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Research Article



## Isolation and Characterization of Petroleum-Degrading Microorganisms from Contaminated Soil for Bioremediation

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### Abstract

Petroleum pollution remains a significant environmental challenge, posing risks to ecosystems, groundwater quality, agricultural productivity, and human health due to the toxicity of hydrocarbons. This study aimed to investigate the bioremediation potential of indigenous microorganisms isolated from petroleum-contaminated soil collected from mechanic workshops at FUTA junction, Akure, Ondo State, Nigeria. Petroleum-polluted soil samples were collected in sterile containers and transported to the microbiology laboratory, where they were stored at 4°C. Microorganisms were isolated and characterized based on their morphological and biochemical properties. Bacterial isolates included *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Enterobacter aerogenes*, *Micrococcus luteus*, and *Staphylococcus aureus*; fungal isolates included *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus ustus*, and *Candida* species. The bacterial load ranged from  $1.87 \times 10^7$  to  $2.52 \times 10^8$  cfu/ml, while the fungal load ranged from  $6.4 \times 10^5$  to  $1.3 \times 10^6$  sfu/ml. Biodegradation potential was assessed by monitoring pH variation, optical density, and free CO<sub>2</sub> production as indicators of hydrocarbon metabolism. *Pseudomonas aeruginosa* and *Aspergillus terreus* exhibited the highest biodegradation efficiency. Biochemical characterization revealed diverse metabolic capabilities among isolates: *Staphylococcus aureus* and *Bacillus subtilis* produced spores; *Micrococcus luteus* and *Enterobacter aerogenes* were citrate

positive; all isolates except *Enterobacter aerogenes* were urease positive; various fermentation patterns were observed across glucose, mannitol, maltose, lactose, and sorbitol substrates. Hydrocarbon-degrading microorganisms can be readily isolated from petroleum-polluted soils, and the high degradative abilities observed, particularly for *Pseudomonas aeruginosa* and *Aspergillus terreus*, demonstrate their potential applicability in large-scale bioremediation efforts.

**Keywords:** *Bioremediation, Hydrocarbon, Microorganisms, Genetic engineering, Enzymes, Pollution*

## Introduction

Petroleum, often known as crude oil, is a complex hydrocarbon mixture, with aromatic and saturated hydrocarbons making up the majority. A mixture of thousands of chemicals, mostly carbon and hydrogen, it is a thick liquid (Moses et al., 2025). Among the world's most vital energy resources, crude oil is paramount. In the petrochemical industry, for example, crude oil is transformed into asphalt, paraffin oils, lubricants, gasoline, vaseline, polymers, and home fuel oil through a series of technological procedures (Agrawal and Shabi, 2017). Over very long periods of time, the breakdown of living things causes crude oil to accumulate underground. The hydrocarbon compounds included in petroleum products can be extremely harmful to various creatures, including humans, when present in high enough quantities (Bautista and Rahman, 2018). Oil pollution occurs when crude oil or its product is released into the environment due to human activities (Bijay et al., 2012). Oil pollution has been observed over the years to have detrimental effects on both plants and animals. Oil spills cause pollution in the soil and are consequently destructive to vegetation due to lack of oxygen and the evolution of H<sub>2</sub>S which kills the roots of most plants (Boboye et al., 2010; Cheesbrough, 2006). Oil pollution occurs when crude oil or its product is released into the environment due to human activities (Das and Chandran, 2010).

Biodegradation which is the breakdown of organic matter by microorganisms such as bacteria and fungi (Fawole and Oso, 2007), helps in the removal of oil spills from the environment. Various microorganisms have been discovered to be of great importance in the degradation of petroleum in the environment. Such microorganisms utilise crude oil as substrate and this results in the biodegradation of crude oil (Geetha et al., 2013). Microbes can return the environment to its natural state by utilizing both synthetic and naturally occurring organic molecules as food and energy sources (Ijah and Antai, 2003). According to Latha and Kalaivani (2012), microbes possess enzyme systems that enable them to break down crude oil and its byproducts, such as gasoline, into carbon and energy. This process is known as biodegradation. Environmental factors, including temperature, pH, and relative humidity, and the make-up of the native microbial population are critical in the bioremediation of contaminants (Makut and Ishaya, 2010). The hydrocarbons provide the native microbes with energy and nourishment, allowing them to develop and multiply (Nwaogo et al., 2008).

Chemical dispersants, surface oil skimming, biological oil agents, and inoculation with relevant bacteria are some of the activities that have been found to be effective in reducing or eliminating the negative effects of oil spillage on the environment and living organisms (Olukunle and Oyegoke, 2016). Among the several studies conducted to mitigate the consequences of large-scale oil spills, the employment of microbes shows the most promise (Sharma et al., 2015). Testing the biodegrading capacities of microorganisms linked to oil spills is thus necessary. So, the goal of this research was to find microbes that could break down petroleum oil and then analyze them in detail.

## MATERIALS AND METHODS

### Sample collection and preparation

The research work was carried out between January 2018 and October 2018. The study area for the collection of samples was mechanic workshops at FUTA junction area of Akure, Ondo State, Nigeria. The petroleum polluted soil was collected into a sterile container then transported immediately to the microbiology laboratory where it was stored in a refrigerator at 4°C.

### Preparation of Culture Media

Nutrient agar (NA) and Potato Dextrose Agar (PDA) were separately prepared according to their manufacturers' specifications in separate conical flasks. The mixtures were further dissolved by heating on a hot plate with frequent agitation until complete dissolution of the agar powders were observed. The conical flasks were then plugged with cotton wool and wrapped with aluminium foil before being sterilized at 121°C for 15 minutes under 15mmHg pressure with the aid of an autoclave. After sterilizing, the media were allowed to cool to about 45°C before chloramphenicol was added to the sterile PDA in order to inhibit the growth of bacteria (Singh et al., 2007).

### Sterilization of materials used

Glass wares including test tubes, conical flask, measuring cylinders, pipettes, beakers and bijou bottles that were used were sterilized by autoclaving at 121°C for 15 minutes. Forceps and inoculating loops were flamed until red-hot with the aid of bunsen burner before use, incubators and inoculating chambers were fumigated with 40% formaldehyde. Work benches were also constantly disinfected using cotton wool previously moistened in 70% ethanol.

