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RESEARCH ARTICLE

Pseudomonas Producing Lipase Assisted Remediation of Petroleum Hydrocarbons

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Abstract

Organisms are indispensable tool in the modern-day biotechnology; their utilities in the various aspect of biotechnology are regarded integral. Studies on physico chemical properties of the contaminated soil collected from petroleum spilled soil from River's state. pH of 5.65, conductivity 892 (Ω-1 Cm-1), Dissolved mineral Cl-, SO4, 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. 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. Lipase assisted degradation of the petroleum hydrocarbon optimized at different conditions showed peak degradation at pH 5.5 and on the 12th day of the incubation. Degradation progresses optimally as enzyme and crude oil concentrations increase from 0-16%v/w.

Keywords: Lipase, degradation, crude oil, Pseudomonas

Background of the Study

Enzymes are integral tool with vast applications in various biotechnology industries [2]. 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 to release glycerol and free fatty acids [2]. In contrast to esterases, lipases are activated only when adsorbed to an oil–water interface [17] and do not hydrolyze dissolved substrates in the bulk fluid.

Lipases are reportedly present in animals, plants and microorganisms [12]. Lipid compound splitting and recovery with lipase as catalyst is advantageous compared to conventional process (chemical and thermal) due to low energy consumption, low cost, high product quality, ease of purification and safer process. Lipases find promising applications in organic chemical processing, detergent formulations, synthesis of bio surfactants, the oleo chemical industry, the dairy industry, the agrochemical industry, paper manufacture, nutrition, cosmetics, and pharmaceutical processing. Development of lipase-based technologies for the synthesis of novel compounds is rapidly expanding the uses of this enzyme.

Nigeria as a sovereign nation with vast mineral deposits of crude oil and gas majorly among others lined within their endowed biosphere [7] Petroleum oil is a complex mixture of several polycyclic aromatic compounds and other hydrocarbons, the major hydrocarbon classes found in this oil are the normal alkanes (rapidly degraded), branched alkanes and cycloalkanes (difficult to identify), the isoprenoids (very resistant to biodegradation), the aromatics (fairly identified and much more soluble than other hydrocarbons), and finally the polar ones containing mainly sulphur, oxygen and/or nitrogen compounds [9]. Non hydrocarbon compounds may also be found in crude oil and they include porphyrins and their derivatives [9].

Attempts to explore these mineral composites have left the country with unique vulnerabilities [9]. These vulnerabilities include human violence, marginalization and of serious health challenges due to environmental pollution [11].

There is presently an unprecedented increase in the upstream and downstream activities of oil and allied industries in this oil rich area [3, 11]. Over the years, these oil companies and their allied have generated myriad of pollutants in the form of gaseous emissions, oil spills, effluents and solid wastes [11] that have polluted the environment beyond sustainability. Abiet the pollutions and different vulnerabilities created during the drilling of these petroleum compounds within the olefiers oil muds, it is recorded that these frontiers companies into the drilling only go with less of the oil barrels as many of the petroleum compounds are lined within the oil muds. Targeted cheap and affordable methods in drilling and recovery of petroleum compounds have being in the target of many upstream companies so as to maximize high petroleum compounds within cheap drilling techniques.

The use of biological materials in phytoremediation and bioremediation offers promising technique for sustainable waste management and recycling [9]. These biological processes are important and effective over physicochemical processes because their end products are non-toxic and eco-friendly [2].

Lipolytic enzymes such as lipases and other esterases are bio molecules produced extracellularly or as part of a cell membrane by a variety of microorganisms ranging from yeast (non-filamentous fungi) bacteria and seeds of plants with wide range of applications. Recently, interests in microbial lipases have increased because of their advantages when compared to the other sources. 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 [10]. Strains of pseudomonas are known hydrocarbonolistic organisms; such that they are ubiquitos in most petroleum spilled areas with unique adaptability in the domicile niche. Oil 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 [13].

Mechanistically, petroleum hydrocarbon hydrolysis starts when the oxygen atom of the catalytic serine attacks the carbon atom of the ester linkage carbonyl group, generating a tetrahedral intermediate that makes hydrogen bonds with backbone nitrogen atoms in the oxyanion hole [10]. This stabilizes the negatively charged transition state that occurs during hydrolysis. An alcohol is released, leaving the acyl-lipase complex behind, which is ultimately hydrolyzed releasing free fatty acid and regenerating the enzyme [10].

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.

