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Research Article

# Purification Profiling and Kinetic Properties of Tri –Responsive Enzymes from Hydrocarbonoclastic Bacteria

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## Abstract

Purification is though a cost implicative aspect in production and application of enzymes. Lipase, urease and peroxidase were produced from strains of Pseudomonas isolated from a crude oil polluted soil using standard microbiology and biochemical techniques. The stress responsive enzymes were produced through submerged fermentation technique. Crude extracts were precipitated at physiologic pH show the crude extract precipitation of lipase with maximum protein precipitation at 70% saturation of the ammonium salt, precipitation of the crude extracts of urease with maximum protein precipitation at 60% saturationof the ammonium salt while precipitation of the crude extracts of peroxidase with maximum protein precipitation at 50% saturation of the ammonium salt. Crude protein of lipase was purified upto 2.32 folds after gel filtration (sephadex G-100) with percentage yield of 2.00%.The specific activity of lipase increased from 211.81 to 490.55 U/m g after gel filtration. Crude urease was purified up to 3.20 folds after gel filtration (sephadex G-100) with percentage yield of 2.30%. The specific activity of urease increased from 170.56 to 546.88U/mg after gel filtration. Crude protein extract of peroxidase was purified upto 2.8 folds after gel filtration (sephadex G-100) with percentage yield of 2.20%. The specific activity of peroxidase increased from 215.38 to 602.20U/mg after gel filtration. lipase, urease and peroxidase activity showed optimum activity at pH of 6.0; 7.0 and 4.5 respectively while 50 ℃;60 ℃ and 40 ℃ were optimum activity of lipase, urease and peroxidase respectively. Michaelis-menten constant (KM) and maximal velocity of catalysis (Vmax) of 0.32mM and 200 µmol/min were obtained at different concentrations of p-NPP for lipase; whereas KM of 0.370mM and velocity maximal of 197.27µmol/min were obtainedat different concentrations of standard urea for urease and Michaelis-Menten constant (Km)and maximal velocity(Vmax) of 3.45mM and 270µmol/min were obtained at different concentrations of o-dianisidine hydrogen peroxide standard substrates for peroxidases. The purification and kinetic properties of enzymes defines their much physiologic stability and efficient catalytic profile for wide application.

## Keywords

Purification, Kinetic Properties, Specific Activities, Precipitation

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# 1. Introduction

Enzymatic properties of biocatalysts are imperative parameters to be considered in bioutilizations of catalysts. Purification of enzymes from their sources is an important protein chemistry technique used for separation of enzymes from their crude mixtures respectively [1-10].

Enzymes are ubiquitous in existence and can be found in plants, animals and microorganisms. There are several studies on enzymes production from bacteria, fungi, yeast and the actinomyce strains [12, 39]. Microbial enzymes with its technologies 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 [14-18, 39].

Housekeeping enzymes, acyl hydrolyases, and nitrification enzymes are examples of signaling protein responses in an ecology known as stress marker proteins. Peroxidases are widely dispersed in plants, animals, and microbes. They protect essential cells and organelles from the deadly effects of oxidative stress, which leads to the death of the affected cells or organelles due to the release of $H_2O_2$ [20, 23, 24]. Lipases, (triacyl glycerol 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 [28] to release glycerol and free fatty acids [3]. In contrast to esterases, lipases are activated only when adsorbed to an oil–water interface [16, 30, 32, 38, 40] and on of hydrolyzed is solved substrates in the bulk fluid. Ureases are *in-situ* inductive enzymes that acts on nitrogenous matters present in organic matter with the release of ammonia and as such sustains the nitrogen cycle of food chains [35].

Since lipases, peroxidases, ureases, and other enzymes are used in both factories and clinical and other biotechnological bias institutes, purification is a costly part of their manufacturing and use. These take care of removal of numerous isoforms of the enzyme so allowing only the required protein to be examined and utilized [11, 20]. Upon doing this, it will be of noteworthy that pure lipase will allow the application of molecular technique to harness their potentials and improvement of their activity.

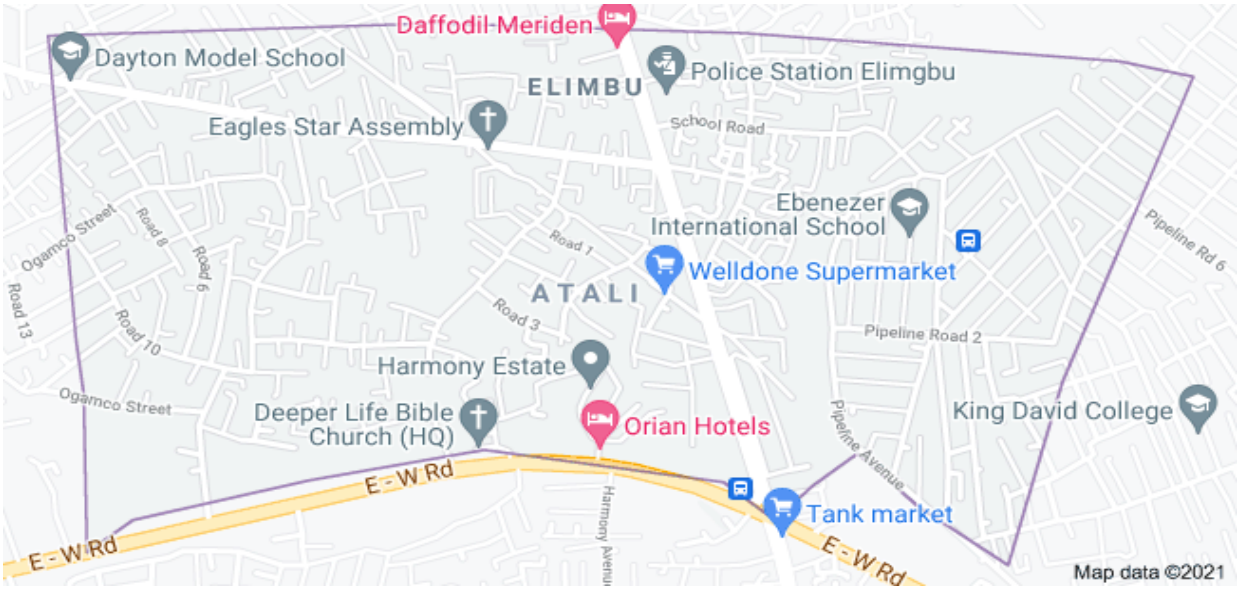
# 2. Materials and Methods

## 2.1. Materials

Materials including those of equipments, apparatus and reagents utilized during the course of the present study were of products of designated companies like BDh, Bristol, May and Bakers; the equipments were calibrated at each use and in good working condition. The reagents were analytical graded and properly stored.