### Isolation of Bacterial Isolates

One gram of the soil sample was microbiologically analyzed by firstly homogenizing with 10ml of sterile water. This was used to make a 6- fold dilution. 1ml each of the dilution factor  $10^6$  and  $10^5$  were then inoculated on nutrient agar via pour plate method. The bacterial load was then enumerated on the nutrient agar after 24 hours of incubation at  $37^{\circ}\text{C}$ .

### Subculturing to Obtain Pure Bacterial Isolates

After 24 hours of incubation at  $37^{\circ}\text{C}$ , the colonies were observed and counted. Five discrete colonies in total were subcultured on freshly prepared nutrient agar plates by the streak plate method and incubated at  $37^{\circ}\text{C}$  for 24 hours. The pure isolates gotten were preserved on freshly prepared agar slant bottle containing nutrient agar before biochemical tests were further carried out (Ajona and Vasanthi, 2020).

### Identification and Characterization of Bacterial Isolates

The bacterial isolates were characterized using morphological and biochemical tests such as gram stain, spore stain, and catalase, coagulase, and indole, urease and sugar fermentation tests (Udeani et al., 2009). The identification of the isolated bacteria was based on their individual colony morphology and biochemical tests which are described below:

#### Gram Staining Procedure

Gram staining was carried out to differentiate the bacterial isolates into either Gram positive or Gram negative based on their interaction with the Gram staining dyes. The test was carried out by making a thin smear of the pure culture on a clean grease-free slide with the aid of sterile inoculating loop. The prepared smear was allowed to air-dry, then heat- fixed by rapidly passing the slide over a gentle flame three times. The smear was thereafter flooded with crystal violet stain (a primary dye) and allowed to stand for 1 minute before being washed away with distilled water from a wash bottle. Lugol's iodine (a mordant) was added to the smear and allowed to stay for 30 seconds before washing off with water. The smear was decolorized with 70% ethanol for 30seconds before being counter stained with safranin solution (a secondary stain) for about 1 minute. The slide was afterward washed with water, cleaned with cotton wool and allowed to dry. The gram-stained slides were then examined microscopically under x40 and x100 oil immersion objectives lens. Gram positive bacteria appeared purple under the microscope as a result of the primary dye whereas Gram negative bacteria stained red or pink as a result of their ability to retain the

secondary dye which is safranin (Akhtar and Mannan, 2020).

#### Spore Staining

Spore staining is a kind of differential staining technique that is used to distinguish between vegetative cells and the endospore (Ani et al., 2021). A smear of the inoculum was made on a clean, grease- free slide and allowed to air-dry. The slide was then heat-fixed. The slide was placed on a wire gauze (with the smeared part facing upwards) which was also placed on a conical flask containing water which was made to boil with the aid of a bunsen burner and tripod stand setting. Malachite green which served as the primary stain was used to flood the smeared slide for about 5 minutes while ensuring that a drop of two of malachite green was added at intervals so as to keep the surface of the smear moist. The slide was afterwards rinsed with water before the addition of safranin which served as the counter stain for about 2 minutes. The slide was then rinsed with water and then allowed to air-dry. The cells were observed under oil immersion using the x100 objective lens of the microscope (Mida et al., 2025).

#### Sugar Fermentation Tests

This test is used to determine the ability of an organism to ferment various sugars. Five ml of a mixture of nutrient broth, sugar and 2 drops of phenol red was pipetted into test tubes aseptically. The sugars used were lactose, sucrose, fructose, glucose, maltose, mannitol and galactose. Durham tubes were inverted into the test tubes after which the test tubes were labelled appropriately and sterilized in an autoclave at  $121^{\circ}\text{C}$  for 15 minutes. After sterilization, each tube was inoculated with the test organism and incubated at  $37^{\circ}\text{C}$  for 24-48 hours. The tubes were afterwards examined for sugar fermentation and gas production. A colour change from red to yellow indicated the ability of the organism to ferment while no colour change indicated inability of the organism to ferment the sugar (Mida et al., 2025). The medium was stabbed and inoculated with the bacterium while an un-inoculated tube was retained as the control. All cultures were incubated at  $35^{\circ}\text{C}$  for up to 24 hours after which observations were made. Acid production in the culture was indicated by a change in colour of the medium from red to yellow. Acid production in both sealed and open tubes suggest a fermentation reaction; acid formation in the open tubes only suggests an oxidative utilisation of the sugar. The sugars used include; mannitol, fructose, galactose, glucose, lactose and sucrose.

#### Citrate test

Simmon's citrate agar is a differential medium that is used to demonstrate the ability of an organism to utilise

citrate as its sole source of carbon and energy. It is used to differentiate between members of the family *Enterobacteriaceae*. The agar contains bromothymol blue which is a pH indicator that turns blue at alkaline pH as a result of the alkaline compound produced by the organism upon utilization of the citrate. The test was performed by inoculating a freshly prepared citrate agar slant with a pure isolate from a 24 hours old culture with the aid of inoculating wire. The agar was incubated at 37°C for 18-24 hours. Colour change from green to blue after appropriate hours of incubation showed a positive result while no colour change indicated negative result (Mayel et al., 2025).

#### Urease test

The test organisms were cultured in a medium containing urea and the indicator phenol red. Positive urease test reaction involves the organisms breaking down urea into ammonia and carbon dioxide. With the release of ammonia, the medium becomes alkaline and is indicated by the colour change of the medium to pink-red. Colour change to pink-red is an indication that the test organism is urease positive. However, a negative urease test is indicated by yellow coloration. Urease slope was prepared in a bijoux bottle. The test organisms were then inoculated into the urease slope and incubated for 24 hours at 37°C (Moses et al., 2025).