Materials and Methods

Materials

All chemicals, reagents and equipments used in the present study were all of analytical grade, standardized and are products from designated renowned companies.

Methods

The study adopted the experimental design

Petroleum Compound Sample Collection

Petroleum compounds were collected from two petroleum drilling sites located at Atali (LONG.7° 03",25°.2" E; LAT. 4° 51",53°.03" N) and Agbada (LONG.7° 00",57°.3" E; LAT. 4° 53",70°.04" N), both in Obio/Akpor L.G.A of Rivers state, Nigeria. The samples were collected from the stated areas at 6:00 am in the morning using a hooked sample bottle. The collections were done at the four perimeters of the marked drilling fronts with average of 9 m apart from each other. The collected petroleum compounds were homogenously pooled together into a clean asceptic container and transferred to the laboratory.

Collection of Soil Sample

Polluted soil sample was collected from the front-line drilling site of Shell Petroleum Company of Nigeria (SPDC), River state, Nigeria as described by [13]. Its physicochemical properties were determined as described by ATSDR (2010).

Microbial Isolation

Strains of Pseudomonas was isolated and identified from the polluted soil using standard microbiology and biochemical techniques as described by [13].

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 [18].

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
Elongation	71	7
Final elongation	72	7

Bacteria Screening for Lipase Production

Strains of *Pseudomonas* were screened using mineral broth medium using [11].

Lipase Production

This was done according to the method described by [7] Submerged fermentation system optimized with fermentation nutrient was employed during the process.

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. Lipase activity was monitored at 410nm whereas enzyme activity was observed in μmole/min.

Estimation of Protein

Protein content was determined as described by of using BSA as standard protein. Quantitative total protein was monitored at 750nm.

Studies on the Degradation of the Petroleum Hydrocarbon

Degradation studies on the crude oil were performed using the produced lipase from *Pseudomonas* sp. This was carried out using the gravimetric method as described by [19, 7] This procedure involves mixing equally a given concentrations of the enzyme solution (%v/v) in a sterile nutrient media optimized for the degradation study. The media was supplemented with known concentration (%w/v) of crude oil and incubated for 16 days at spinning rate of 180 rpm. Afterwards extraction was done using n-Hexane to determine the weight of the oil effluent remaining after the incubation.

Degradation work outplan:

Work Out-plan for the Study Equation

Gravimetric method of determining percentage and rate of degradation of oil:

W2 is the weight of residual crude oil after evaporation.

W1 is the weight of empty beaker

W" weight of the conical flask and the crude oil

W"-W1= weight of residual crude oil (W2)

Percentage Degradation

The percentage of oil degraded = weight of crude oil degraded/original weight of POME used x 100

Weight of crude oil degraded = original weight of crude oil used – Weight of residual crude oil obtained after evaporating the extract.

Rate of Degradation = weight of crude oil degraded/Time taken

Optimization of Physiologic Parameters During the Degradation Study

The following physiologic conditions were varied during the study for optimal determination of the crude oil: pH, incubation days, enzyme concentration (%v/v) and crude oil concentrations (%w/v).

Effect of pH

Different pH ranging from 4.5- 8.0 were prepared using respective buffer salts (sodium acetate, sodium phosphate and tris-HCl) of 1 M concentration. The mineral salt medium constituted for the POME degradation include: crude oil 5%, phosphate salt 0.5%, 5% enzyme solution, deionize water 200ml. These were incubated at 37^{O}C for 16 days.

Effect of Incubation Days

Mineral salt medium constituted for crude oil degradation was Incubation at different days of 0-16 days. The mineral salt medium contain: crude oil 5%, phosphate salt 0.5%, 5% enzyme solution, deionize water 200ml. The cultivated media were incubated at 37°C and pH 5.5.

Effect of Lipase Concentrations (%v/v) on the Degradation of Crude oil

Enzyme solution concentrations ranging from 0-20% (v/v) were varied during the degradation studies of crude oil. The mineral salt medium constituted include: crude oil 5%, phosphate salt 0.5%, deionized water 200 ml. The cultivated media were incubated at 37°C and pH 5.5.

Effect of Crude oil Concentrations (% w/v) During the Degradation Studies

Crude oil concentrations ranging from 0-20% (w/v) were varied during the degradation studies of crude oil. The mineral salt medium constituted include: enzyme suspension 5%, phosphate salt 0.5%, deionized water 200 ml. The cultivated media were incubated at 37°C and pH 5.5.

