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Research Article
Biodegradation Potential of Tropical Hydrocarbon Degrading *Providencia stuartii*

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Abstract

Background and Objective: Cleaning up petroleum hydrocarbon contaminated sites has been a major challenge, this has led to the exploration of many approaches to affect the cleanup of the polluted soils. The direct use of microorganisms in biodegradation of oil polluted sites may be technically difficult, hence the use of microbial enzymes is considered as an alternative. This study investigated the isolation of bacteria from crude oil contaminated site, optimized different condition parameters for maximum lipase production and subsequently degraded the petroleum hydrocarbons. **Materials and Methods:** Bacterial isolates were screened on Mineral Salt Medium (MSM) containing 1% Premium Motor Spirit (PMS) and diesel oil as carbon source. One bacterial isolate showed maximum zone of clearance on the medium containing 1% PMS and was identified by molecular evolutionary genetic analysis 16S RNA gene sequence to be *Providencia stuartii*. It was cultured for lipase production in a submerged medium. The crude enzyme extracted was further used for the degradation of petroleum hydrocarbons. **Results:** The results from the optimization study showed that maximum lipase activity was achieved when MSM having 2% (v/v) olive oil was used for 48 h at 35°C and neutral pH. Optimum degradation of PMS was 60.89% and diesel was 56.19% after 20 days of degradation. **Conclusion:** This study showed that lipase from *P. stuartii* was able to degrade PMS and diesel which could serve as a simple and effective biodegradation tool.

Key words: *Providencia stuartii*, hydrocarbon, biodegradation, bacteria lipase, optimization

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

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

INTRODUCTION

Petroleum hydrocarbons are considered the main energy source and materials for different industries[1]. Petroleum oil contains aromatic compounds that are toxic for most life forms. They poses many threats and major environmental pollutants are generated when it use in accidental spilling, coastal oil refining, transport, shipping activities and offshore oil production[2]. Such major environmental problems arises from various human activities e.g., agriculture sector, petroleum industry, nuclear technology and petrochemical sector. The release of wastes generated due to those activities is of a particular concern in the environment[3]. Human activities, such as municipal run-offs and liquid release and industrial, cause petroleum hydrocarbon pollution which impacts the environment and poses a direct or indirect health hazard to forms of life[4]. Hydrocarbon components have been known to belong to the family of carcinogens and neurotoxic organic pollutants. In an accidental leak, on-site removal, treatment or recovery of contaminants is facilitated but contaminants in petrol stations and spills may persist because the amount of leakage is small. Petroleum leakage due to frequent accidental and illegal disposal of oil waste at sea severely harms various ecosystems. Petroleum hydrocarbons are toxic compounds classified as priority pollutants[5]. This is why various techniques have to be developed to degrade these compounds into non-toxic or less toxic compounds. A biological treatment is an alternative pollutant removal method because this technique does not elicit deleterious effects on the environment and may also be less expensive than other techniques. Chemical and mechanical conventional procedures generally used for treating hydrocarbon contaminants on oil polluted sites have limited effectiveness and incur more cost.

Biodegradation is the degradation caused by biological activity, especially by enzymatic action, leading to a significant change in the chemical structure of the exposed material and resulting in the production of carbon dioxide, water, mineral salt and new microbial cellular constituents (biomass)[6]. In the microbiological sense, "biodegradation" means the decaying of all organic materials that is carried out by life forms comprising mainly bacteria, fungi, protozoa and other organisms[3]. Through this, biologically natural process, hazardous toxic contaminants are transformed into less toxic or non-toxic substances. The secondary metabolites, intermediary molecules or any degradation products from one organism can become the nutrient for others, substantiate carbon source and energy. They can further work on breaking down the remaining organic matter.

Biodegradation has an immense role to play in the treatment of hazardous wastes from petroleum. The bioprocesses for treating the hazardous contaminants is a promising technology, since it is cost effective and can lead to complete removal of hazard and their mineralization. Biodegradation is nature's way of recycling wastes or breaking down organic matter into nutrients that can be used by other organisms or transformed into less toxic or non-toxic substances. By harnessing these natural forces of biodegradation, some types of environmental contaminants can be reduced and completely removed[3]. However, the direct use of microorganisms in biodegradation of oil polluted sites may be technically difficult, hence the need to focus on microbial enzymes is considered as an alternative[7]. The purpose of this study was to screen for potential microbes capable of producing lipase, optimize the condition parameters for lipase production and to biodegrade petroleum hydrocarbons using lipase from *Providencia stuartii*.

