Original Research Article

Kinetic properties of *Pseudomonas* sp. producing lipase

Abstract

Pseudomonas sp was isolated from petroleum spilled soil using culture dependent techniques. The organism was confirmed *Pseudmonas aeruginosa* using molecular typing (PCR and sequencing). Lipase producing capability of the organisms was screened in an optimized culture broth in the presence of p-NPP. Yellow coloration of the broth confirms the exo-lipase secretory of the bacteria. Lipase production peaked on day 5 of the fermentation; pH 7 was best for the lipase production. 60% ammonium sulphate was used in precipitation of proteins with peak lipase activity. Protein was dialyzed against gradient (0.01/0.1M) for 12 hrs for salt removal. Native sephadex G100 was used in separation of the dialysate into sizes and weight; fraction tubes of 16-25 were recorded as the elution volume of the chromatogram (VE). pH 4.5 and 60°C were characteristics of the purified protein where as Michaelis-Menten and velocity maximal (Km and Vmax) were 3.45mg/ml and 275 µmole/min. *Pseudmonas aeruginosa* seen from the study as a competitive bacteria for prolific lipase production to meet with the current biotechnological demand of the next generational enzyme.

Keywords: Lipase; *Pseudmonas aeruginosa,* Optimization

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Introduction

Lipases (generally rregarded as triacylhydrolyase) are vast enzymes of considerable physiological significance and biotechnological potentials. They catalyze the hydrolysis of triacylglycerols at oil-water interface (Kiyouta *et al.*, 2011) to release glycerol and free fatty acids (Abolemonaem *et al.,* 2011). Apart from other biotechnological application of lipase, ecology impact assessment studies have suggested great potentials of lipase in waste treatment systems (Ladero *et al*., 2003). The enzyme ability in hydrolyase of organic matters to simple easily amenable forms of less eco-implicativeness makes them promising in environmental monitoring such as eco toxicology. Development of lipase-based technologies for the synthesis of novel compounds is rapidly expanding the uses of this enzyme (Liese *et al*., 2000). Lipases can be produced from plants, animal and microbiology. Lipases from plants suffer low turn out due to technological incompetence in the plant tissue cultivation where as animal and microorganism lipases suffers setback in the purification system of the biomass from the proteins. Advantages which include their ability to display a high level of biocompatibility, biodegradation, biodigestibilty, ease of preparation and ability to serve in a wide range of physicochemical parameter such as: temperature, pH and salt concentration (Chen *et al*., 2007).

Bacteria are known for short doubling time in cultivation process and much very biotechnological relevance in production of metabolites.

Strains of *Pseudomonas* are known hydrocarbonolistic organisms; such that they are ubiquitos in most petroleum spilled areas with unique adaptability in the domicile niche. Crude oil degradation and recovery using lipses is an uncommon practice but of high promising advantages with regards to the potentials of the enzyme and the larger family of esterases towards hydrophobic compounds such as lipids etc (Chai-wei *et al*., 2013).

The use of emulsifying bacteria with potential physiologic activities in production of enzymes may have an improve activity on weathered petroleum hydrocarbon and significantly may contribute to mid- long term hydrocarbon degradation/ recovery.

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Material and Method

Soil Sample Collection

Soil samples were collected from crude oil polluted soil from Shell surge pipeline as described by Ezenwelu *et al.,* (2022).

Microbial Isolation

Strains of *Pseudomonas* was isolated and identified from the polluted soil using standard microbiology and biochemical techniques as described by Ezeonu *et al*. (2013).

Heterotrophic Counting and Microbial Standardization

Total heterotrophic colonies from both the nutrient media and the differential media were counted from the grown media plate as described as follow:

TCFU/ml=

$$microbial\ colonies\ observed\ X\ innoculum\ dilution\ factor\ X\ innoculum\ volume\ pippetted$$

The microbial cells were standardized using the Marcfarland standard solution (BaCl2/H2SO4 aq.) at λ 610 nm.

Molecular Identification of *Lactobacilli* sp.

Genomic DNA (gDNA) from the selected isolate was obtained using the QIA amp DNA Mini Kit. The 16S rDNA gene was amplified by RT-PCR (the conditions for the amplification stated below) using the forward (5'-AGTTTGATCATGGTCAG-3') and reverse (5'-GGTTACCTTGTTACGACT-3') primers. The amplified DNA sequence was compared to the Gen Bank database of National Center for Biotechnology Information (NCBI) using the BLAST program (Kumar *et al*., 2016).

Table 1 Conditions for Amplification of the Bacteria Genome using RT- PCR

TREATMENT	TEMPRETURE (°C)	TIME (Min)
Pre-denaturation	95	5
Denaturation	94	1
Annealing	52	1

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Elongation	71	7
Final elongation	72	7

Bacteria screening for lipase production

Strains of *Pseudomonas* were screened using mineral broth medium using Bhuwal *et al*. (2013).

Lipase Production

This was done according to the method described by Allam *et al*.(2016).

Assay of Lipase Activity

This was done according to the method of Lotrakul and Dharmsthiti (1997) using p-nitrophenyl palmitate (p-NPP) as a substrate.

Estimation of Protein

Protein content was determined as described by of Lowry *et al*. (1951) using BSA as standard protein.

Protein Purification

The crude extracts were passed through four purification steps which include:

- Ammonium sulphate precipitation
- Dialysis
- Gel filtration described by Allam *et al*. (2016); Chen *et al*. (2008) respectively.

Characterization of Lipase

Optimum pH determination: The optimum pH value of lipase activity was determined using various prepared organic buffers as described by Chilaka *et al*. (2002)

Optimal temperature determination

The optimum temperature value of lipase activity was determined as described by Sighn *et al*., 2014.

Determination of Kinetic Properties of the Lipase

Michealis menten constant (Km) and velocity maximal of the protein were deduced from the reciprocal curve of the michealis menten plot. Lipase was assayed in the presence of 0.0-05mM of p-NPP at the recorded optimum conditions.