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 (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. 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, River's 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^{0}C	43.0^{0}C

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 25-40oC.

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

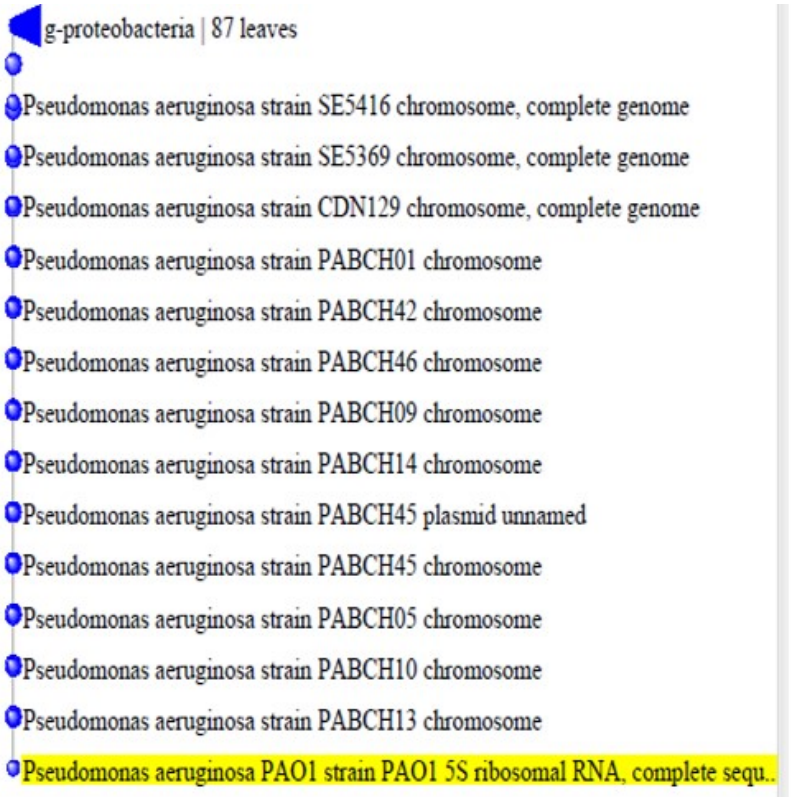

Figure 1: Phylogenetic evolutionary tree of strains of Pseudomonas aeruginosa obtained afer geneomic blasting of the aplicons using NCBI blast tools

Figure 2 and 3 below showed the effect of incubation days and physiologic pH on the production of lipase from the strains of Pseudomonas. Lipase activity peaked at day 5 of the 9 days incubation days and at pH 7.0 respectively.

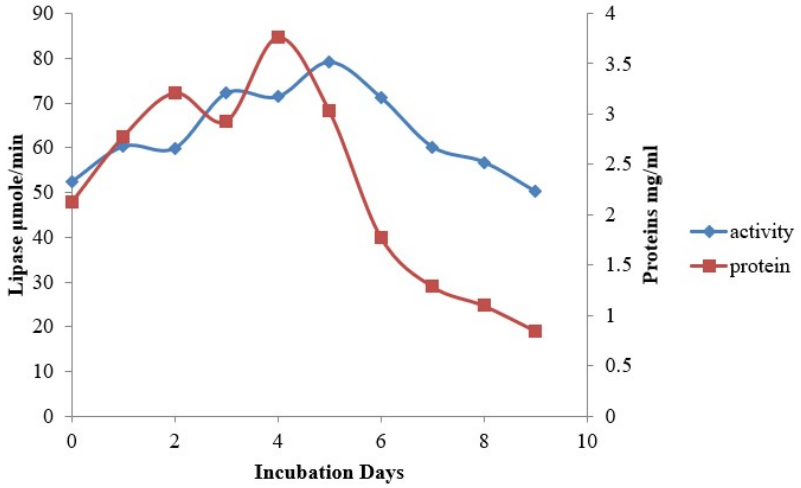

Figure 2: Effect of incubation days for Lipase production from strains of Pseudomonas sp