MATERIALS AND METHODS

Materials

Sample collection: Soil sample was collected from 2-3 cm depth of an oil polluted site at Abual, Abua/Odual Local Government Area, Rivers state, Nigeria. The study was carried out between March-July, 2019. The collected sample was packed in sterile bags to the laboratory. The entire sample was stored in the refrigerator before commencement of the experimental study.

Isolation of bacteria: Isolation of both potential bacterial cultures capable of degrading petroleum was carried out using serial dilution technique and spread plate method on nutrient agar medium and were incubated at 37°C for 24 h. Pure cultures of the isolates were maintained on nutrient agar slants and were sub-cultured from time to time to maintain its viability in the laboratory.

Screening of hydrocarbon degrading bacteria: The isolated bacteria were inoculated on an enrichment medium that contains Mineral Salt Medium (MSM) supplemented with single hydrocarbon compound as sole carbon source (1% petrol and diesel). The MSM composition was made up of basal salt medium and trace element solution. The basal salt medium contained (g L^{-1}): K_2HPO_4, 1.8, KH_2PO_4, 1.2, NH_4Cl, 4.0, $MgSO_4.7H_2O$, 0.2, NaCl, 0.1, yeast extract, 0.1 and $FeCl.4H_2O$, 0.05. The trace element solution contained: H_3BO_3, 0.1,

$ZnSO_4.7H_2O$, 0.1, $CuSO_4.5H_2O$, 0.05 and $MnSO_4.H_2O$, 0.04 with pH[8] of 6.5. Agar powder was added to the medium to solidify it and then was poured into 6 plates. The plates were incubated at 37°C for 7 days.

Molecular identification of the isolate: The extraction of total genomic DNA and Polymerase Chain Reaction (PCR) using standard methods to determine the phylogenetic grouping of genomic DNA of sample, their DNA was amplified by using standard PCR and DNA sequencing were carried out at the Bioscience Laboratory, International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. Nucleotide sequences were determined by analysis of fluorescently labeled 16S RNA products generated by PCR cocktail mix on an AB 373a Strech (short gun) DNA sequencer. Primers 16SF: GTGCCAGCAGCCGCGCTAA and 16SR: AGACCCGGGAACGTATTCAC were used in all sequencing reactions. The obtained sequences of 16S RNA were aligned by submitting them to the non-reductant nucleotide database at Genbank using the FLINCH TV program in order to determine the identity of the isolate (http://www.ncbi.nlm.nch.gov). The 16S rRNA DNA sequence was submitted to the National Center for Biotechnology Information (NCBI) data based and the sequence was compared to other available 16S rRNA sequence using an automatic alignment tool (Blast). The construction of the phylogenetic tree was generated by Phy ML and the visualization of the tree by Tree Dyn using the online program www.Phylogeny.fr. The MEGA program was used for drawing the tree.

Lipase production and assay: Lipase production was carried out in a submerged medium containing peptone 0.2% (w/v), $NH_4H_2PO_4$ 0.1, NaCl 0.25, $MgSO_4.7H_2O$ 0.04, $CaCl_2.2H_2O$ 0.04, olive oil 2.0 (v/v), pH 7.0, 1-2 drops of Tween 20 as emulsifier. Overnight cultures were suspended in 5 mL of deionized water and used as inoculums. Submerged microbial cultures were incubated in 500 mL Erlenmeyer flasks containing 100 mL of liquid medium on a rotary shaker[8] (150 rpm) at 36°C. After 24 h of incubation, the culture was centrifuged at 10,000 rpm for 20 min at 4°C and the cell free culture supernatant fluid was used as the source of extracellular enzyme.

The culture from the lipase production was centrifuged at 10,000 rpm for 15 min at 4°C. After which the supernatant was collected in sterile Mccathney bottles as crude extracellular enzyme that was used in the lipase activity assay. Lipase assay was determined by titrimetric method using olive oil as a substrate according to the method of Pualsa *et al.*[9]. Olive oil (10% v/v) was emulsified with Gum Arabic (5% w/v) in 0.1 M potassium phosphate buffer pH 7.0. 0.1 mL of enzyme was added to the emulsion and was incubated for 15 min at 37°C. The reaction was stopped and fatty acids were extracted by the addition of 1.0 mL of acetone: ethanol solution (1:1). The amounts of fatty acids liberated were estimated by titrating with 0.1 N NaOH using a phenolphthalein indicator until a light pink coloration appeared. The reading for the blank was also taken the blank had no enzyme in it.