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Results

Physicochemical Analysis of the Soil

Studies on physico chemical properties of the contaminated soil collected from petroleum spilled soil from Rivers state. pH of 5.65, conductivity 892 ($\Omega^{-1\ Cm-1}$), Dissolved mineral Cl-, SO_4, K, Ca, Mg were: 1182.91, 14.44, 16.25 36.21 and 24.12 mg/g respectively; heavy metals of Pb, Fe, Cu were: 17.30, 24.15 and 23.12 mg/g respectively while Hg, Cd and As were below detectable limit. TOC and TOM contents were 281.93 and 346.25 mg/ml respectively. Total petroleum hydrocarbon content was 21234.10 mg/g.

Table 2. Physico chemical properties of the petroleum contaminated soil from Onne drilling site, Rivers state.

Physiochemical parameters	**Control experiment**	**Soil sample**
pH	7.51	5.65
Soil Conductivity ($\Omega^{-1}cm^{-1}$)	588	892
Chloride ion (Mg/g)	778	1182.91
Phosphorus (Mg/g)	3.72	8.91
Magnesium (Mg/g)	12.17	24.12
Potassium (Mg/g)	10.02	16.25
Calcium (Mg/g)	28.93	36.21
SO_4 (Mg/g)	16.89	14.44

Iron (Mg/g)	16.55	24.15
Cadmium (Mg/g)	BDL	BDL
Mercury (Mg/g)	BDL	BDL
Arsenic (Mg/kg)	BDL	BDL
Lead (Mg/g)	9.23	17.30
Copper (Mg/g)	4.28	23.12
Total Organic Carbon (TOC) (mg/g)	106.85	281.93
Total Organic Matter (mg/g)	131.42	346.25
Total Petroleum Hydrocarbon (TPH) (Mg/g)	413.12	2123.410
Soil temperature	34.5^0C	43.0^0C

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Pure colonies of *Pseudomonas* 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 2.7 x 10^8 CFU/ml (Plate 1) and 3.1 x 10^7 CFU/ml (Plate 2).

Plate 1 Pure colonies of strains of Pseudomonas sp. on nutrient media plate

The micrograph of the bacteria (*Pseudomonas* sp) suspensions 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 (plate 2).

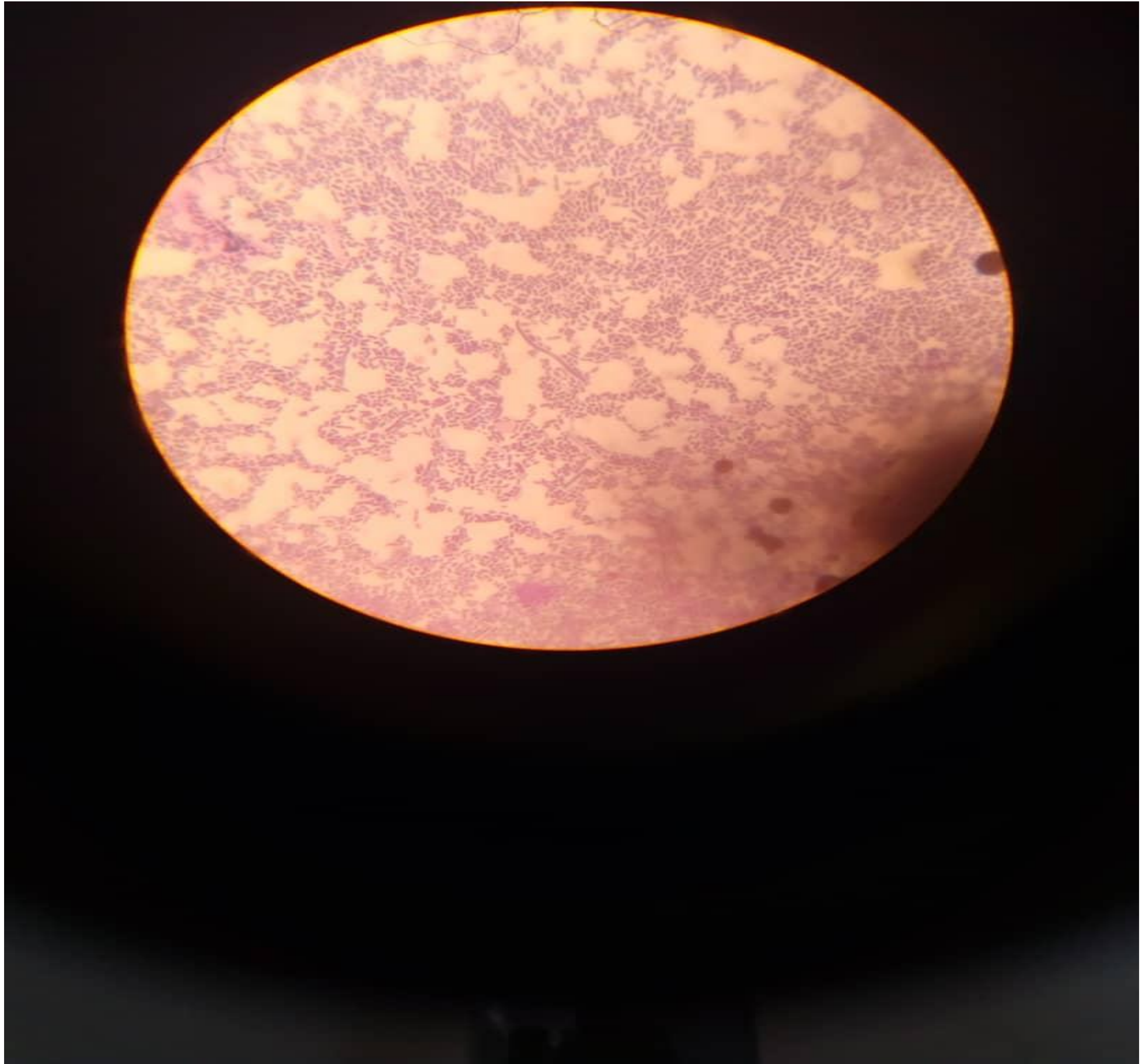

Plate 2. The micrograph of the bacteria (*Pseudomonas* sp) suspensions under an objective magnification of x40.

The basic morphology and biochemical features of strains of *Pseudomonas* sp. isolated from waste water and polluted soil (Table 3). The basic morphological features of the bacteria showed that the isolate *Pseudomonas*: rod shaped, non sporulating and non motile 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°C.