### 2.1.1. Soil Sample Collection

Soil samples were collected from petroleum drilling sites located at Atali (Figure 1) (LONG.7°03",25°.2" E;LAT.4°51",53°.03"N). Atali was known with illegal pipeline bunkering activities by oil hooligans. The samples were collected as described by [22] from the stated areas at 6:00 am in the morning using a spade. The collections were done at the four perimeters of the marked soils with average of 9m apart from each other.




Source: [18, 19, 33].

***Figure 1.*** *Map of Atali oil bunkering site in Rivers state, Nigeria.*

### 2.1.2. Microbial Isolation from the Polluted Soil

Microbial ecology of the polluted soil was determined according to the method described by [21, 23-29]. From the isolates, strains of the bacteria were identified using standard microbiology and biochemical techniques.

## 2.2. Methods

*Enzyme Production*

Submerged fermentation system was utilized for the enzyme production as described by [41]. The conical flasks(250ml)were supplemented with nutrient media necessary for protein production whereas optimized conditions for peak production were employed during the fermentation process. The whole-setups were sterilized at 121 ℃/15 psi for 20 minutes using the electronic autoclave.

*Determination of Enzyme Activity*

Activity of peroxidase was monitored using o-dianisidine and hydrogen peroxide at spectrophotometric wavelength of 460nm as described by [20].

Lipase activity was determined using the colorimetric method as described [22] using p-nitrophenylpalmitate (p-NPP) as the standard substrate.

Urease activity in the soil will be determined in the presence of standard urea as described by [15].

*Purification of the Enzyme Crude Extracts*

Crude extracts of the enzymes will be precipitated using different saturations of ammonium sulphates and thereafter desalted against concentration gradients using dialysis bag as described by [20]. The dialysate will be loaded on tosephadexG100 packed column.

Protein concentration will be determined using [31] method with BSA as the standard protein.

*Characterization of Peroxidase and Urease Activity*

Optimum pH and temperature of the enzymes will be determined at different pH (3.0-8.5) using 0.1m buffers (sodium acetate, phosphates and tris-HCl salts) and water bath (30-80 ℃) as described by [42]. The effect of substrate concentrations on the activity of enzymes were determined by incubating the enzyme with 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 0.9mM of O-dianisidine (peroxidase), and 0.1, 0.5, 1.0mg/lof urea (urease) solution. The $V_{max}$ and $K_m$ values of the enzyme were determinedusing the double reciprocal plot as described by [22].

## 2.3. Statistical Analysis

Data obtained are expressed as mean±SD. Microsoft excel software was employed for analysis of the enzyme s mean activities.

# 3. Results

*Microbial Isolation and Biochemical Characterization*

Heterotrophic counts of the total viable cells and coliform numbers of the bacteria and hydrocarbon utilizing bacteria as shown in Table 1.

Pure colonies of *Pseudomonas* sp. were obtained using differential nutrient culture medium from the polluted soil. From the cultured plate, distinct colonies of the organisms were observed with total heterotrophic number of $3.9x10^8$CFU/ml (Figure 2).

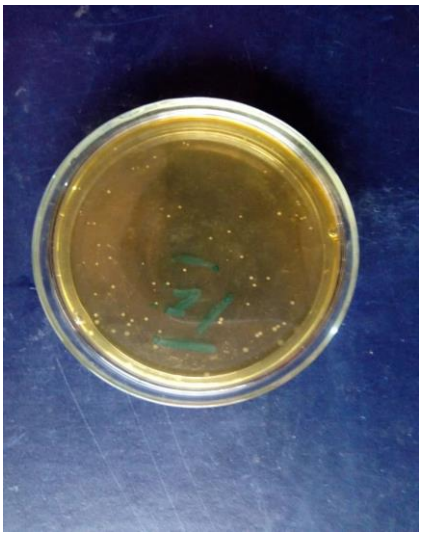


***Figure 2.*** *Pure colony of strains of Pseudomonas sp. on nutrient media plate.*

***Table 1.*** *Heterotrophic Counts (CFU/ml) of the bacteria.*

| Coliform counts | ControlAtali | H-C Bacteria |
|---|---|---|
| Total Viable Counts ($10^{-2}$) | $2.7X10^5$ $4.5x10^7$ | $2.65x10^3$ |
| Total Coliform Counts ($10^{-2}$) | $0.6x10^2$ $1.2x10^3$ | |

Among the microbes isolated from the crude oil polluted soil, bacteria kingdom was optimum in diversity.Total heterotrophic counts of 4.5 x $10^7$ CFU/g bacteria on nutrient media plate and that of $2.65x10^3$CFU/g on differential media of BushNellHaas

Agar of the bacteria isolate were identified on media plates respectively plated from dilution factor of $10^{-2}$ folds.These finding showed a relatively higher correlation with that of Corral *et al.*(2006)on ecological relevance in whey surge polluted water. They reported heterotrophic microbial population of $3.5x10^{6}$CFU/g bacteria on nutrient media plate and that of $2.0x10^{3}$CFU/g on differential media of BushNellHaas Agar.

Basic morphological and biochemical screening were used to identify the isolate as *Pseudomonas*. Basic morphological features of the bacteria showed that strains of *Pseudomonas* under an objective magnification of x40, showed clusters of rod shaped, non-sporulating cream coloured non-motile bacteria cells in a lactophenol blue stained background bacteria; biochemically, they are obligate gram-negative, non-starch hydrolyzing, catalase (+), oxidase (+) and lactic acid forming organisms with optimum growth at temperature of 25-40 ℃.