The lipase activity was calculated using:

$$\text{Lipase activity } (\text{U mL}^{-1}) = \frac{\text{Milliliter NaOH for sample} - \text{Milliliter NaOH for blank}}{\text{Milliliter of lipase} \times \text{Reaction time}} \times N$$

where, N is normality of NaOH.

One unit of enzyme is defined as the amount of enzyme required to hydrolyse 1 µmol of fatty acids from triglycerides equivalent per minute under the assay conditions.

Optimization studies

Optimization of culture conditions for maximum lipase production: Four different media viz Mineral Salt Medium (MSM) contained (g/100 mL): K_2HPO_4 (0.18) KH_2PO_4 (0.12), NH_4Cl (0.4), $MgSO_4.7H_2O$ (0.02), NaCl (0.01), yeast extract (0.01), $FeCl.4H_2O$ (0.005), H_3BO_3 (0.01) $ZnSO_4.7H_2O$ (0.01) $CuSO_4.5H_2O$ (0.005), $MnSO_4.H_20$ (0.004) and 2% (v/v) olive oil according to the modified method of Kareem *et al.*[7], Maltose Peptone Yeast Extract (MPYE) medium contained 1% (v/v) olive oil, Maltose (1 g), Peptone 0.5% (w/v), yeast extract (0.1 g), K_2HPO_4 (0.1 g), $MgSO_4.7H_2O$ (0.02 g) and Na_2CO_3 (1.5 g)[10], tryptic soy broth (1.6 g) containing K_2HPO_4 (0.18 g) KH_2PO_4 (0.12 g), $MgSO_4.7H_2O$ (0.02 g), $FeCl.4H_2O$ (0.005 g) and medium A contained peptone 0.5% (w/v), $NH_4H_2PO_4$ (0.1), NaCl (0.25), $MgSO_4.7H_2O$ (0.04 g), $CaCl_2.2H_2O$ (0.04 g), 2% (v/v) olive oil, Tween 80 (1-2 drops)[11] were investigated for maximum lipase production by the selected isolate. Each medium was incubated at 35°C and 120 rpm for 48 h. Then they were centrifuged at 10,000 rpm for 20 min at 4°C after which the supernatant of the culture was assayed as described above for lipase yield. The best medium was used for further studies of various parameters for maximum lipase activity of the selected isolate.

Effect of incubation period on lipase activity: The selected isolate was cultured in Mineral Salt Medium containing 2% (v/v) olive oil at 37°C for 5 days by centrifugation at 10,000 rpm for 20 min at 4°C. The supernatant collected was used as crude enzyme solution and was assayed for lipase activity in an in orbital shaker at agitation speed of 150 rpm. The culture was harvested at 24 h interval.

Effect of temperature on lipase activity: For selection of the optimum temperature for lipase activity, temperatures varying from 25-65°C were selected by keeping the remaining parameters same.

Effect of ph on lipase activity: The optimum pH for lipase activity was selected by varying the pH of the Mineral Salt Medium from 5.5-9.5 while keeping the other parameters unaltered.

Gravimetric analysis: The amount of oil in culture was estimated using the Gravimetric method. Diethyl ether and acetone were taken in 1:1 ratio and was mixed with the culture. The mixture was allowed to vaporize at room temperature. The oil residue obtained was weighed and taken as the gravimetric value for further calculation[7]:

$$\text{Amount of oil in culture} = \frac{\text{Weight of oil degraded}}{\text{Weight of oil added to the medium}} \times 100$$

where, weight oil degraded can be calculated by using:

$$\text{Oil degraded weight} = \text{Original weight of PMS oil - weight of residual PMS oil obtained after evaporating the extract}$$

Determination of lipase biodegradation of petroleum hydrocarbons: The degrading activities of the crude enzyme were obtained using Mineral Salt Broth (MSB) in which 4 mL of each hydrocarbon (petrol and diesel) was added and incubated at room temperature for 20 days. The enzyme activity was measured by taking the Optical Density (OD) readings at 600 nm every 5 days till the 20th day against Mineral Salt Medium (MSM) as blank[7].