Table 3. Basic morphology and biochemical features of strains of *Pseudomonas* sp. isolated from polluted soil.

morphology	observation	Biochemical test	observation
shape	rod	Gram reaction	Positive
sporulation	none	Starch hydrolysis	negative
texture	Smooth and raised	heamolysis	Positive
colour	cream	catalase	positive
motility	none	oxidase	Positive
capsulation	none	Lactic acid formation	negative
		Urea hydrolysis	positive
		Glucose fermentation	positive
		Indole utilization	negative
		Gelatin hydrolysis	Positive
		Voges proskaeur	positive
		Hydrogen sulphide production	negative

Electrophoretogram of the amplified genome of *Pseudomonas* showed a typical band at 750 bp as shown in the figure 1; *Pseudomonas aeroginosa* was identified after the genomic sequencing with ascribed NCBI accession number of NR_075116.1 as shown in figure 2.

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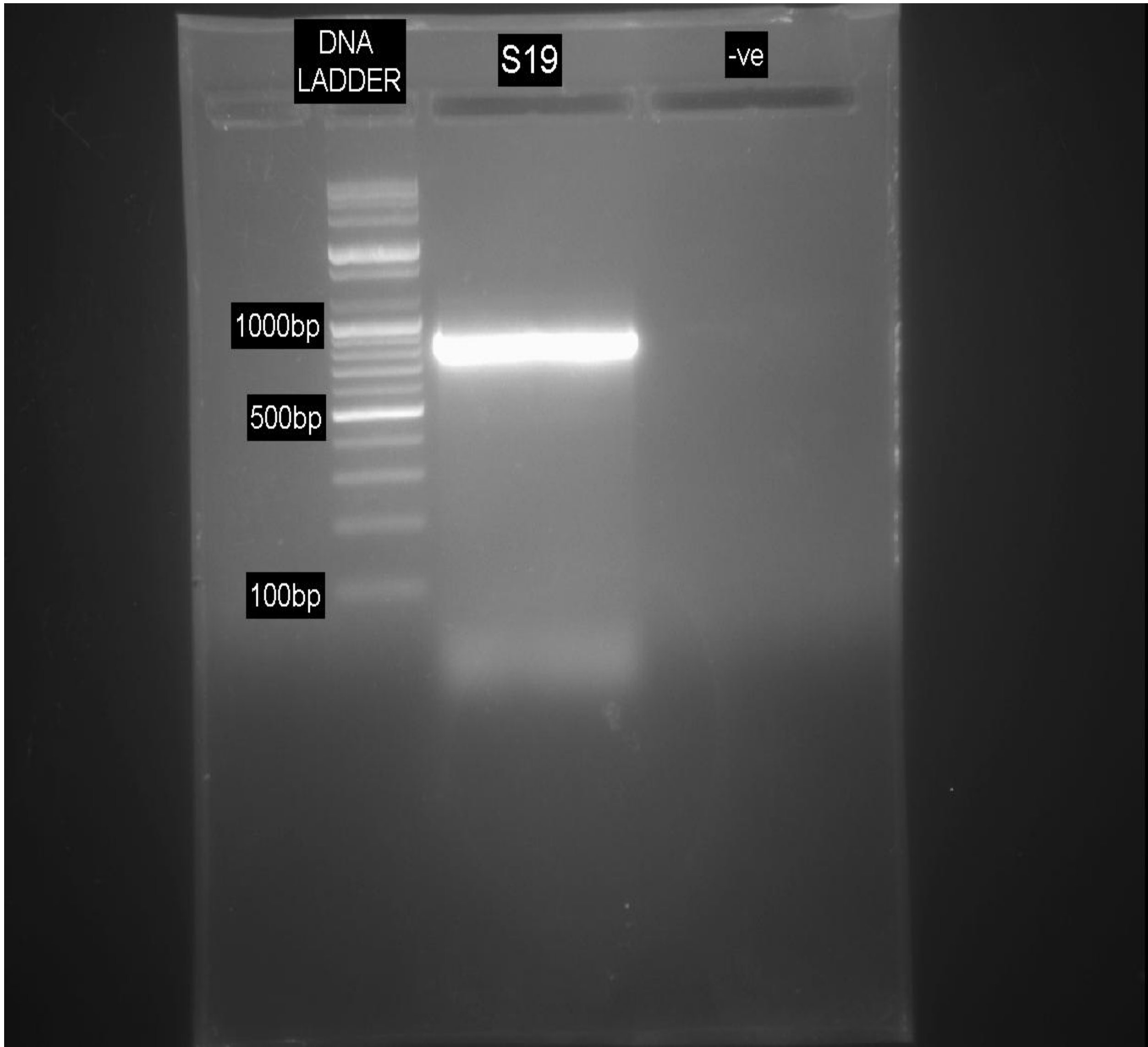

Fig 1 Electrophoretogram of the amplified genome of *Pseudomonas* sp. showed a typical band at 750 bp.

g-proteobacteria | 87 leaves

Pseudomonas aeruginosa strain SE5416 chromosome, complete genome

Pseudomonas aeruginosa strain SE5369 chromosome, complete genome

Pseudomonas aeruginosa strain CDN129 chromosome, complete genome

Pseudomonas aeruginosa strain PABCH01 chromosome

Pseudomonas aeruginosa strain PABCH42 chromosome

Pseudomonas aeruginosa strain PABCH46 chromosome

Pseudomonas aeruginosa strain PABCH09 chromosome

Pseudomonas aeruginosa strain PABCH14 chromosome

Pseudomonas aeruginosa strain PABCH45 plasmid unnamed

Pseudomonas aeruginosa strain PABCH45 chromosome

Pseudomonas aeruginosa strain PABCH05 chromosome

Pseudomonas aeruginosa strain PABCH10 chromosome

Pseudomonas aeruginosa strain PABCH13 chromosome

Pseudomonas aeruginosa PAO1 strain PAO1 5S ribosomal RNA, complete sequ..

Fig 2. Phylogenetic evolutionary tree of strains of *Pseudomonas aeruginosa* obtained afer geneomic blasting of the aplicons using NCBI blast tools.