These findings corroborate with that of basic manual for organisms isolations and identifications written by Ezeonu*et al*. (2013) was used to identify the pure isolates of *Pseudomonas.*

# 4. Protein Purification

## 4.1. Ammonium Sulphate Precipitations

Figure 1, Figure 2 and Figure 3 showed the precipitation profile of the crude extracts of lipase, urease and peroxidase from the solution using ammonium sulphate salt of various percentages forming the precipitation profile. Crude extracts were precipitated at physiologic pH of optimum production Figure 1 show the crude extract precipitation of lipase with maximum protein precipitation at 70% saturation of the ammonium salt, Figure 2 show the precipitation of the crude extracts of urease with maximum protein precipitation at 60% saturation of the ammonium salt while Figure 3 show the precipitation of the crude extracts of peroxidase with maximum protein precipitation at 50% saturation of the ammonium salt.

## 4.2. Gel Chromatography Purification of the Protein

Figure 4, 5 and 6 show the gel chromatogram of lipase, urease and peroxidase using sephadex G-100 of bed height 75 and column volume of 235.65$cm^3$;from the figure, proteins eluted from the column from fraction tube number14-22forlipase,10-18 for urease and 11-19 for peroxidase.

## 4.3. Purification Table of the Protein

Crude protein of lipase was purified upto 2.32 folds after gel filtration (sephadex G-100) with percentageyieldof2.00%.The specific activity of lipase increased from 211.81 to 490.55U/mg after gel filtration (Table 2). Crude protein urease was purified upto 3.20 folds after gel filtration (sephadex G-100) with percentage yield of 2.30%.The specific activity of urease increased from 170.56 to 546.88U/mg after gel filtration (Table 3). Crude protein extract of peroxidase was purified upto2.8 folds after gel filtration (sephadexG-100) with percentage yield of 2.20%. The specific activity of peroxidase increased from 215.38 to 602.20U/mg after gel filtration (Table 4).