Statistical analysis: Data was expressed as mean ± SEM for all assays. Graphs and bar charts were used to express data in a more comprehensive form.

RESULTS

Isolation, screening and identification of microorganisms Isolates: Nine microorganisms (six bacteria and three fungi) were isolated from the soil sample. One bacterial microorganism showed maximum zone of clearance as shown in Plate 1 on mineral salt medium having petroleum as its carbon source. The selected isolate was identified by morphological, biochemical and molecular characterization using 16S rRNA sequencing-based methods. The 16S rRNA sequence of the isolate was submitted to NCBI and identified as *Providencia stuartii* MRB-44 with the accession number KY568710.1 and percentage identity 99.87%. The phylogenetic tree of *Providencia stuartii* MRB-44 in relation to close species is presented in Fig. 1.

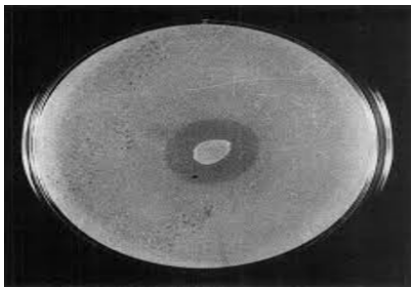

Plate 1: *Providencia stuartii* MRB-44 showing zone of clearance on MSM 1% (v/v) petrol as carbon source

Table 1: Biodegradation of petroleum hydrocarbons

Test enzyme+hydrocarbons	OD at (600 nm)	Oil degradation (%)
Free lipase+PMSs	0.28	60.89*
Free lipase+diesel	0.40	56.19*
PMS with no enzyme (Control)	1.76	0.00

*Value of triplicate samples

Determination of lipase biodegradation of petroleum hydrocarbons: Results of Table 1 showed the biodegradation of petroleum hydrocarbons by lipase from *Providencia stuartii* over a period of 20 days. The lipase enzyme from *Providencia stuartii* was able to degrade the hydrocarbons to 60.89%. The oil layer slowly emulsified and disappeared with incubation.

Optimization of culture conditions and effect of variable conditions for maximum lipase production: From the results shown in Fig. 2, it was found that MSM with 2% (v/v) olive oil medium facilitated maximum lipase yield by *Providencia stuartii* with lipase activity of 0.033 U mL^{-1} and hence was used throughout the study.

Lipase activity was at its maximum at 48 h having lipase activity of 0.033 U mL^{-1} and then declined with longer incubation time as shown in Fig. 3. The temperature preference of this enzyme revealed optimal activity value at temperature 35°C with activity of 0.05 U mL^{-1} (Fig. 4). This showed that the enzyme was mesophilic. The effect of pH on the activity of lipase was determined in different buffers covering the range of 5.5-9.5. The enzyme had optimal pH 7 with lipase activity of 0.047 U mL^{-1} (Fig. 5).