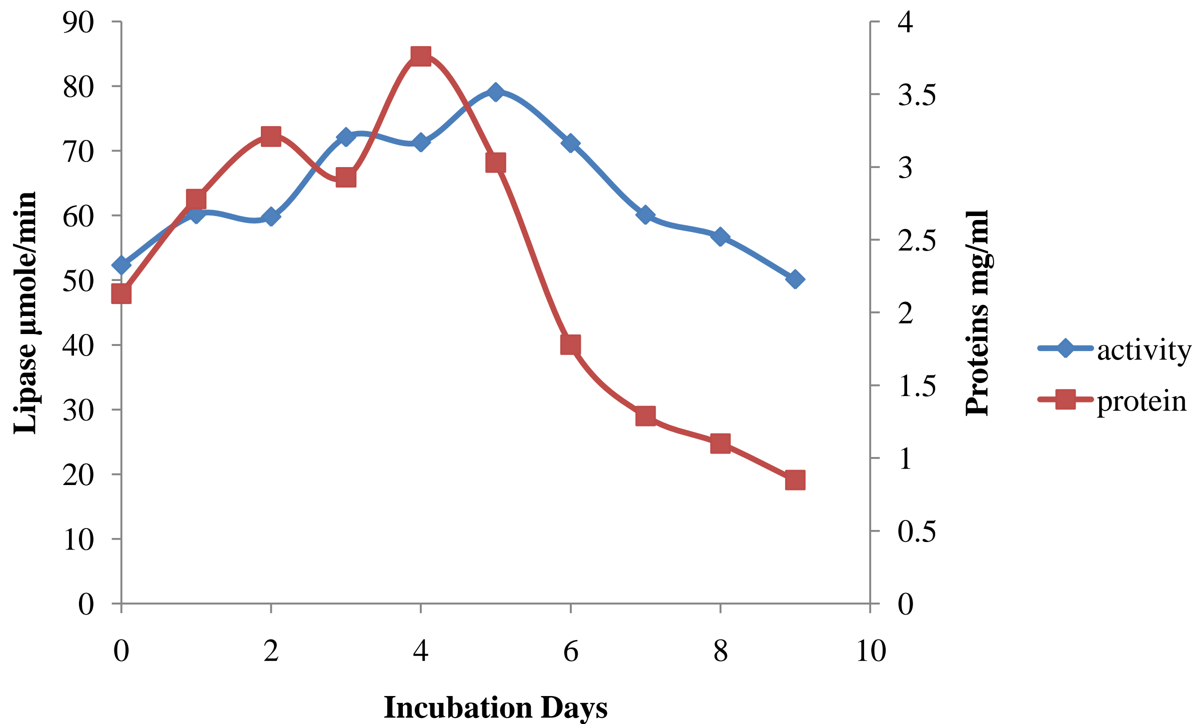

Fig 3. Effect of incubation days for Lipase production from strains of *Pseudomonas* sp.

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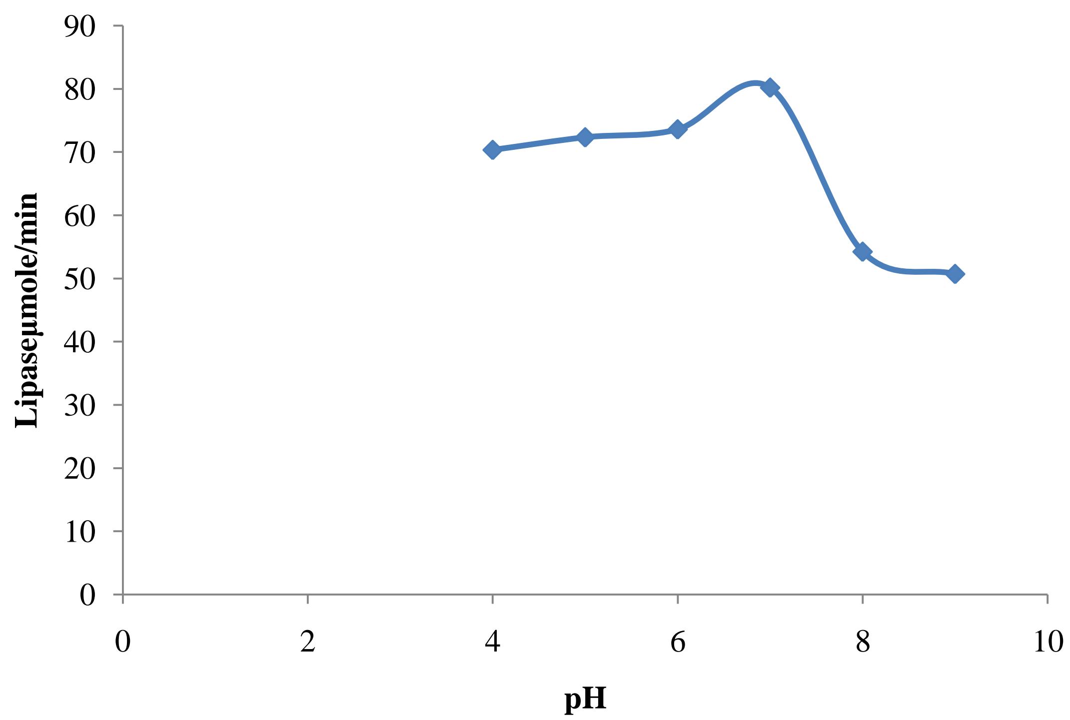

Fig 4. pH optimization for Lipase production from strains of *Pseudomonas* sp.

Studies on column chromatographic separation of the proteins using sephadex G-100 of bed height 75 and column volume of 235.65cm^3; figure 4, one distinct peak from the chromatogram of lipase from seeds of *Pseudomonas*; 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 20-39. Void volume (Vo) of the elution profile was recorded from tube number 0-19.