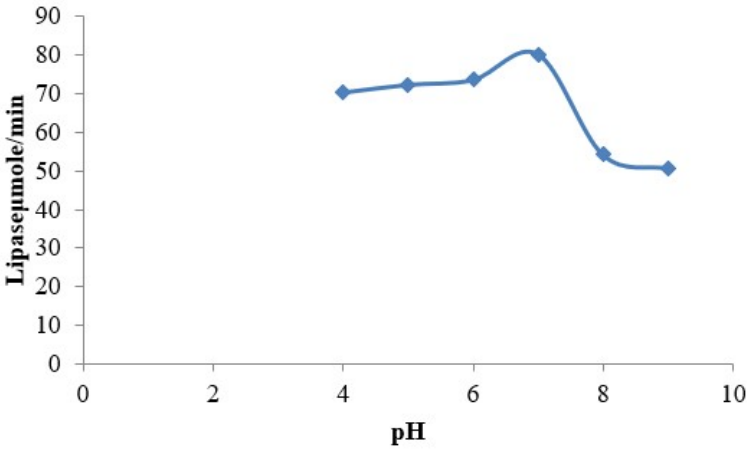

Figure 3: pH optimization for Lipase production from strains of Pseudomonas sp

Lipase assisted degradation of the crude oil hydrocarbons demonstrated below showed the hydrocarbonolistic evident of the enzyme on the petroleum hydrocarbons. Optimum degradation was seen at pH 5.5 and at day 12 of the incubation. However progressive degradation was observed as the enzyme concentration increases per the culture broth