#### Catalase test

Catalase test was carried out for the gram positive organisms. The test was used to characterize organisms based on their ability to produce the enzyme catalase which splits hydrogen peroxide into water and oxygen gas. A drop of hydrogen peroxide was added on a clean, grease free slide with the aid of a dropping pipette. With the aid of an applicator stick, a discrete colony of the test organism was picked from the 24-hour old plate and drop on the hydrogen peroxide. It was then observed for effervescence. Immediate bubbling indicates that the organism is catalase positive. The absence of bubbles of gas however indicates that the test organism is catalase negative (Moses et al., 2025).

#### Coagulase Test

Two drops of normal saline were placed on two clean, grease-free slides. With the aid of a sterile wire loop, a colony of the test organism was emulsified in each of the drops to make thick suspensions. A drop of plasma was then added with the aid of a sterile syringe and then, the contents on the slide were gently rocked. Observation for immediate clumping of the mixture was done within 5-10 seconds. The clumping indicates positive coagulase test. No plasma was added to the other suspension placed on the other slide which served

as a negative control to the test and to differentiate non-specific granular appearance from true coagulase clumping. The presence of clot indicates that the organism is coagulase-positive while the absence of clot indicates the presence of other species of *Staphylococcus* (Moses et al., 2025).

#### Indole Test

The test organisms were inoculated into peptone water and incubated at 35-37°C for 24 hours. After incubation, about two drops of Kovac's reagent was added and shaken. The appearance of red ring at the uppermost layer indicates that the organism is indole positive while the absence of red ring indicates that the organism is indole negative (Emochone et al., 2023).

#### Motility Test

A little amount of immersion oil was introduced round the edges of the depression of a glass cavity slide. A loopful of the bacterial isolate was transferred to the centre of a clean dry cover slip placed on a clean dry sheet of paper. The cavity slide was then inverted over the cover slip such that the culture drop was in the centre of the slide depression, the slide was pressed down carefully but firmly so that the oil seals the cover slip in position. The slide was quickly and carefully inverted so that the culture drops appear hanging. The slide was examined immediately for motility under the microscope (Timothy et al., 2022).

#### Isolation of Fungi

1 ml each of dilution factor  $10^4$  and  $10^5$  were inoculated on Potato Dextrose Agar via the pour plate method. The agar plates were incubated at 27°C for 72 hours.

#### Sub Culturing to Obtain Pure Fungal Isolates

Five fungal isolates were obtained from the pour-plating and each of the isolates was sub cultured on Potato Dextrose Agar (PDA) plates and incubated at 27°C for 72 hours by picking from their spores and transferring aseptically on a freshly prepared PDA plates with the aid of a sterile inoculating needle. Agar slants were thereafter prepared in sterile bijoux bottles and pure cultures were aseptically transferred into the bottles before incubation at 27°C for 72 hours was carried out. The cultures in slants were then refrigerated at 4°C.

#### Identification and Characterization of Fungal Isolates

The fungal isolates were identified by using the lactophenol cotton blue technique. A drop of cotton blue in lactophenol was placed on a clean grease-free

slide, a tiny mycelium was taken from the sub-cultured plate with an inoculating needle and placed on the clean grease- free slide, teased out to make a smear.

### Biodegradative Activities of the Bacterial Isolates

The Bushnell Haas broth containing the bacterial isolates was incubated at 37°C for seven (7) days in a rotary shaker at 120 oscillations per minute. The conical flasks containing the isolates in the Bushnell-Haas medium were incubated. The variation in pH, free CO<sub>2</sub> generated, and optical density was observed all throughout the period of incubation. The pH of the medium was determined with the aid of a pH metre which had been calibrated with buffer solutions before use, the optical density was measured using a spectrophotometer which was put on for about 15 minutes so as to allow it to stabilise before use. The free CO<sub>2</sub> generated was calculated using the formula:  
Free CO<sub>2</sub> (mg/L) = Titre value X Normality of NaOH X 1000 X MM of CO<sub>2</sub>

Volume of the sample  
Where:

Titre value = Volume of NaOH used Normality of NaOH = 0.1M  
1000ml = 1L

The slide was covered with a cover slip and examined under the microscope with x40 objective lens for morphological structures. The fungal isolates were also characterized based on the colour of the colony and vegetative parts (Ibraheem et al., 2025).

### Biodegradative Activities of the Microbial Isolates

#### Preparation of Selective Medium for the Bacterial Isolates

The pure bacterial isolates were inoculated into 100ml conical flasks containing Bushnell Haas broth; a special medium which is used to evaluate the ability of bacteria to degrade hydrocarbon. The Bushnell Haas medium consisting of MgSO<sub>4</sub> (0.2g/L), CaCl<sub>2</sub> (0.02g/L), KH<sub>2</sub>PO<sub>4</sub> (1.0g/L), FeCl<sub>2</sub>(0.05g/L) was prepared by dissolving the reagents in 1 litre of clean water and sterilised at 121°C for 15 minutes in an autoclave. The broth was supplemented with 1% v/v of petrol as the source of carbon. The petrol was sterilised by passing through a sterile syringe filter.

MM of CO<sub>2</sub> = 44g/mol Volume of the sample = 5ml

### Biodegradative Activities of the Fungal Isolates

The inoculated Czapek Dox broth containing the pure fungal isolates was incubated in a rotary shaker at 27°C for seven (7) days at 120 oscillations per minute. It was ensured during the period of incubation that the variation in pH, optical density and the free CO<sub>2</sub> generated was observed. The pH of the medium was determined with the aid of a pH metre which had been calibrated with buffer solutions before use, the optical density was measured using a spectrophotometer which was put on for about 15 minutes so as to allow it to stabilise before use. The free CO<sub>2</sub> generated was calculated using the formula:

Free CO<sub>2</sub> (mg/L) = Titre value x Normality of NaOH x 1000 x MM of CO<sub>2</sub>

Volume of the sample  
Where:

Titre value = Volume of NaOH used Normality of NaOH = 0.1M  
1000ml = 1L

MM of CO<sub>2</sub> = 44g/mol Volume of the sample = 5ml

#### Preparation of selective medium for fungi

The pure fungal isolates were inoculated into 100ml conical flasks containing Czapek Dox medium; a selective enriched medium was used to evaluate the ability of fungi to degrade petrol. Czapek Dox medium consists of KH<sub>2</sub>PO<sub>4</sub> (1g/L), NH<sub>4</sub>NO<sub>3</sub> (1g/L), MgSO<sub>4</sub>(0.2g/L), KH<sub>2</sub>PO<sub>4</sub> (1g/L), CaCl<sub>2</sub> (0.02g/L), FeCl<sub>3</sub> (0.05g/L) and distilled water(1L). It was then sterilized in an autoclave at 121°C for 15 minutes after which 1 ml of 1% v/v filter sterilised petrol which served as the carbon source, was added into each conical flask containing the medium.