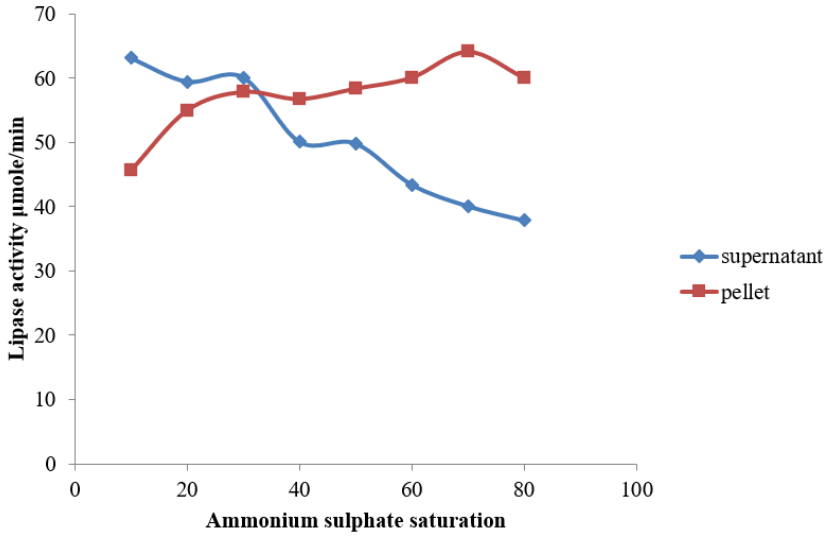



***Figure 3.*** *Ammonium sulphate precipitation profile of lipolytic enzyme from strain of Pseudomonas sp.*

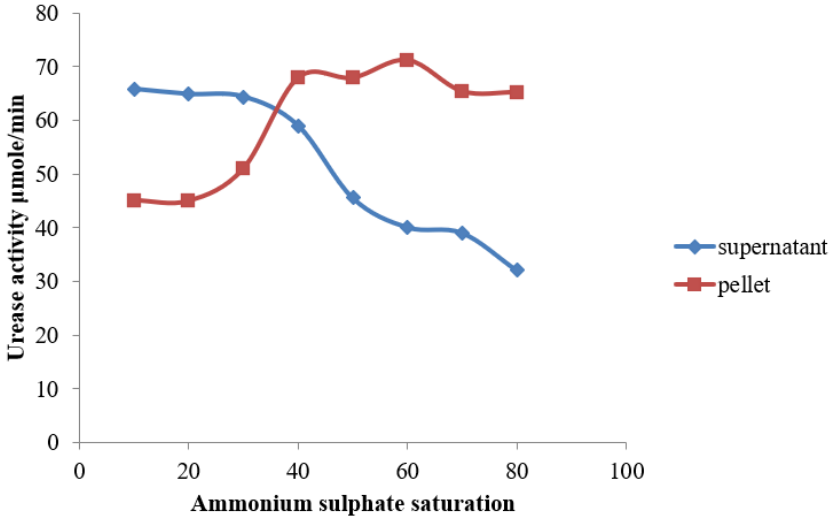



***Figure 4.*** *Ammonium sulphate precipitation profile of crude extracts of Urease from strain of Pseudomonas sp.*

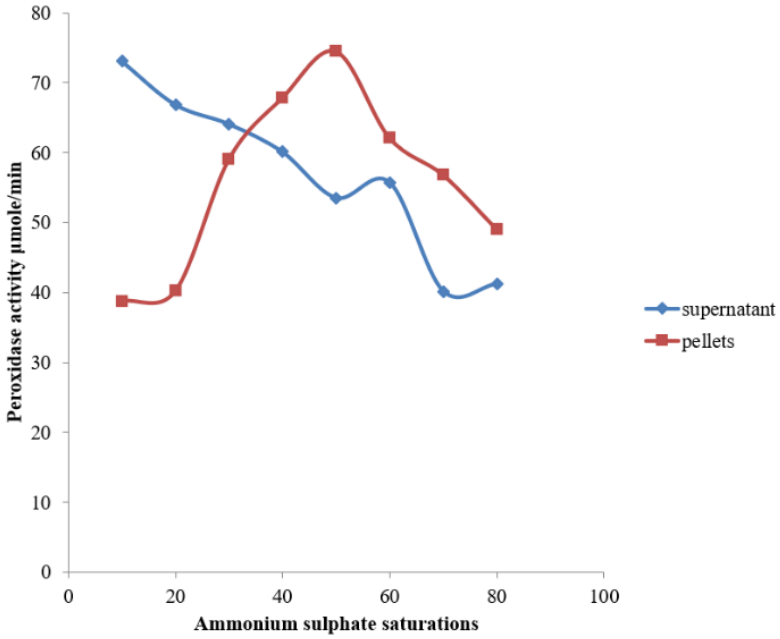



***Figure 5.*** *Ammonium sulphate precipitation profile of crude extracts of peroxidase from strain of Pseudomonas sp.*

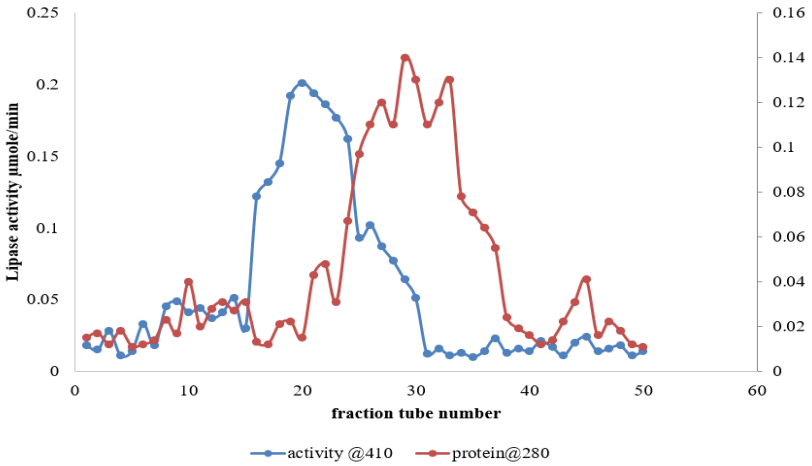



***Figure 6.*** *Chromatogram of Lipase produced from strain of Pseudomonas on sephadex G-100.*

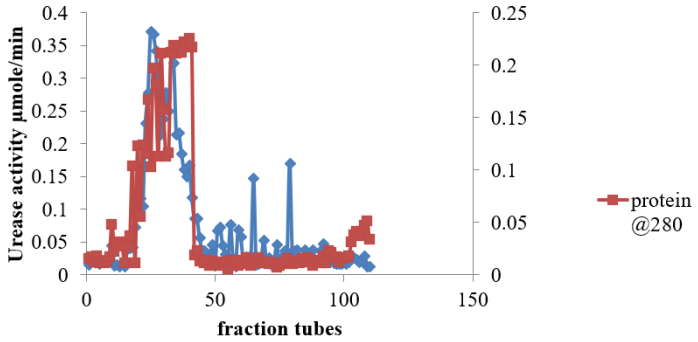



***Figure 7.*** *Chromatogram of Urease produced from strain of Pseudomonas on sephadex G-100.*

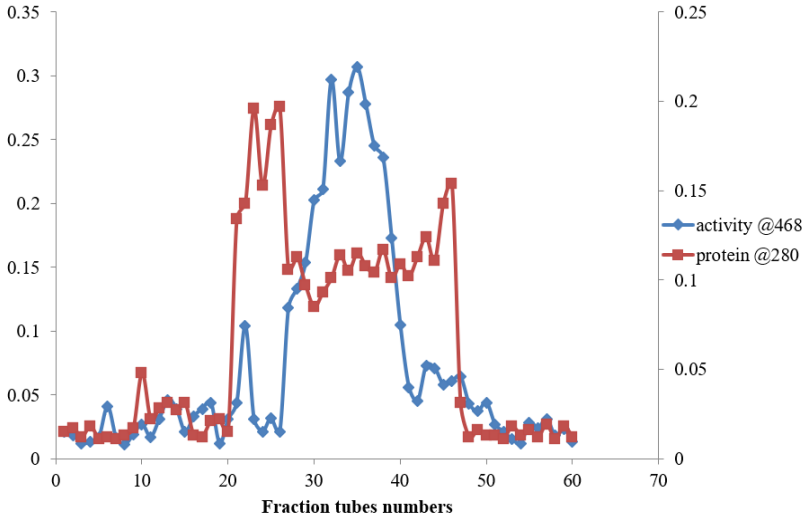



***Figure 8.*** *Chromatogram of Peroxidase produced from strain of Pseudomonas on sephadex G-100.*

***Table 2.*** *PurificationTable of Lipase from strain of Pseudomonas.*

| Lipase | Vol-ume(ml) | Pro-tein(Mg) | Total protein | Activi-tyµmol/min | Total activ-ityU/ml | Specific ac-tivityU/mg | Purifica-tion folds | Percentage yield |
|---|---|---|---|---|---|---|---|---|
| Crude enzyme | 1000 | 0.64 | 640 | 109.16 | 109160 | 170.56 | 1.00 | 100 |
| Ammonium sulphate precipitation | 250 | 0.401 | 100.25 | 81.49 | 20372.5 | 203.22 | 1.19 | 18.66 |
| Gel filtration(G-100) | 45 | 0.104 | 4.68 | 56.46 | 2540.7 | 546.88 | 3.20 | 2.3 |

***Table 3.*** *PurificationTable of Urease from Strain of Pseudomonas.*

| Urease | Vol-ume(ml) | Pro-tein(Mg) | Total protein | Activi-tyµmol/min | Total activi-tyU/ml | Specific ac-tivityU/mg | Purifica-tion folds | Percentage yield |
|---|---|---|---|---|---|---|---|---|
| Crude enzyme | 1000 | 0.52 | 520 | 110.14 | 110140 | 211.81 | 1 | 100 |
| Ammonium sul-phate precipitation | 200 | 0.398 | 79.6 | 86.49 | 17298 | 217.31 | 1.03 | 15.71 |
| Gel filtration(G-100) | 40 | 0.110 | 4.56 | 53.96 | 2098.4 | 490.55 | 2.32 | 2.00 |

***Table 4.*** *Purification Table of Peroxidase from Strain of Pseudomonas.*

| Peroxidase | Volume(ml) | Protein(Mg) | Total protein | Activityμmol/min | Total activity U/ml | Specific activityU/mg | Purification folds | Percentage yield |
|---|---|---|---|---|---|---|---|---|
| Crude enzyme | 1000 | 0.52 | 520 | 112 | 112000 | 215.38 | 1 | 100 |
| Ammonium sulphate precipitation | 300 | 0.298 | 89.4 | 96.54 | 28962 | 323.41 | 1.50 | 25.85 |
| Gel filtration(G-100) | 30 | 0.131 | 4.53 | 78.86 | 2365.80 | 602.00 | 2.80 | 2.11 |