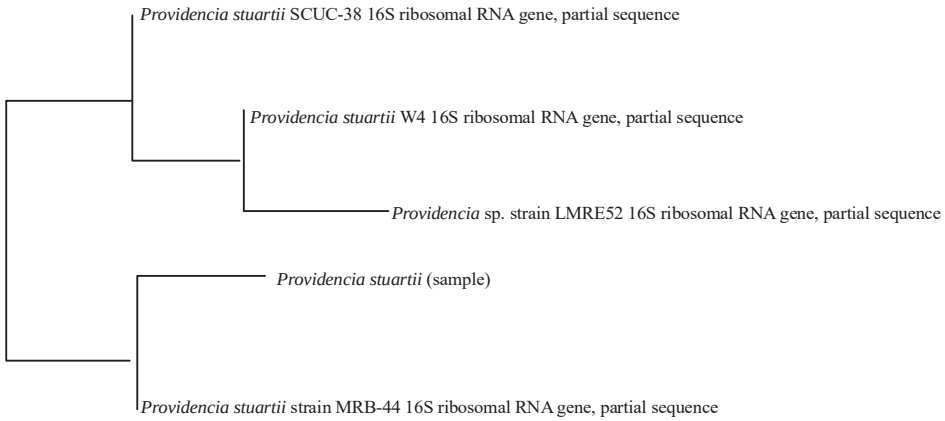

Fig. 1: Phylogenetic tree of *Providencia stuartii* in relation to close species

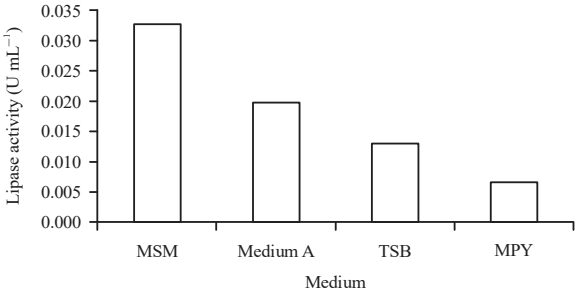

Fig. 2: Effect of culture medium on lipase activity
MSM: Mineral salt medium with 2% olive oil, MPY: Maltose peptone yeast medium, TSB: Tryptic soy broth medium

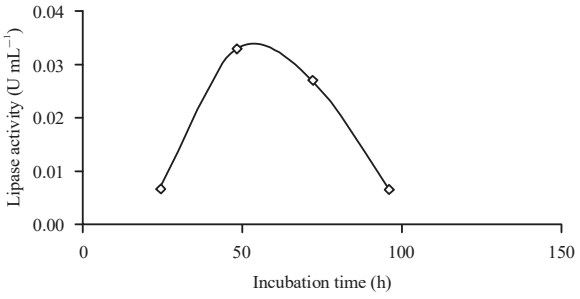

Fig. 3: Effect of incubation period on lipase activity

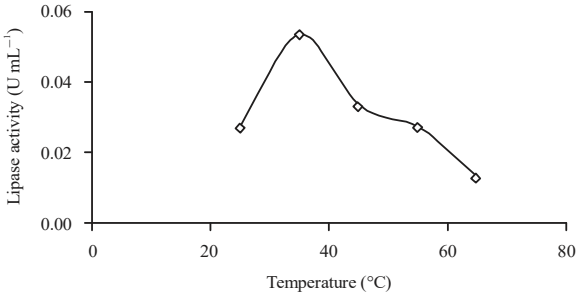

Fig. 4: Effect of temperature on lipase activity

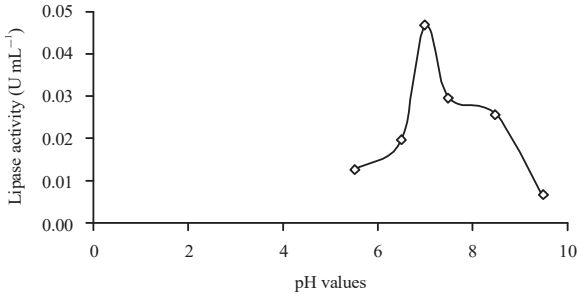

Fig. 5: Effect of pH on lipase activity

DISCUSSION

Hydrocarbon-degrading microorganisms are widely distributed in marine, freshwater and soil ecosystems. The ability to isolate certain oil degrading microorganisms from an environment is commonly taken as evidence that those organisms are the active degraders of the constituents of that environment. *Providencia stuartii* MRB-44 was prominent among the bacteria isolated from an oil contaminated soil in Rivers state. This agrees with earlier reports by Kumar *et al.*[12] which stated that *Bacillus* sp., *Pseudomonas* sp., *Micrococcus* sp. and *Aeromonas* sp., are among bacteria found in oil-contaminated areas. *Providencia stuartii* is a Gram-negative bacillus that is commonly found in soil, water and sewage. *P. stuartii* is the most common of the 5 species found in the genus Providencia. It is motile via flagella, non-sporulating, non-lactose fermenting, catalase positive and oxidase negative. It can also grow in anaerobic conditions. The presence of *Bacillus* species could be attributed to their ability to produce spores which enable them to survive in a different environment including hydrocarbon polluted soils[13].

The bacterial cultures initially isolated from the soil were inoculated on mineral salts medium with 1% PMS and diesel as the carbon source each to determine their biodegrade ability. The organism that showed maximum zone of clearance was discovered to be *Providencia stuartii* MRB-44. It was able to utilize the PMS as its carbon source. This corresponds to the findings of Kareem *et al.*[7] where the bacterial isolates were able to utilize PMS as their carbon source.

The organism was identified based on its rRNA gene sequence. The sequence matched the nucleotide sequence present in Gen Bank at NCBI database using FLINCH TV program. The nucleotide sequences showing similarity to the query sequence were retrieved and a neighbor-joining phylogenetic tree was created. The tree showed that the closest taxa were obtained as *Providencia stuartii* strain FC297, hence the query organism was identified as *Providencia stuartii* MRB-44.