## RESULTS

#### Determination of Microbial Load of Isolates

Observed growth of bacteria and fungi was enumerated on nutrient agar plates and Potato Dextrose Agar plates respectively after incubation, as illustrated in Table 1 below. The bacterial load that was observed on the nutrient agar plates was 1.87 x 10<sup>7</sup> cfu/ml and 2.52 x 10<sup>8</sup> cfu/ml. The observed fungal load on the incubated Potato Dextrose Agar plates was observed to be 1.3 x 10<sup>6</sup> sfu/ml and 6.4 x 10<sup>5</sup> sfu/ml.

**Table 1.** Microbial load of isolates

Agar	Dilution Factor	Plate count
Nutrient Agar	10 <sup>5</sup>	2.52 x 10 <sup>6</sup> cfu/g
	10 <sup>6</sup>	1.87 x 10 <sup>7</sup> cfu/g
Potato Dextrose Agar	10 <sup>4</sup>	6.4 x 10 <sup>5</sup> sfu/g
	10 <sup>5</sup>	1.3 x 10 <sup>6</sup> sfu/g

### Identification of Bacterial Isolates

The bacterial isolates were identified based on their reaction to gram stain, morphological and biochemical characteristics. Bacterial isolates that were identified are *Micrococcus luteus*, *Staphylococcus aureus*, *Enterococcus aerogenes*, *Pseudomonas aeruginosa* and *Bacillus subtilis*. All of the bacterial isolates were observed to have raised elevation on nutrient agar plates except *Enterobacter aerogenes* which had a flat elevation. *Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus subtilis* and *Enterobacter aerogenes* were observed to be cream in colour while *Pseudomonas aeruginosa* appeared white on nutrient agar plates. *Micrococcus luteus* and *Staphylococcus aureus* were observed to be gram positive cocci while *Enterobacter aerogenes* and *Bacillus subtilis* were observed to be gram positive bacilli while *Pseudomonas aeruginosa* was gram negative bacilli.

Only *Staphylococcus aureus* and *Bacillus subtilis* were observed to produce spores when subjected to spore staining test. *Micrococcus luteus* and *Enterobacter aerogenes* were observed to be citrate positive while the other bacterial isolates were negative. All the isolates were positive to urease except *Enterobacter aerogenes*. Only *Bacillus subtilis* and *Enterobacter aerogenes* fermented mannitol, while all except *Staphylococcus aureus* fermented glucose.

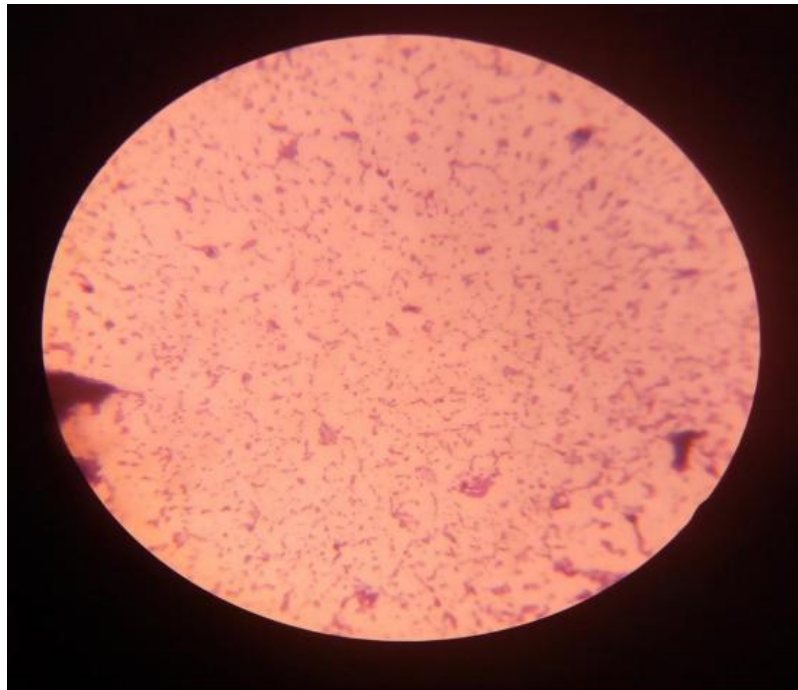
*Micrococcus luteus* and *Enterobacter aerogenes* fermented maltose, all of the bacterial isolates fermented lactose but none fermented sorbitol. Table 2 shows the morphological and biochemical characteristics of the bacterial isolates. All of the bacterial isolates were able to ferment lactose while none of them were able to degrade sorbitol. This is because they were all able to utilise lactose for their metabolism but could not utilise sorbitol. Plate 1 below shows gram positive reaction for cocci organism viewed under the microscope.