Precipitation profile of the crude extracts of lipase, urease and peroxidase from the solution using ammonium sulphate salt of various percentages forming the precipitation profile as shown in fig1-3.Crude extracts were precipitated at physiologic pH show the crude extract precipitation of lipase with maximum protein precipitationat70% saturation of the ammonium salt, precipitation of the crude extracts of urease with maximum protein precipitation at 60% saturation of the ammonium salt while precipitation of the crude extracts of peroxidase with maximum protein precipitation at 50% saturation of the ammonium salt. High salt saturations for precipitation of lipase is attributed to the nature of the enzyme as hydrophilic protein and so require much of the ion effect concentrations of the differential salt for salting out from surrounding liquid solution when compared to urease and peroxidase which depicted precipitation at 60 and 50% saturations of the precipitating salt [14].

Size exclusion chromatography was used for further purification of the protein to various molecular sizes and weight (fig4-6).This was done using sephadex G-100 packed into a column of bed height 75cm and column volume of 235.65cm$^3$.During the process, large molecular weight protein excludes first from the column through the inter-bead spaces of the stationary phase (sephadex gel) while small molecular weight protein exclude last from the column through the intra-bead spaces.Elution was done with 0.1M of sodium phosphate buffer of pH 6.0 at flow rate of 5ml/18min.

One distinct peak was seen from the chromatograms were observed in the profiles of the enzymes: lipase and urease while an adjourning peak was seen in the chromatogram of peroxidase respectively; proteins eluted from the column from fraction tubenumber 14-22forlipase,10-18forureaseand11-19 for peroxidase. As reported by Chilaka*et al*. (2002), 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.

Crude protein of lipase was purified upto 2.32 folds after gel filtration (sephadexG-100) with percentage yield of 2.00%. The specific activity of lipase increased from 211.81 to 490.55U/mg after gel filtration. Crude proteinureasewaspurifiedupto3.20 folds after gel filtration (sephadex G-100) with percentage yield of 2.30%.The specific activity of urease increased from 170.56 to 546.88U/mg after gel filtration. Crude protein extract of peroxidase was purified up to 2.8 folds after gel filtration (sephadex G-100) with percentage yield of 2.20%. The specific activity of peroxidase increased from 215.38 to 602.20U/mg after gel filtration.

Multiple peaks of the enzyme activity could be attributed to ionic scrambling encouraged by dialysis and this leads to formation of aggregates within correct ionic bond pairs.

## 4.4. Characterization of the Enzymes Activity

### 4.4.1 Optimum pH Determination

Studies on the effect of pH on lipase, urease and peroxidase activity showed optimum activity at pH of 6.0; 7.0 and 4.5 respectively (Figures 7, 8 and 9).

### 4.4.2 Optimum Temperature Determination

Studies on the effect of temperature on lipase, urease and peroxidase activity showed optimum activity at 50 ℃; 60 ℃ and 40 ℃ respectively (Figures 10, 11 and 12).

### 4.4.3 Determination of kinetic Constants ($K_m$and $V_{max}$)

Michaelis-menten constant ($K_M$) and maximal velocity of catalysis ($V_{max}$) of 0.32mM and 200μmol/min were obtained at different concentrations of p-NPP for lipase; whereas $K_M$ of 0.370mM and velocitymaximalof197.27μmol/min were obtained at different concentrations of standard urea for urease and Michaelis-Menten constant ($K_m$) and maximal velocity ($V_{max}$) of 3.45mM and 270μmol/min were obtained at different concentrations of o-dianisidine hydrogen peroxide standard substrates for peroxidases.