The lipase produced by *Providencia stuartii* from the lipase production medium showed biodegradable activities and values of the degraded PMS and diesel varied after 20 days of incubation. Table 1 presented degradation of PMS and Diesel with free lipase of *Providencia stuartii.* The free lipase degraded 60.89% PMS and 56.19% diesel. Optimization of culture medium for maximum lipase production was carried out. The result showed that MSM having 2% olive oil had the maximum lipase yield of 0.0033 U mL^{-1} and hence was used throughout the study. The major factor for the expression of lipase activity has always been carbon, since lipases are inducible enzymes[14] and are always produced in the presence of a lipid source such as oil or any other inducer such as; triacylglycerols, fatty acids, glycerols etc. Among the different carbon sources used, olive oil was found to be the most suitable source as shown in Fig. 3. Most published experimental data have shown that lipid carbon sources (especially natural oils) stimulate lipase activity. High levels of lipase activity were reported by Eltaweel *et al.*[15] in the presence of olive oil as carbon source in the culture medium.

The presence of maltose in the cultivation medium depressed the production of lipase. Maltose supplementation to the basal medium inhibits lipase production, which reveals that carbohydrates are not effective in enhancing lipase production. The loss of activity in the presence of a carbohydrate source might be due to catabolic repression[16]. Beside the carbon source, the type of nitrogen source in the medium also influenced the lipase yield in the production medium. *Providencia stuartii* released maximum lipase when organic nitrogen sources like yeast extract and peptone were used in the MSM medium and medium A, which have been used for lipase production by various thermophilic *Bacillus* sp. and *Staphylococcus* sp.[17]. Other media component such as K_2HPO_4, $MgSO_4.7H_2O$ and NH_4Cl increased lipase production.

Providencia stuartii exhibited its maximum lipase activity at 48 h at the early stationary phase. The activity of the enzyme gradually decreased after 72 h. This finding was also supported in *Staphylococcus pasteuri* according to Aruna and Karim[10], had its maximum lipase activity at 48 h. An incubation period of 12 h was optimum for lipase production by *Acinetobacter calcoaceticus*[18].

The temperature preference of *Providencia stuartii* reveals higher lipase activity value at temperature 35°C as shown in Fig. 4. Physically, the medium appeared to be most turbid at this temperature, being followed closely by 45°C. Then it decreased with increasing temperature, perhaps due to reduced growth or lower survival. This shows that the enzyme was mesophilic. This finding supports the data by Zhang and Zeng[19] in *Pseudomonas* sp. 7323. It has been observed that in general, lipases are produced in the temperature[20] range 20-45°C.

The lipase activity was observed from 5.5-9.5 when the effect of pH was determined. The enzyme was most active at pH 7 as seen in Fig. 5, showing preference from pH 7-8.5. It can be characterized as a neutral enzyme. A comprehensive review on all bacteria lipases done by Gupta *et al.*[20] which stated that maximum activity of lipases at pH values higher than 7 has been observed in many cases. Bacterial lipases have a neutral or alkaline optimum pH, with the exception of lipase from *Pseudomonas fluorescens* SIK W1 that has an acidic optimum pH[21] of 4.8.

CONCLUSION

It is evident from the outcome of this study that hydrocarbon utilizing and/or degrading microorganisms could readily be isolated from oil contaminated soil samples and possibly used for bioremediation of hydrocarbon polluted soil. It also showed that *Providencia stuartii* which is generally known as an opportunistic and pathogenic microorganism has lipase producing potentials and can be used in hydrocarbon degrading processes. Finally, the enzymatic degradation of petroleum hydrocarbon using lipase from *Providencia stuartii* is an effective and eco-friendly biotechnological approach. Lipase proved to be an excellent biodegradation tool in remediating oil spills.

SIGNIFICANCE STATEMENT

This study discovers the potential of lipase enzyme from *Providencia stuartii* which serves as a simple and effective

biodegradation tool for degradation of hydrocarbon products-gasoline and diesel. This study will help the researcher to uncover the critical areas of environmental microbiology in combating the oil polluted areas. This study discovers the use of microbial enzymes as an alternative means as oppose the direct use of microorganisms in biodegradation of oil polluted sites which may be technically difficult.

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