**Table 2.** Morphological and biochemical characteristics of the bacterial isolates

Suspected organism	<i>Micrococcus luteus</i>	<i>Staphylococcus Aureus</i>	<i>Enterobacter aerogenes</i>	<i>Bacillus cereus</i>	<i>Pseudomonas aeruginosa</i>
Elevation	Raised	Raised	Flat	Raised	Raised
Margin	Entire	Entire	Entire	Entire	Wavy
Colour	Yellow	Cream	Cream	Cream	White
Texture	Smooth	Smooth	Shiny	Dry	Mucoid
Shape	Cocci	Cocci	Bacilli	Bacilli	Bacilli
Gram	+	+	+	+	-
Spore	-	+	-	+	-
Motility	-	-	+	+	+

Citrate	+	-	+	-	-
Urease	+	+	-	+	+
Indole	-	+	-	-	+
Catalase	-	-	+	-	-
Coagulase	-	+	-	-	-
Mannitol	AG	A	-	-	AG
Glucose	AG	G	A	A	A
Fructose	-	A	A	A	-
Sucrose	A	-	A	A	-
Maltose	-	A	-	A	A
Lactose	A	A	AG	AG	AG
Galactose	A	A	A	G	A
Sorbitol	-	G	-	-	-

Key + = Positive - = Negative, A = Acid production AG = Acid and Gas production G = Gas production



**Plate 1. Gram positive cocci organism viewed under the microscope**

**Identification of Fungal Isolates**

All the fungal isolates were identified based on morphological appearances on Potato Dextrose Agar

plates and their reactions to lactophenol cotton blue stain. *Aspergillus niger* was observed to have a conidial head which was uniseriate and a tree like branching when observed after the lactophenol cotton blue

staining, it was also observed to have a powdery, black appearance on the agar plate. *Aspergillus terrus* was observed to have a small, round hyaline conidia attached to vegetative hyphae, it was also observed to have a powdery, dark brown appearance on the Potato Dextrose Agar plate. *Aspergillus ustus* had an elongated conidial head with bent hulle cells scattered throughout the mycelium, it also appeared to be

powdery and had a grey appearance on agar plate. *Candida* sp. appeared as a single cluster of blastoconidia which was round and elongate; it also had a whitish appearance with flat elevation on the PDA plate. Table 3 and Plate 2 shows the microscopic appearance and morphological characteristics of the fungal isolates.

**Table 3: Identification of fungal isolates**

Isolate	Microscopic Examination	Morphological characteristics	Suspected Fungal Isolate
A	Conidial head, uniseriate, tree- like branching	Powdery in nature and very black in colour. from the reverse sidethere is a graduation from white to yellow.	<i>Aspergillus niger</i>
B	Small, round hyaline conidia attached to vegetative hyphae	Powdery in nature and dark brown in colour. When viewed from the reverse side there is a graduation from white to cream	<i>Aspergillus terrus</i>
C	Elongated conidial head, bent hulle cells scattered throughout the mycelium.	Powdery in nature and grey in colour. When viewed from the reverse side, there is a graduation from white to pale yellow.	<i>Aspergillus ustus</i>
D	Single cluster of blastoconidia, which is round and elongate	Whitish appearance, flat elevation	C <i>Candida</i> sp.



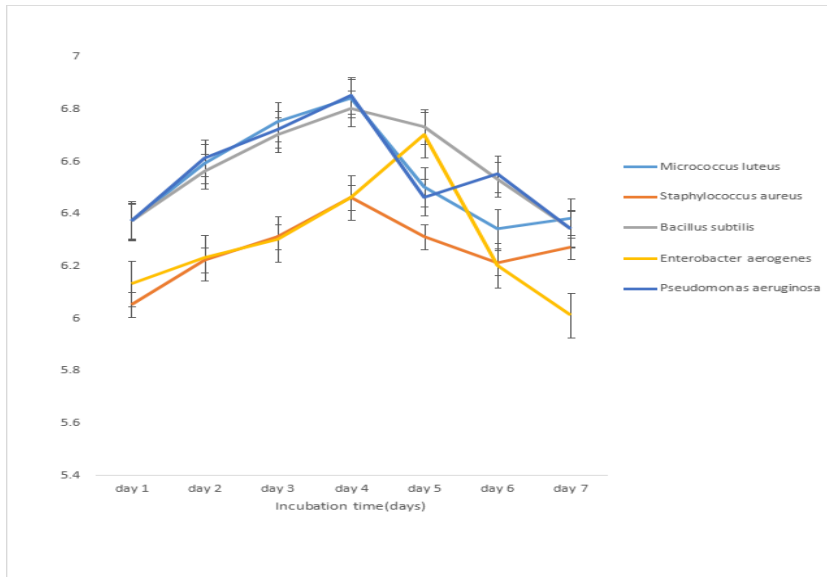
**Plate 2. Morphological structure of fungi mycelia under microscope**

**Degradative Activities of Microbial Isolates**

**pH of the Bacterial Isolates**

The pH of the bacterial isolates throughout the period of incubation in Bushnell Haas Medium is illustrated in Figure 1 below. *Bacillus subtilis*, *Pseudomonas*

*aeruginosa*, *Micrococcus luteus* and *Staphylococcus aureus* were observed to have their highest pH on the fourth day of incubation, while *Enterobacter aerogenes* was observed to have its highest pH on the fifth day. A general reduction in the pH of the bacterial isolates was observed on the seventh day of incubation

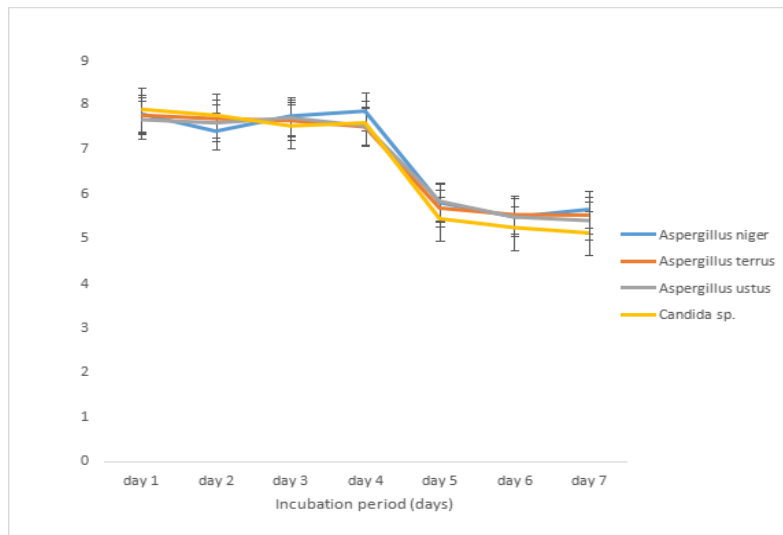


**Figure 1. pH of bacterial isolates during the incubation period**

**pH of Fungal Isolates**

The pH of the fungal isolates throughout the period of incubation in Czapek Dox Medium are illustrated in Figure 2 below. The pH of the fungal isolates were

observed to be at the highest on the fourth day and lowest on the seventh day of incubation. *Aspergillus niger* had the highest value of pH while *Candida* sp. had the lowest pH value during the incubation period.



