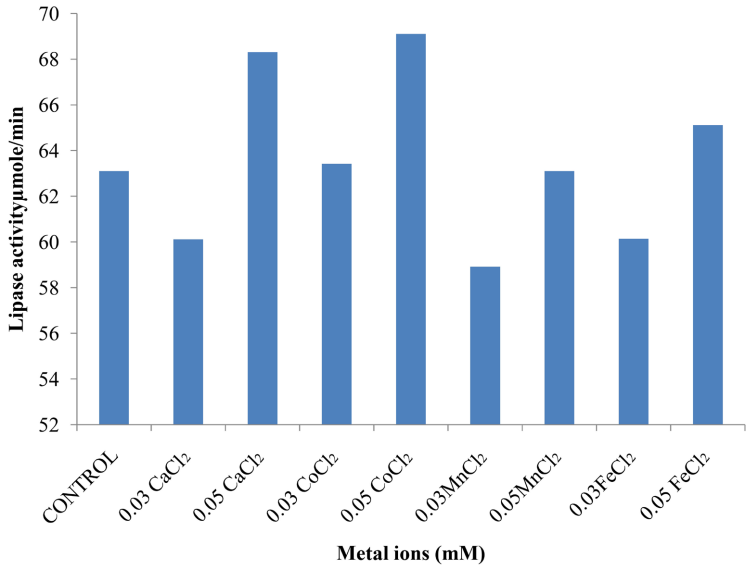

Figure 9. Effect of Divalent metal ions on Lipase from strains of *Aspergillus* sp. at various concentrations of the metal ions.

5. Conclusion

Effective utilization of biocatalyst largely depends on their specific activity quotients and purification folds clarity. Pure enzymes are scarcely available due to the cost implications associated with the down-stream processing of enzymes from their crude solutions. Lipase is a multi-purpose enzyme that has found its way into many producing industries including those of foods, clinical and environmental-based research industries. The present study has shown the purification properties of the enzyme through precipitation using ammonium sulphate, dialysis and chromatographic techniques (ion exchange and gel filtrations). There is an appreciable increase in the specific activity of lipase per its protein

concentration as the purification progresses with minute loss in the yield of the enzyme. Stability of the pure lipase is a promising adventure in utilization of the biocatalyst for various endeavours.

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Author's Contributions

Ezenwelu Chijioke O. (PhD): Conceived and designed the experiments, performed the experiment and processed the data, analyzed the data and wrote the manuscript.

Obiano Anthony Ugochukwu: Revised the manuscript and performed the experiment.

Okoro Promise Nzubechi: Performed the experiment and guided the experimental design.

Oladejo Afeez A.: Guided the experimental design and processed the data.

Ude-ezika Mmmezoma A.: Guided the experimental design.

Oparaji, Emeka H.: Processed the data and wrote the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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