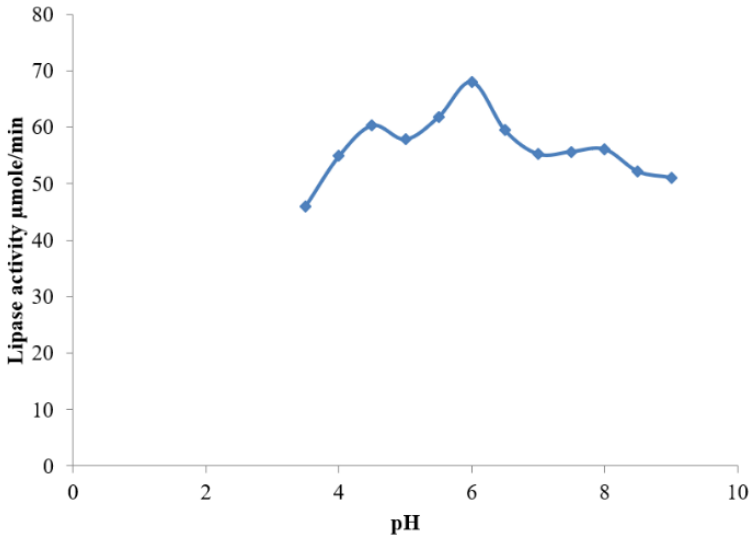



***Figure 9.*** *Effect of pH on Lipase produced from Strain of Pseudomonas.*

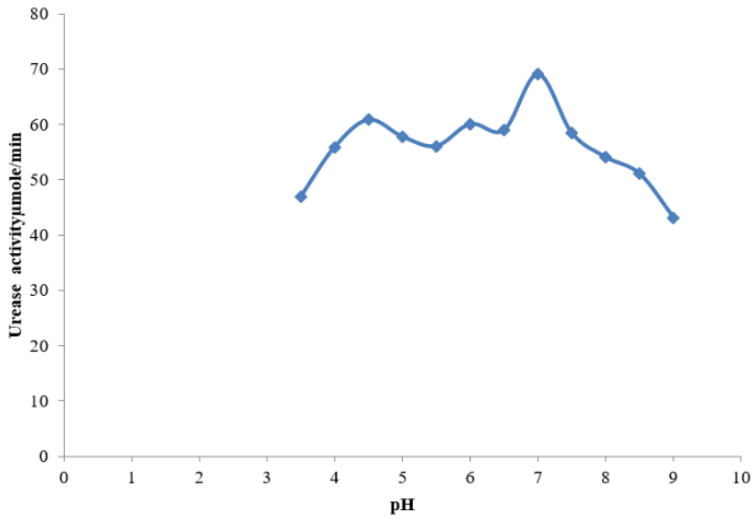



***Figure 10.*** *Effect of pH on Urease produced from Strain of Pseudomonas.*

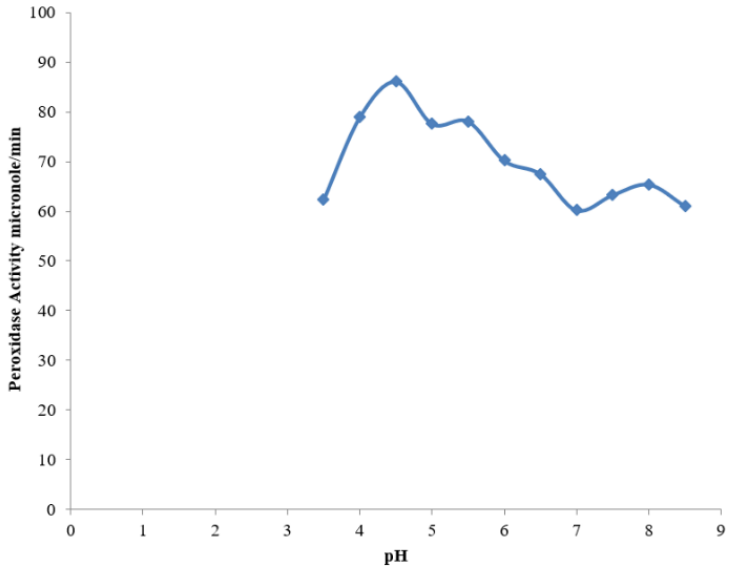



***Figure 11.*** *Effect of pH on peroxidase produced from Strain of Pseudomonas.*

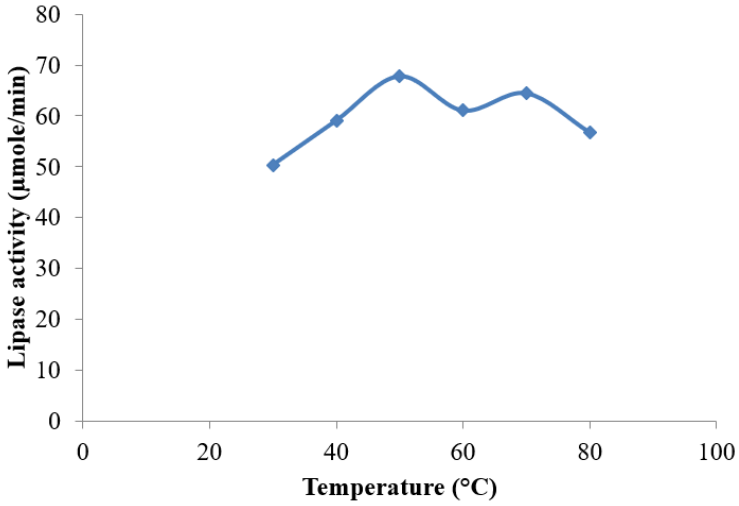



***Figure 12.*** *Effect of temperature on lipase produced from Strain of Pseudomonas.*

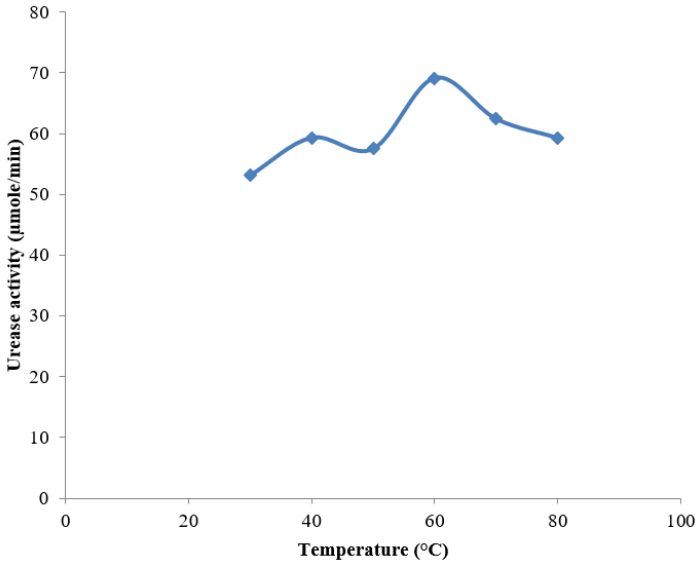



***Figure 13.*** *Effect of temperature on urease produced from Strain of Pseudomonas.*

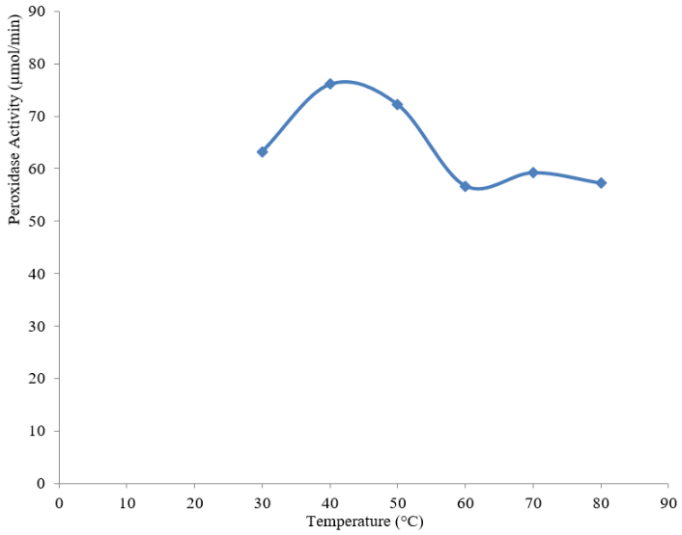



***Figure 14.*** *Effect of temperature on peroxidase produced from Strain of Pseudomonas.*

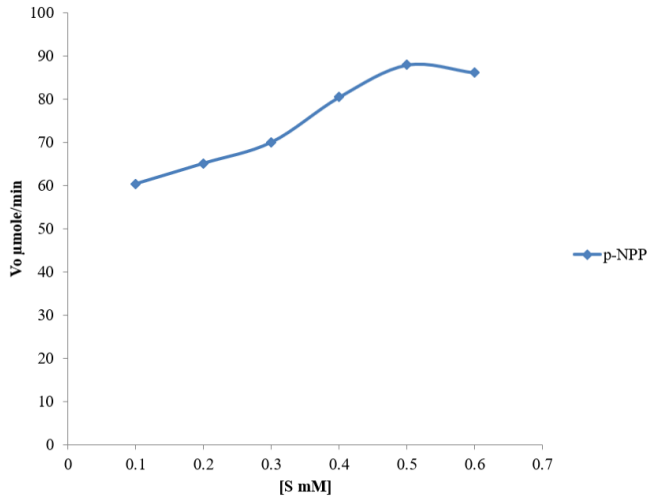



***Figure 15.*** *Michaelis-mentenplotofinitialcatalyticactivityoflipasefromstrainof Pseudomonas.*

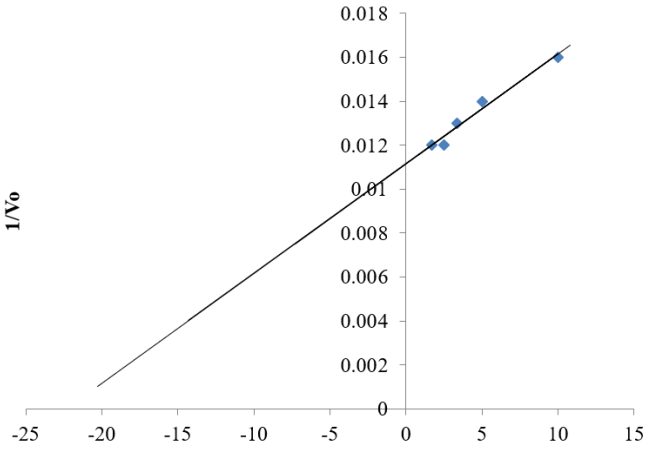



***Figure 16.*** *Linear weaver-burke plots of Lipase activity produced from strain of Pseudomonas.*

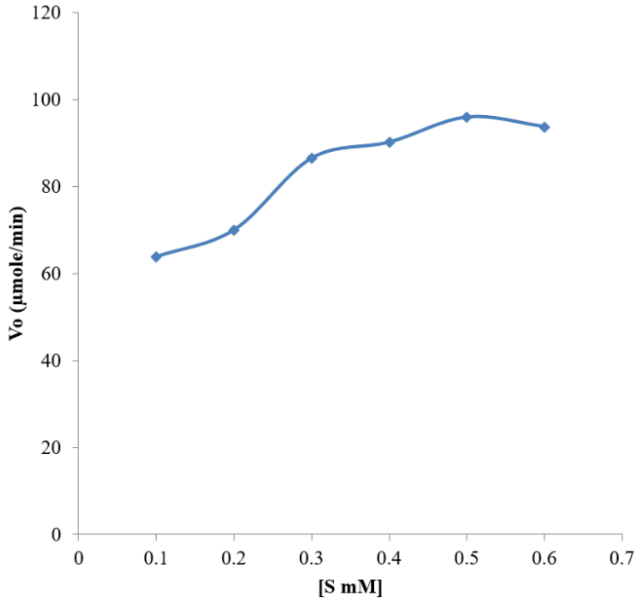



***Figure 17.*** *Michaelis-menten plot of urease in corresponding urea [1Mm] from strains of Pseudomonas.*

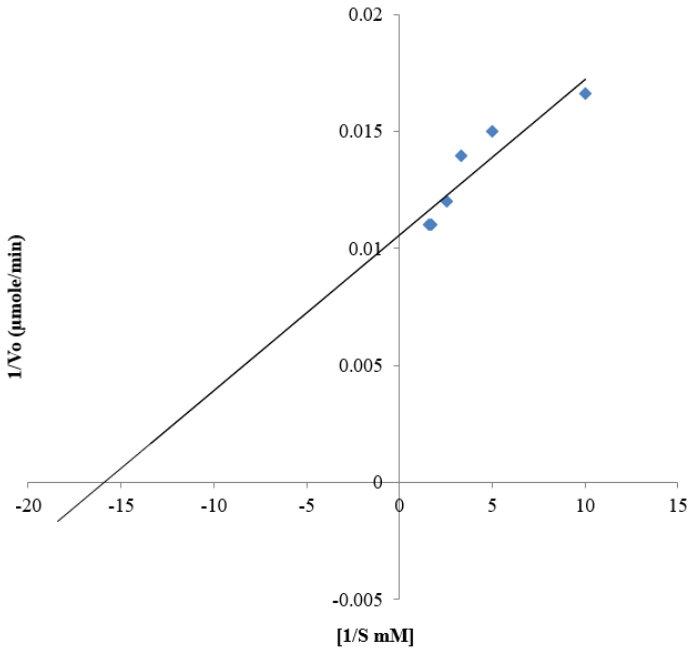



***Figure 18.*** *Line weaver burke plot of urease from strains of Pseudomonas.*

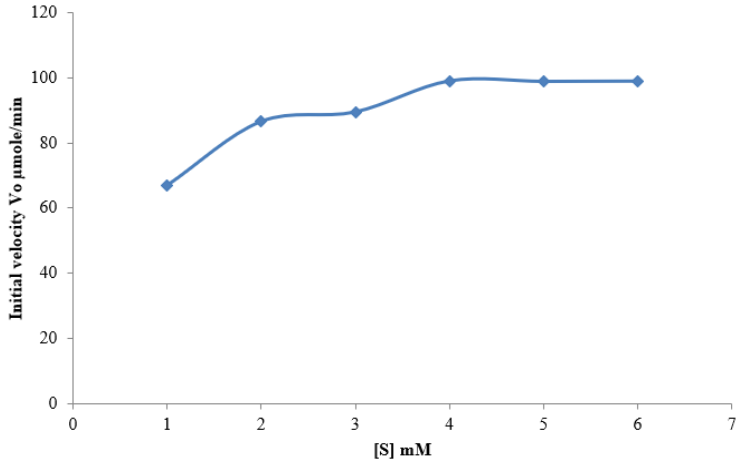



***Figure 19.*** *Michaelis-Menten plot of peroxidase activity at various concentrations of o-dianisidine.*

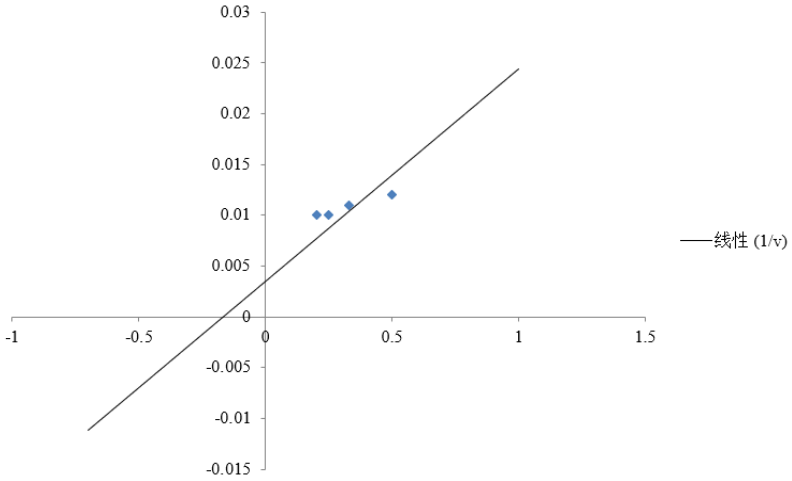



***Figure 20.*** *Lineweaver burke plot of peroxidase activity at various concentrations of o-dianisidine.*

## 5. Discussion

Studies on the effect of pH on lipase, urease and peroxidase activity showed optimum activity at pH of 6.0;7.0 and 4.5 respectively. [20] reported optimum pH of 8.0 forisolatedfrombacteriastrainsisolatedfromalocalpalmoilprocessingsitewhile [6] reported an optimum pH of 7.0 for lipase produced from coconut seeds under different production conditions. [22] reported optimum pH for peroxidase A produced from *Aspergillus tamari* was4.5.Peroxidase activity peaked at 50 ℃ while [27] reported optimum activity of urease from seeds of *P. macrophylla*at pH8.0.