**Figure 2. pH of fungal isolates during the incubation period**

### Optical Densities of Bacterial Isolates

The optical densities of the bacterial isolates during the incubation period are shown in Figure 3. *Pseudomonas aeruginosa* was observed to have its highest optical density on the fourth day of incubation; *Bacillus subtilis* and *Enterobacter aerogenes* had their highest

optical densities on the sixth day; *Micrococcus luteus* had its highest optical density on the fifth day while *Staphylococcus aureus* was observed to have its highest optical density on the seventh day of incubation. The optical densities of the bacterial isolates generally increased on the seventh day of incubation.

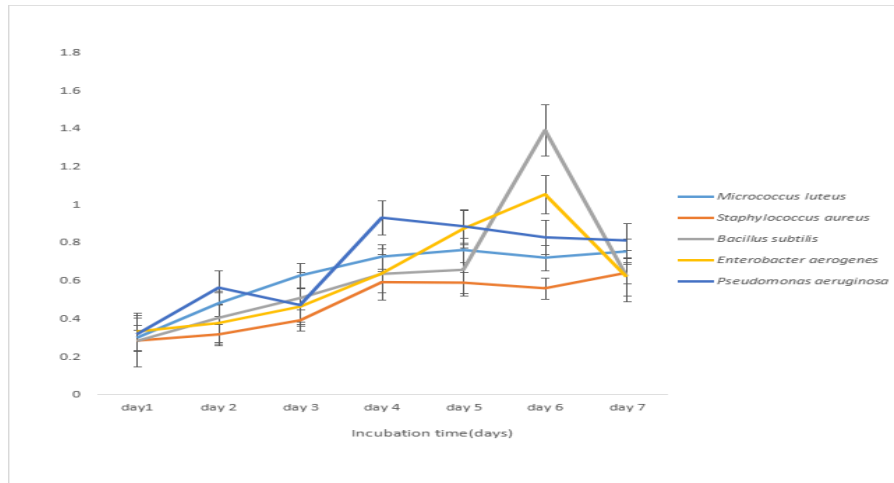


Figure 3. Optical densities of bacterial isolates during the incubation period

### Optical densities of Fungal isolates

The optical densities of the fungal isolates generally increased on the seventh day of incubation as illustrated in Figure 4. *Aspergillus niger* was observed to have its highest optical density on the third day; the optical

density of *Aspergillus terreus* was highest on the second day while *Aspergillus ustus* and *Candida* sp. had their highest optical densities on the seventh day of incubation. *Aspergillus terreus* generally had the highest optical density while *Candida* sp had the lowest optical density during the period of incubation.

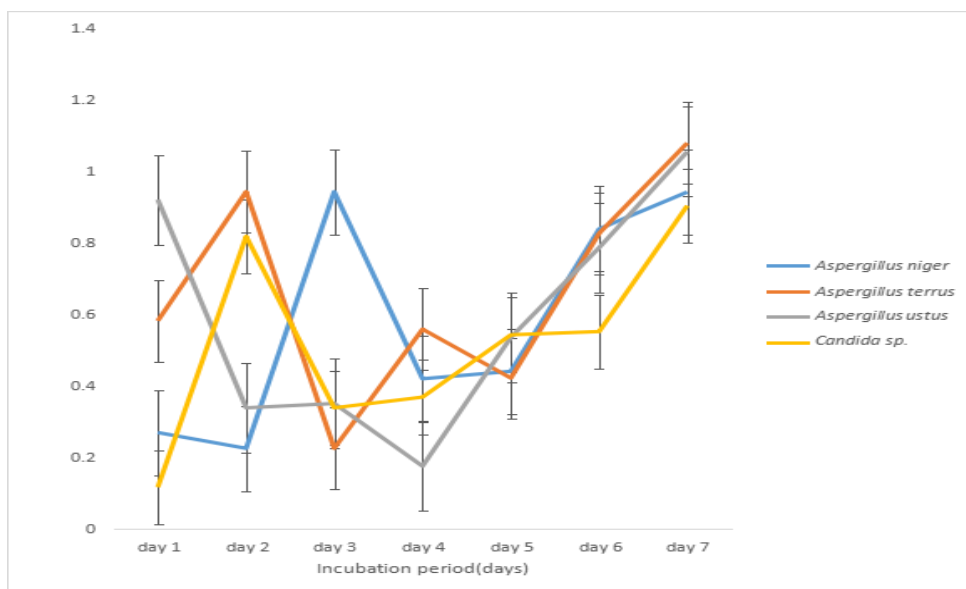
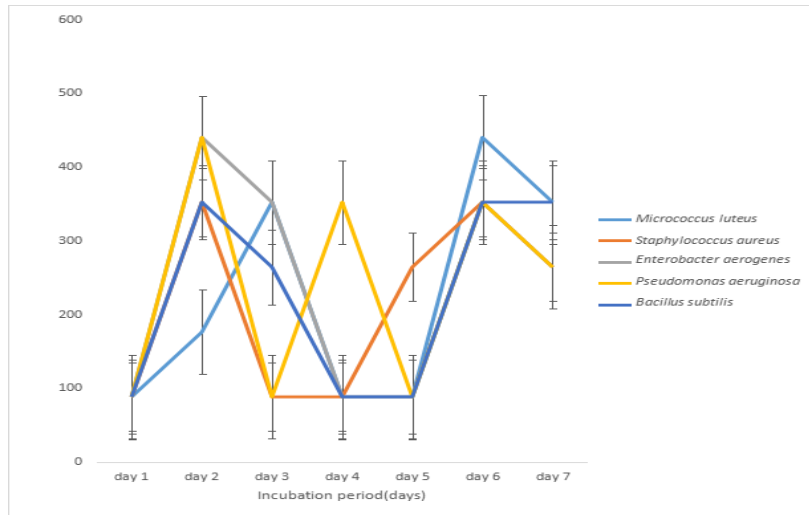