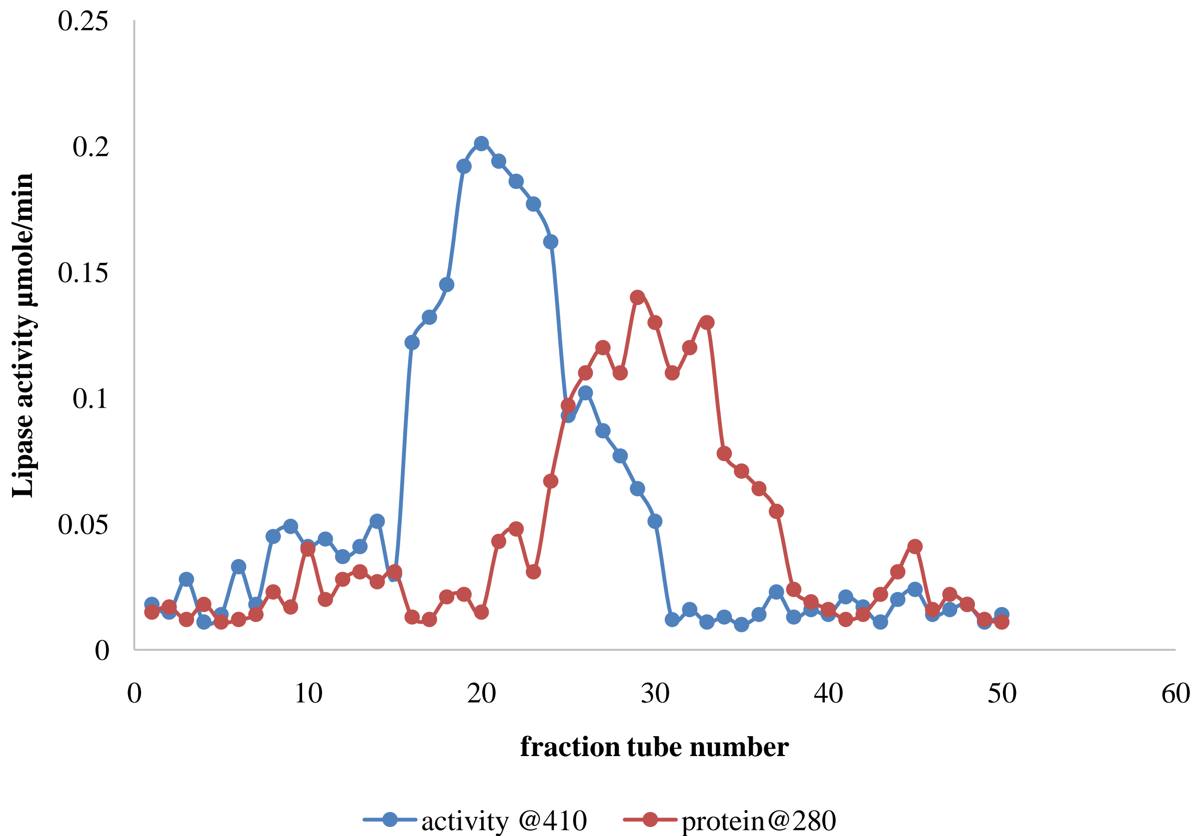

Fig. 5 Chromatogram of Lipase produced from *Pseudomonas* sp using sephadex G-100

Crude protein from strains of Pseudomonas was purified upto 2.32 folds after ion exchange (DEAE-cellulose) and gel filtration (sephadex G-100) with percentage yield of 2.00%. The specific activity of lipase increased from 211.81to 490.55 U/mg after gel filtration (Table 3).

Table 4 Purification Table of Lipase from *Pseudomonas* sp.

Lipase Profiles	**Volume (ml)**	**Protein (Mg)**	**Total protein**	**Activity µmol/min**	**Total activity U/ml**	**Specific activity U/mg**	**Purification folds**	**Percentage yield**
Crude enzyme	1000	0.64	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
Dialysis	70	0.281	19.67	76.96	5387.2	273.88	1.61	5.00
Gel filtration (G-100)	45	0.104	4.68	56.46	2540.7	546.88	3.20	2.3

Optimum pH and Temperature Determination

Lipase optimum activity peaked at pH of 4.5 while 60°C was the optimum temperature for the lipase activity.

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

Michaelis-menten constant (K_M) and maximal velocity of catalysis (V_{max}) of 3.45mg/ml and 275 µmol/min were obtained for the *Pseudomonas* producing lipase at different concentrations of p-NPP.

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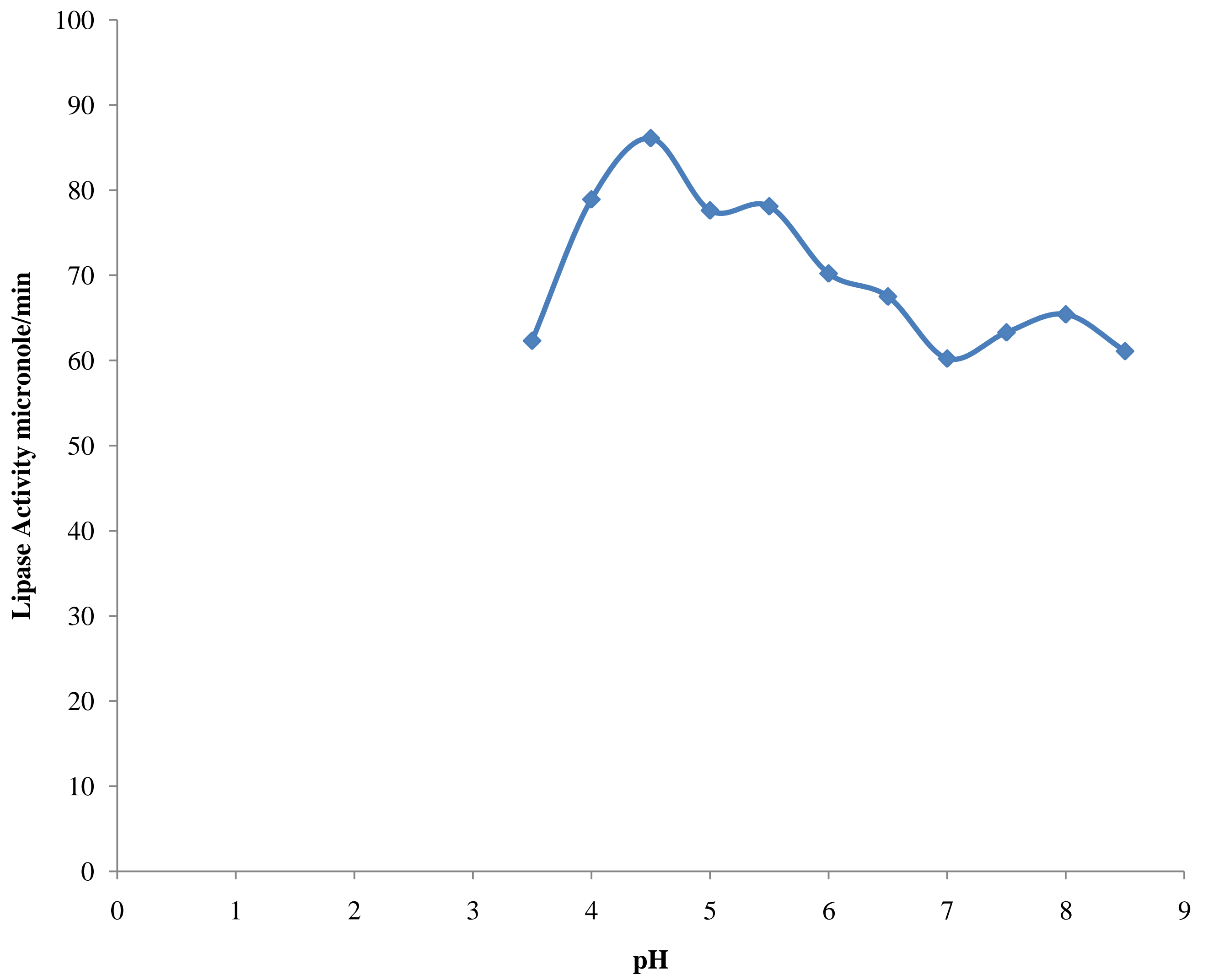