Temperature represents the heat (enthalpy change) content of a chemical system. For most chemical reactions (invivo/invtro), every 10 ℃ rise in temperature results to double increase of the process. Heat content of a system increases the kinetic energy of atoms and molecules of the system thus resulting in an increase in the number of effective collisions of the there in particles. Increase in catalysis by enzymes in a biological system is mediated by effective collision between an enzyme's desired substrate(s) at its active site [39]. Effect of temperature on lipase, urease and peroxidase activity showed optimum activity at 50 ℃; 60 ℃ and 40 ℃ respectively. [20] reported a moderate thermo-tolerant peroxidase of peak activity at 70 ℃. Reports from [6] stated an optimum temperature of 50 ℃ for lipase produced from seeds of *Jatropha cucas*while Marjanovic reported 55 ℃ forpeak activity of bacteria producing lipase. [32, 34] reported an optimum temperature of 60 ℃ for urease activity produced from thermo tolerant *Bacillus* undergoing di- chroism in the presence of 5Mmercurychloride.

The single most important characteristics of an enzyme is to accelerate the rate of reaction (catalysis) occurring in living organisms; in the presence of a given amount of enzyme, the rate of catalysis increases as the substrate concentration increases until a limiting state is reached, after which further increase in the substrate concentration produce no significant rate in the rate of turn-over [36-38].

Michaelis-menten constant ($K_M$) and maximal velocity of catalysis ($V_{max}$) of 0.32mMand 200µmol/min were obtained at different concentrations of p-NPP for lipase; whereas $K_M$ of 0.370mM and velocity maximal of 197.27µmol/min were obtained at different concentrations of standard urea for urease and Michaelis-Menten constant ($K_m$) and maximal velocity($V_{max}$)of 3.45mM and 270µmol/min were obtained at different concentrations of o-dianisidine hydrogen peroxide standard substrates for peroxidases. Substrate specificity determination of lipase, urease and peroxidase from microbes respectively showed that p-NPP, o-dianisidne/guacol and urea gave better kinetic properties of the enzymes Kinetic constants ($K_m$ and $V_{max}$) of lipase determined during the study showed $K_m$ of 0.32.