Figure 4. Optical densities of fungal isolates during the incubation period

**Free CO<sub>2</sub> Generated by Bacterial Isolates**

The free CO<sub>2</sub> generated by the bacterial isolates throughout the incubation period are illustrated in Figure 5. The free CO<sub>2</sub> generated by *Staphylococcus aureus*, *Micrococcus luteus* and *Enterobacter aerogenes* were observed to be at their highest on the

sixth day of incubation while *Bacillus subtilis* and *Pseudomonas aeruginosa* had their highest free CO<sub>2</sub> yield on the second day of incubation. The free CO<sub>2</sub> yield of the bacterial isolates were observed to generally reduce on the seventh day of incubation.

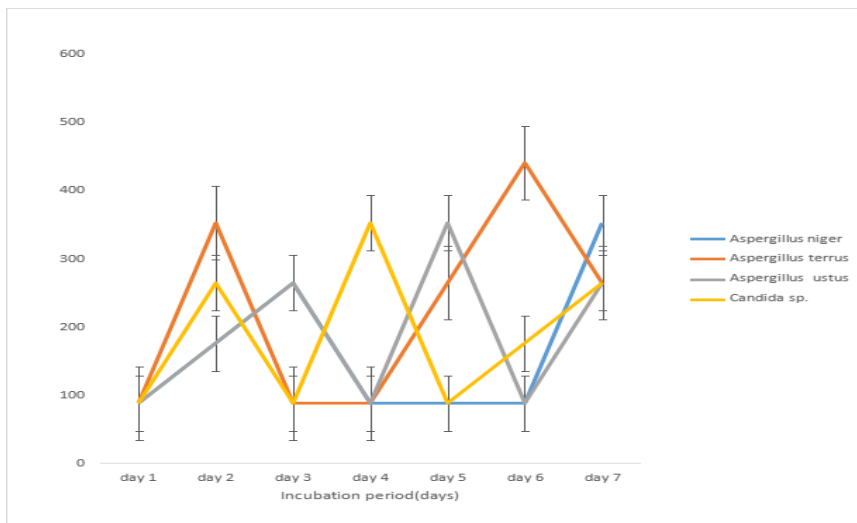


**Figure 5. Free CO<sub>2</sub> produced by the bacterial isolates during the incubation period**

**Free CO<sub>2</sub> Generated by Fungal Isolates**

The observation of the free CO<sub>2</sub> generated by the fungal isolates as illustrated in Figure 6 shows that *Aspergillus niger* had its highest yield of free CO<sub>2</sub> on the seventh day of incubation. *Aspergillus terrus* which

had the overall highest yield of free CO<sub>2</sub> throughout the period of incubation was observed to have its highest value of free CO<sub>2</sub> on the sixth day; *Candida* sp. and *Aspergillus ustus* had their highest free CO<sub>2</sub> on the fourth and fifth days of incubation respectively.



**Figure 6. Free CO<sub>2</sub> produced by the fungal isolates during the incubation period**

## Discussion

Microorganisms are capable of using organic substances whether natural or synthetic as their sources of nutrients and energy thereby restoring the original condition of the environment<sup>26</sup>. Biodegradation process is made possible because microorganisms have enzyme systems that helps to degrade and utilise crude oil and its products such as petrol as a source of carbon and energy (Ani et al., 2021). According to Singh et al. (2007), among the several studies conducted to mitigate the consequences of large-scale oil spills, the employment of microbes shows the most promise. Testing the biodegrading capacities of microorganisms linked to oil spills is thus necessary. Isolating, characterizing, and identifying microorganisms capable of decomposing petroleum oil was the primary goal of this investigation.

From this study, the result of the microbial load revealed a higher number of bacteria than fungi. This indicates that bacteria are more abundant than fungi in hydrocarbon polluted soil. Suspected bacterial isolates that were observed are *Micrococcus luteus*, *Staphylococcus aureus*, *Enterobacter aerogenes*, *Bacillus cereus* and *Pseudomonas aeruginosa*.

*Micrococcus luteus*, *Staphylococcus aureus*, *Enterobacter aerogenes* and *Bacillus subtilis* were observed to be gram positive bacteria while only *Pseudomonas aeruginosa* was gram negative. This indicates that gram positive bacteria were able to survive better in the hydrocarbon polluted soil. This could be because of the thicker layer of peptidoglycan which gram positive bacteria possess. Latha and Kalaivani (2012) in their research reported that the presence of a peptidoglycan cell wall makes gram positive organisms tolerant to high concentration of hydrocarbons. Unlike Gram positive bacteria, the cell wall of gram negative bacteria contains porins which aids its survival in the petroleum polluted environment. This account for the reason why *Pseudomonas aeruginosa* was able to survive in the petroleum polluted soil (Makut and Ishaya, 2010).

*Bacillus subtilis* is a known spore former. The ability to produce spores also accounts for its survival in the petroleum polluted soil. It was shown that hydrocarbon polluted soils, when challenged with *Bacillus subtilis* showed a reduction in the quantity of hydrocarbon pollutant with time (Nwaogu et al., 2008). The suspected fungal isolates are *Aspergillus niger*, *Aspergillus terrus*, *Aspergillus ustus* and *Candida* sp. The presence of varieties of species of *Aspergillus* indicates that they have a greater ability to survive in the petroleum polluted soil than other fungal isolates. The indigenous bacteria were metabolically active, since oil-degrading organisms were found in the contaminated soil. The bioremediation of environmental contaminants is a direct outcome of these microbes' actions (Olukunle and Oyegoke, 2016).