Figure 6: Effect of pH on lipase activity.

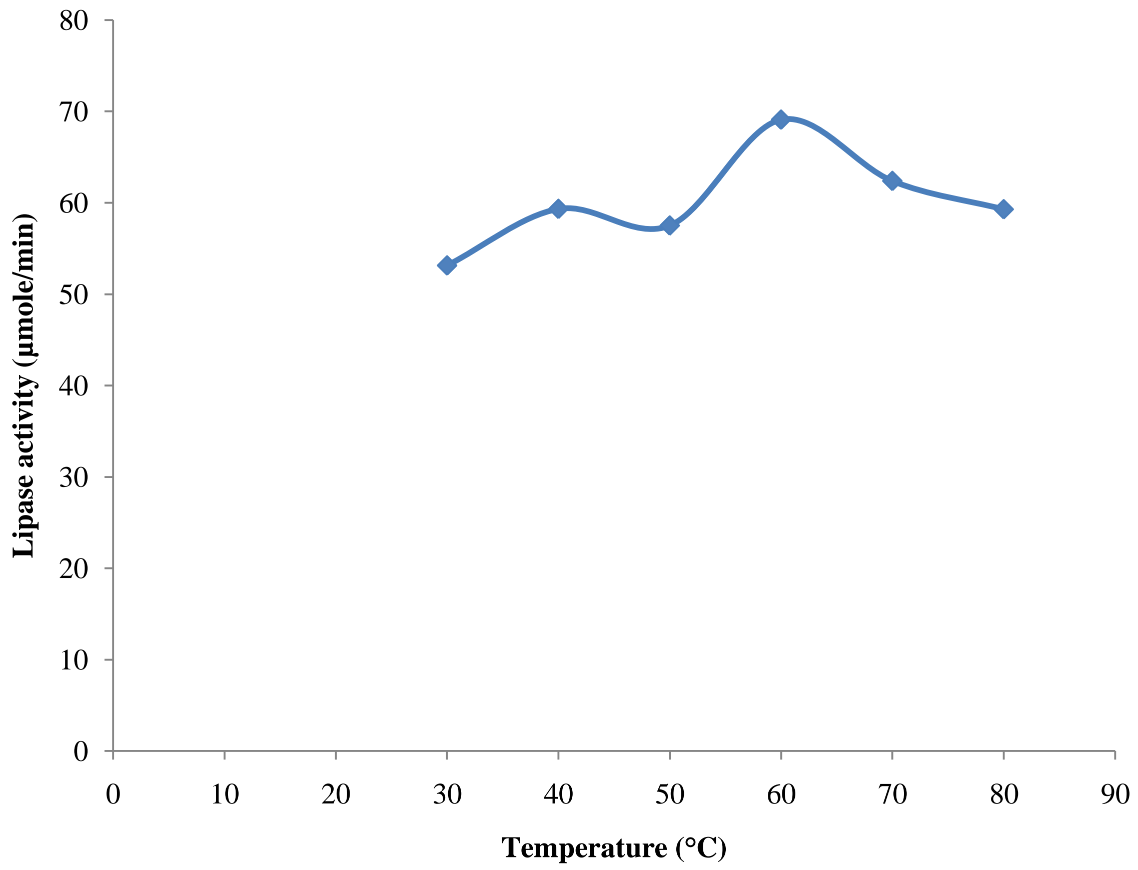

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Figure 7: Effect of temperature on lipase activity.