$K_m$which is the substrate concentration at half the maximum velocity during enzyme catalyzed reaction shows the affinity of the enzyme to its available substrates [13]. It is shown that the higher the $K_m$ value for each enzyme catalyzed reaction the lower the affinity of the enzyme to its available substrate and vice-versa. [16, 24] reported Km and Vmax of 1.94mM and 255 µmole/min respectively for lipase activity at various concentration of p-NPP. Kinetic constants ($K_m$ and $V_{max}$) of peroxidase A and B determined during the study at various concentrations of 2,6DMP showed $K_m$ of 3.45mM as reported by [22].

## 6. Conclusion

Pure enzymes are scarce due to the cost implications associated with the downstream processing of enzymes from their crude solutions. Among the other assayed proteins (peroxidase and urease), lipase is a multipurpose enzyme that has found its way in many producing industries, including those of food, clinical, and environmental-based research. The effective use of biocatalysts largely depends on their specific activity quotients and purification folds clarity. 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 subtle loss in the yield of the enzyme. Stability of the pure lipase is a promising adventure in utilization of the biocatalyst for various endeavours.

## 7. Recommendations

For further studies, research path should be channeled to the following areas:

1) Stability studies of the enzymes at respective pH and temperatures.
2) Site directed mutagenesis of the proteins amino acyl residues responsible for catalysis.

## Abbreviations

| | |
|---|---|
| mM | Mili Mole |
| µmol/min | Micro Mole Per Min |
| CFU/ml | Coliform Unit Per MiliLitre |
| Km | MichealisMenten Constant |
| Vmax | Velocity Maximal |

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## Conflicts of Interest

The authors declare no conflicts of interest.

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