The pH values of all the bacterial isolates as illustrated in Figure 1 were observed to generally reduce on the seventh day of incubation in the Bushner Haas Broth. *Bacillus subtilis*,

*Pseudomonas aeruginosa*, *Micrococcus luteus* and *Staphylococcus aureus* were observed to have their highest pH on the fourth day of incubation while *Enterobacter aerogenes* was observed to have its highest pH on the fifth day. This shows that bacterial growth increased with decreasing pH over the seven days of incubation. This is in agreement with the work of Makut and Ishaya (2010) who showed in his studies that the reduction in the pH in cultures within the incubation period was as a result of chemical changes of the hydrocarbon substrate which must have been precipitated by microbial enzymes. The reduction in pH might also be due to the fact that microbial degradation of hydrocarbons often leads to the production of organic acids and other metabolic products (Singh et al., 2007).

A general reduction in the pH values of all the bacterial isolates as illustrated in Figure 2 was observed on the seventh day of incubation in the Czapek Dox Medium. *Aspergillus niger*, *Aspergillus terrus*, *Aspergillus ustus* and *Candida* sp. while the highest pH values were observed on the seventh day. This indicates fungal growth increased with decreasing pH over the seven days of incubation. This is in agreement with the work of Makut and Ishaya (2010) who showed in his studies that the reduction in the pH in cultures within the incubation period was as a result of chemical changes of the hydrocarbon substrate which must have been precipitated by microbial enzymes. The reduction in pH might also be due to the fact that microbial degradation of hydrocarbons often leads to the production of organic acids and other metabolic products (Udeani et al., 2009).

*Bacillus cereus* and *Enterococcus aerogenes* were observed to have their highest optical densities on the sixth day of incubation. *Pseudomonas aeruginosa* had its highest optical density on the fourth day of incubation. The optical density of *Staphylococcus aureus* was at its highest on the seventh day of incubation; *Micrococcus luteus* and *Bacillus cereus* had their highest value for optical density on the sixth day of incubation. This showed that all the bacterial isolates were able to utilise the hydrocarbon substrate for their growth even though the level of utilization varied (Nwaogu et al., 2008). Microbial cells are able to multiply within the days of study indicating that they were able to utilize the hydrocarbon substrate for their growth and development, hence the observed increase in the turbidity of the broth (Olukunle and Oyegoke, 2016). This increase in the optical densities of the bacterial isolates indicates bacterial growth and hence, their biodegradative abilities. *Pseudomonas aeruginosa* was observed to have the highest degradative ability among all the bacterial isolates while *Staphylococcus aureus* had the least degradative ability.

From the illustration on Figure 4 above which shows the variation in the optical densities. The optical densities of all the fungal isolates were observed to be at the highest on the seventh day of incubation. This showed that the rate of degradation by the fungal isolates were at their highest on the 7<sup>th</sup> day of incubation. *Aspergillus niger* had the highest optical

density while *Candida* sp was observed to have the least optical density (Ajona and Vasanthi, 2020).

The isolates involved in biodegradation have the ability to degrade a hydrocarbon substrate to give a free CO<sub>2</sub> yield. From the Figure 5 above, the free CO<sub>2</sub> yield of the bacterial isolates generally reduced on the seventh day of incubation. This indicates that the degradation process was completed on the sixth day of incubation. Variation in the amount of free CO<sub>2</sub> produced were also observed for the fungal isolates. *Aspergillus niger* had its highest free CO<sub>2</sub> release on the seventh day of incubation. *Aspergillus usus* had its highest yield of free CO<sub>2</sub> on the fifth day of incubation. *Aspergillus terrus* had its highest yield of free CO<sub>2</sub> on the sixth day of incubation. *Candida* sp. had its highest yield of free CO<sub>2</sub> on the fourth day of incubation. The free CO<sub>2</sub> released is an indication of the degradative action of the fungal isolates on the hydrocarbon substrate (Emochone et al., 2023). *Aspergillus terrus* had the highest yield of free CO<sub>2</sub> while *Candida* sp. had the lowest throughout the incubation period. This indicates that *Aspergillus terrus* was the most effective degrader of all the fungal isolates while *Candida* sp. had the least degradative ability of all.

## Conclusion

This study demonstrated that petroleum-contaminated soil harbors a diverse community of indigenous microorganisms with the capacity to degrade hydrocarbons and contribute to environmental restoration. Bacterial populations were more abundant than fungal populations, indicating a greater adaptability of bacteria to hydrocarbon-polluted environments. Among the bacterial isolates, *Pseudomonas aeruginosa* and *Bacillus* species exhibited superior biodegradative potential, attributed to their metabolic versatility, cell wall characteristics, and, in the case of *Bacillus*, spore-forming ability. Fungal isolates, particularly species of *Aspergillus*, also showed strong degradative activity, as evidenced by increased optical density and CO<sub>2</sub> evolution. Observed reductions in pH, increases in turbidity, and measurable CO<sub>2</sub> production confirmed active microbial metabolism and effective utilization of petroleum hydrocarbons. Overall, the findings highlight the importance of indigenous petroleum-degrading microorganisms and support their potential application as cost-effective and environmentally friendly agents for the bioremediation of hydrocarbon-contaminated soils.

## Significance Statement

Petroleum contamination of soils poses serious environmental and public health challenges, particularly in oil-impacted regions where effective remediation strategies are limited. This study identified and characterized indigenous bacteria and fungi capable of degrading petroleum hydrocarbons from contaminated soil, with *Pseudomonas aeruginosa*, *Bacillus* species, and *Aspergillus* species showing notable biodegradative efficiency. The observed reductions in pH,

increased microbial growth, and CO<sub>2</sub> evolution confirm active hydrocarbon metabolism by these microorganisms. These findings are significant because they demonstrate the inherent bioremediation potential of native microbial communities without the need for external inoculation. Broadly, this research supports the development of cost-effective, sustainable, and eco-friendly bioremediation approaches, contributing valuable insights to environmental microbiology and pollution management efforts globally.

## Authors' Contributions

The authors of this research have significantly contributed to the study's conception, data collection, and manuscript development. All authors were involved in writing the manuscript or critically reviewing it for its intellectual value. They have reviewed and approved the final version for submission and publication and accepted full responsibility for the content and integrity of the work.

## Conflicts of Interest

All authors declare that they have no conflict of interest associated with this research article.

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