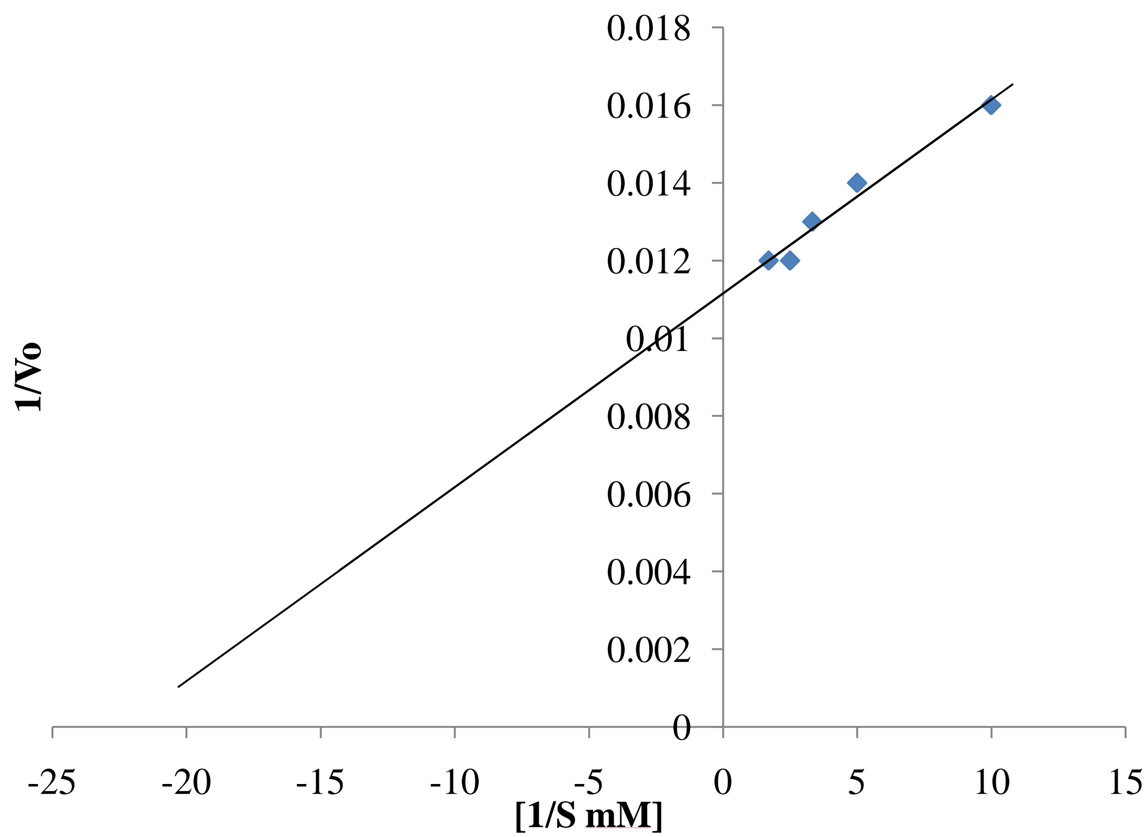

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Fig.8 Linear weaver-burke plots of Lipase activity produced from strains of *Pseudomonas*

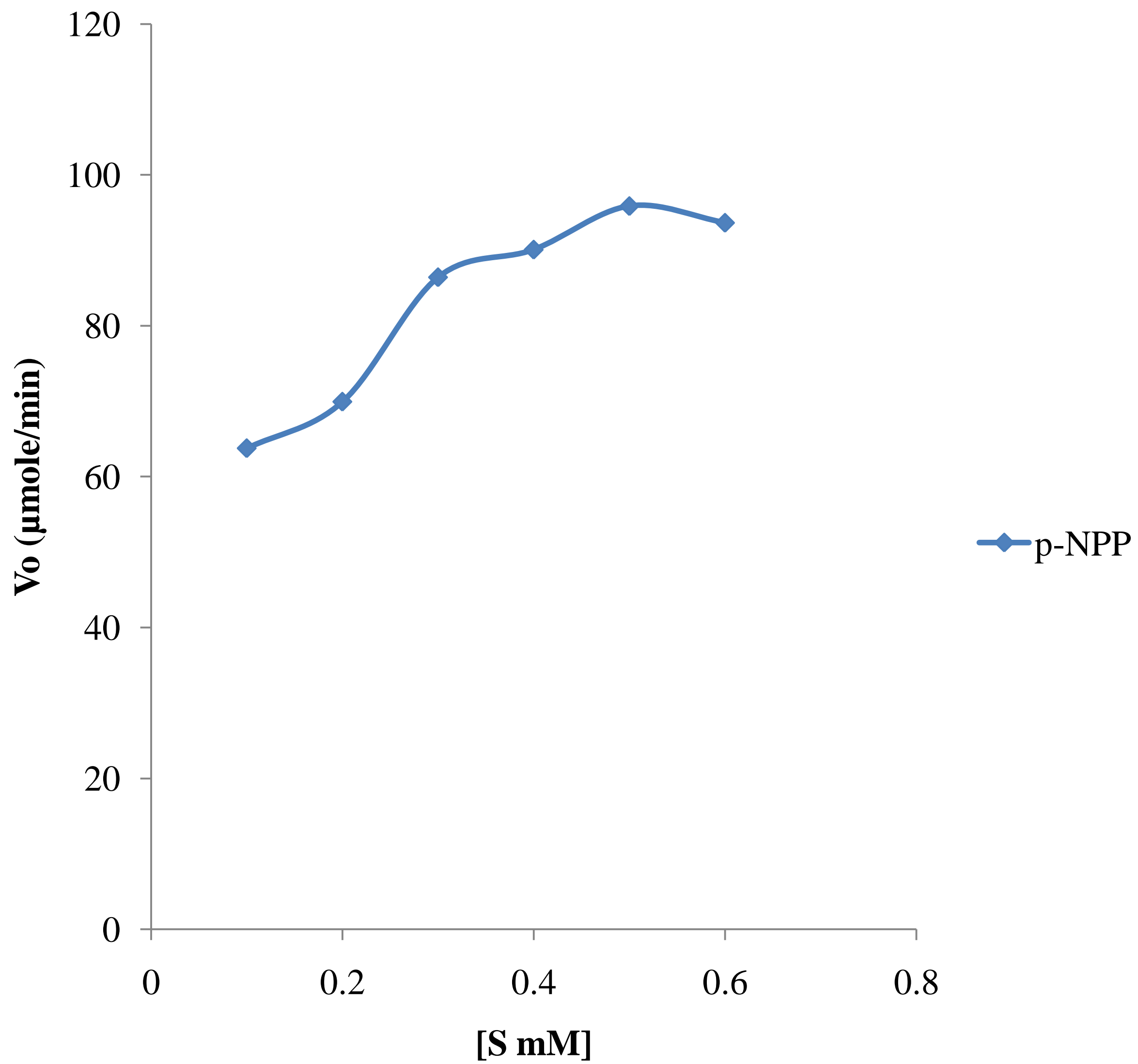

Fig 9 Michaelis- menten plot of Lipase from Pseudomonas at various concentrations of p-NPP.