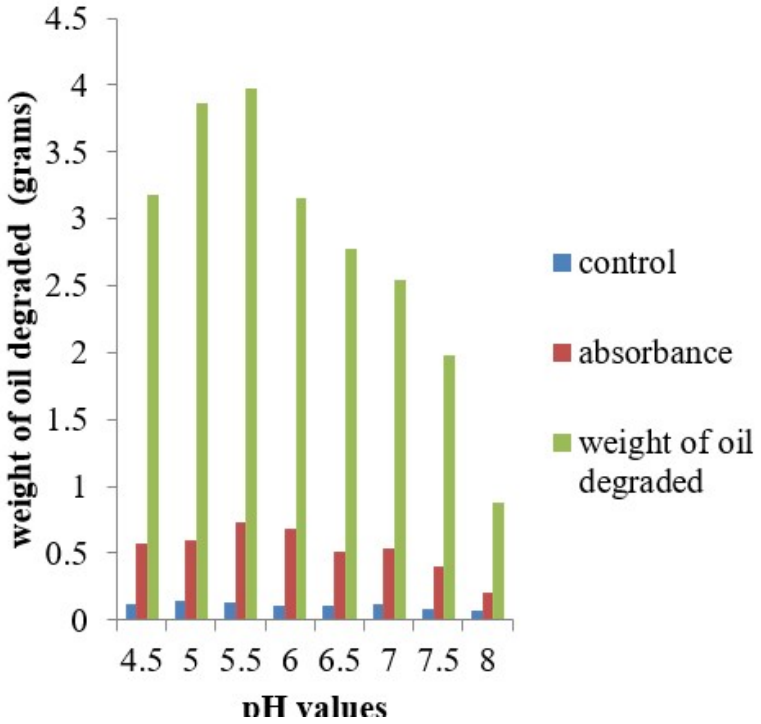

Figure 4: Effect of pH on degradation of crude oil by lipase

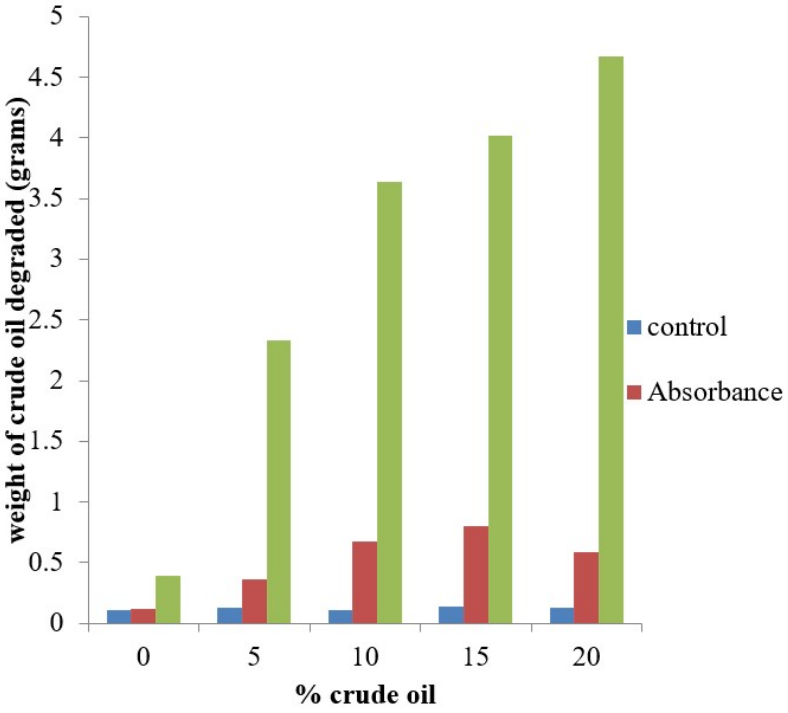

Figure 5: Effect of Lipase concentrations on degradation of crude oil by lipase

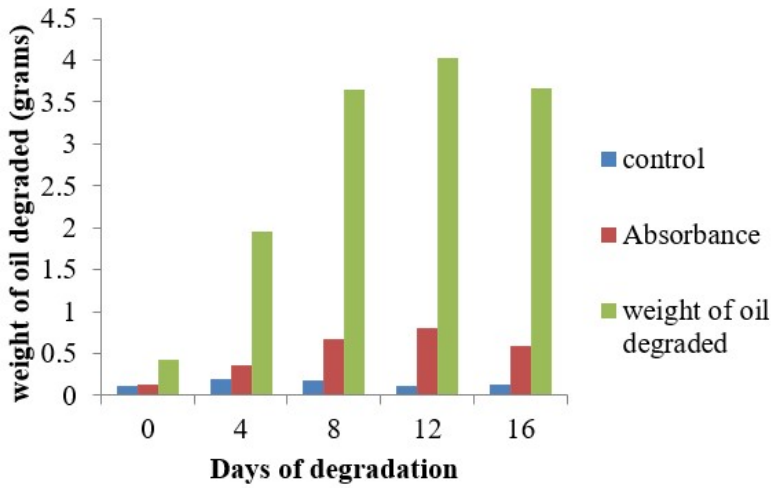

Figure 6: Effects of incubation period on crude oil degradation at 10% (v/v) of the lipase incubated at pH 5.5

Discussion

In the present study, lipase produced from strains of Pseudomonas sp. isolated from a petroleum spilled soil assisted in the biodegradation of petroleum hydrocarbons. Studies on the physicochemical properties of the polluted soil showed pH of 5.65, conductivity 892 (Ω-1 Cm-1), Dissolved mineral Cl-, SO4, 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/g respectively. [7] 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. 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.

Morphological features of the bacteria showed that strains of Pseudomonas under an objective magnification of x40, showed clusters of rods 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 25-40oC. These findings corroborate with that of basic manual for organisms isolations and identifications written by [13]. 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. Peak lipase activity (106μmole/min) was obtained at the fifth day of the seven days fermentation process. pH 7 was optimum physiologic range for lipase production from the Pseudomonas strain 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, 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.

Effect of pH on the degradation of the total petroleum hydrocarbon using the lipase was optimum at 5.5 while studies on effect of incubation days on lipase utilized degradation of petroleum hydrocarbon was day 12 of the 16 days study. As described by [19] most degradation studies using biological means are optimum at lower pH and a given lengthy stipulated time. This owe to the fact of the nature of petroleum hydrocarbon and other adjourning composite of the compound.

Conclusion

Lipase is a multi-purpose enzyme that has found its way in many producing industries including those of foods, clinical and environmental based research industries. The present study has shown the basic enzymology and kinetic properties of lipases produced from Luffa aegyptica and Citrullus lanatusthrough the significance of its large range of catalytic peak conditions and stability. Low Michaelis-menten and high velocity maximal of the enzyme confirmed their potential applications in industrial and environmental bias sector. Impact of lipase from Pseudomonas sp. in fostering degradation of petroleum hydrocarbons also have pave way for another plausible utilization of the enzyme in eco-monitoring and toxicological studies. Evidently from the studies are peak biohydrolysis of carbon catenation of crude oil at optimized degradation condition(s).

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Author's Contributions

Nworie MB: Conceived and designed the experiments, performed the experiment and processed the data, analyzed the data and wrote the manuscript.

Oyibo ON: Revised the manuscript and performed the experiment.

Olokor AN: Performed the experiment and performed the experiment.

Mere CS: Guided the experimental design and processed the data.

Ayantse LM: Guided the experimental design.

Odo CP: Performed the experiment and revised the manuscript.

Duru I: Co-concieved the research and guided the experiment

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

Ethics

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

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