Discussion

In the present study, lipase was produced from strains of *Pseudomonas* sp. isolated from a petroleum spilled soil using a submerged fermentation system. Studies on the physicochemical properties of the polluted soil showed pH of 5.65, conductivity 892 (Ω^{-1} Cm^{-1}), Dissolved mineral Cl-, SO_4, K, Ca, Mg were: 1182.91, 14.44, 16.25 36.21 and 24.12 mg/g respectively; heavy metals of Pb, Fe, Cu were: 17.30, 24.15 and 23.12 mg/g respectively while Hg, Cd and As were below detectable limit.

Khalid *et al.,* 2017 in their assessment study on soil pollution and Lead (Pb) accumulation revealed higher quotients of heavy metals like Fe, Pb and Cu in the soil while heavy metals of Hg, As, Cd were found below detectable limits in the soil.

TOC and TOM contents were 281.93 and 346.25 mg/g respectively. Mbachu *et al*. (2016) stated that total organic carbon and organic matter content of a medium reveal the carbon catenation oxidizable in the sampled area and organic matter show the degradable composite of the oxidizable carbon. The two enlisted components revealed the presence of carbon in an ecosystem. They reported a TOC and TOM of 196.71 and 241.95 mg/g Total petroleum hydrocarbon content was 2123.410 mg/g. Chikere *et al*. (2012); Okpokwasili and Adieze (1988) reported a total petroleum hydrocarbon contents of 1987.21 and 1786.51 mg/g respectively on soil from Bonny island, Portharcourt and Gokana L.G.A all in Rivers state. Chikere *et al*. (2012) stated that petroleum hydrocarbon concentration with soil and water bodies fluctauates with season and prevailing physiologic pH of the medium.

Basic morphological and biochemical screening were used to identify the isolate as *Pseudomnas*. 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°C. These findings corroborate with that of basic manual for organisms isolations and identifications written by Ezeonu *et al*. (2013). Molecular test (16S rDNA) was used to identify the pure isolates of *Pseudomonas*. Electrophoretogram of the amplified genome of *Pseudomonas* showed a typical band at 750 bp as shown in the figure 4. *Pseudomonas aeroginosa* was identified after the genomic sequencing with ascribed NCBI accession number of NR_075116.1. Yellow colouration of the screening broth after three days of incubation of the bacteria in the presence of 2mM of p-NPP showed the capability of the organisms to produce the protein. Peak lipase activity (106µmole/min) was obtained at the fifth day of the seven days fermentation process.

Lipase activity progressively decreases after the day 5 to the seventh day of the fermentation. Protein concentration of the enzyme peaked on the day 4 of the 7 days incubation.

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pH 7 was optimum physiologic range for lipase production from the *Pseudomonas* strain. Ciafardini *et al*. (2006) reported an optimum production of lipase from yeast on day 5. He went further to state that pH 8.0 was very favorable for optimum lipase production. However, Immanuel *et al*. (2008) reported that lipases from seeds are mostly produced within the peak time of the seeds germination which are usually 3-4 days after seed imbibitions.

Precipitation of the protein from the crude extracts solution using ammonium sulphate salt was carried out. Enzyme precipitation generally serves as an initial stage in protein purification; however it is an essential technique used in concentration of proteins from their solutions. The principle of protein precipitations using salts concentration (ammonium/calcium sulphate) is known as Common Ion Effect (Li *et al.*, 1975). During this process as reported by Li *et al.* (1975), as ammonium sulphate concentration increases, the hydrophobic surfaces on the protein are exposed continually with the salt present. These exposed hydrophobic patches on the protein aggregates through hydrophobic interaction. This leads to precipitation of the desired protein out from the solution. As stated, the presence of the precipitating salt stabilizes the various charged groups on the protein, this attracts protein into the solution and increases their solubility (salting-in).

However, as the salt concentration increases, the protein stabilizes more as there is less available water for its solubilization. The net result is the precipitation of these compounds from the surrounding environment (salting out) (Li *et al*., 1975).

60% saturation of ammonium sulphate at pH 6.0 was found suitable for precipitation of proteins with highest lipase activity. 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 (Anosike, 2002).

Salt removal from precipitated macromolecules is considered an essential step in protein purifications as it help in stabilization of desired protein seen evidently in the activity after desalting. Desalting of precipitated proteins through dialysis is done on the principle of Reverse osmosis. The precipitated proteins were de-salted for 12 hours using dialysis bag (with pore size of 2mm) in an ice pack container with buffer exchange after six hours of the dialysis. After dialysis, the dialysate specific activities were found to be 343.20.

Column chromatography (gel filtration) was used for further purification of the protein to various molecular sizes and weight. This was done using sephadex G-100 packed into a column of bed height 75 cm 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.

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One distinct peak was seen from the chromatograms respectively on tubes number 18-29 and 22-39 before a drop-in activity. 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 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. 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 211.81to 490.55 U/mg, respectively after gel filtration.

Optimum pH for lipase produced from strains of *Pseudomonas* was 4.5. Ertugrul *et al*. (2007) reported optimum pH of 8.0 for isolated from bacteria strains isolated from a local palm oil processing site while Ejedegbe *et al.* (2007) reported an optimum pH of 7.0 for lipase produced from coconut seeds under different production conditions.

Temperature represents the heat (enthalpy change) content of a chemical system. For most chemical reactions (invivo/invtro), every 10°C rise in temperature results to double increase of the process (Anosike, 2001). Optimum temperature for lipase produced from strains of *Pseudomonas* was 60 ^{o}C. Reports from Abigor*et al*. (2003) stated an optimum temperature of 50°C for lipase produced from seeds of *Jatropha cucas*.

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 (Adalberto *et al*, 2010). Kinetic constants (K_m and V_{max}) of lipase determined during the study showed K_m of 3.45. 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 (Chilaka *et al.,* 2002). 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. Velocity maximal (V_{max}) which shows the catalytic efficiency (rate of turnover) of lipase during catalysis shows V_{max} of 275 µmole/min. Abigor *et al*. (2002) reported Km and Vmax of 1.94 mM and 255 µmole/min respectively for lipase activity at various concentration of p-NPP.

Conclusion

The present study has shown the catalytic properties of lipase from *Pseudomonas* from polluted site through its kinetics. Biotechnological advances largely rely on the use of catalyst from non-noscomial source(s) as *Pseudomonas* showed high prolific production of lipase for next generational biotechnological and green chemistry advancement.

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Ethics

Authors declared no ethical issues that may arise after the publication of this manuscript.

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