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# **Characterization of Hydrocarbon Utilizing Bacteria and Fungi Associated with Crude Oil Contaminated Soil**

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## **Authors' contributions**

*This work was carried out in collaboration among all authors. Author DNO designed the study. Author VGA performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author SID managed the analyses of the study and literature searches under the strict supervision of author DNO. All authors read and approved the final manuscript.*

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## **ABSTRACT**

Many substances known to have toxic properties are regularly introduced into the environment through human activity. These substances which include hydrocarbons range in degree of toxicity and danger to human health. Frequent oil spills incidents have become a problem to ecological protection efforts. Conventional methods to remove, reduce or mitigate toxic substances introduced into soil via anthropogenic activities suffer setbacks due to the level of risk involved but bioremediation offers an alternative method to detoxify contaminants especially if the soil conditions are amended with organic nutrients or growth enhancing co-substrates. This study was therefore aimed characterizing hydrocarbon utilizing microorganisms associated with crude oil contaminated soils. Soils were obtained from the Rivers State University Agricultural farm contaminated deliberately with crude oil and allowed for 21 days to mimic the natural polluted soil. Sample collection and analyses were carried out according to standard microbiological procedures while characterization of the isolates was done using genomic studies. The results of microbial counts

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obtained from the soil samples for total heterotrophic bacteria ranged from  $2.10 \times 10^8$  to  $2.58 \times 10^8$  cfu/g, Total heterotrophic fungi had  $1.6 \times 10^5$  to  $2.0 \times 10^5$  cfu/g while the hydrocarbon utilizing bacteria ranged from  $8.0 \times 10^3$  to  $5.0 \times 10^4$  cfu/g and total hydrocarbon utilizing fungi ranged from  $9.0 \times 10^3$  to  $7.0 \times 10^4$  cfu/g in the contaminated soil. Five hydrocarbon utilizing bacterial species were identified as *Staphylococcus saprophyticus*, *Bacillus amyloliquefaciens*, *Pseudomonas aeruginosa*, *Comamonas testosteroni* and *Chryseobacterium cucumeris* while five hydrocarbon utilizing fungal species were identified as *Penicillium citrinum*, *Penicillium brocae*, *Fusarium solani*, *Kodamaea ohmeri* and *Lentinus squarrosulus*. *Bacillus* and *Penicillium* species were predominantly isolated from the soil. This may be due to the ability of the organisms to produce spores, which may shield them from the toxic effects of the hydrocarbons. Since these organisms are able to utilize crude oil as their sole carbon source. Hence, can be used for bioremediation of crude oil polluted environment.

**Keywords:** Hydrocarbon utilizing microbes; crude oil; contaminated soils; genomic studies; pollution.

## 1. INTRODUCTION

Crude oil is a naturally occurring complex mixture of hydrocarbon and non-hydrocarbon compounds which at appropriate concentration; possess a measurable toxicity towards living systems [1,2]. The toxicity of crude oil or petroleum products varies widely, depending on their composition, concentration, environmental factors and on the biological state of the organisms at the time of the contamination [3]. Leaks and accidental spills occur regularly during the exploration, production, refining, transport, and storage of petroleum and petroleum products. According to [4], an average of 240,000 barrels of crude oil are spilled in the Niger Delta yearly giving 31.85%, the third party activity records 20.74%, while mechanical failure of equipment has 17.04%. The oil spills contaminate the surface water, ground water, ambient air, and crops. The release of hydrocarbons into the environment whether accidentally or due to human activities is a main cause of water and soil pollution [3]. Oil spills have become a global problem particularly in industrialized countries and developing countries like Nigeria [5]. This crude oil pollution over the years has been a challenge to terrestrial and aquatic environments [6] and this has caused the greatest challenges to humanity especially the endangering of biota including the fauna and flora. Thus putting virtually every plant species and animals located at these contaminated sites in grave danger and also interfere with the ecological system and the environment at large [4]. Besides, the contamination of these habitats constitutes public health and socio-economic hazards [7]. Most of the lands and shorelines in the oil producing communities are important agricultural resources and are under continuous cultivation. Any damage will affect the soil

properties and plant communities due to associated changes in soil condition for example, when nutrient elements essential for plant growth are made unavailable [7,8]. At high concentrations of oil in the soil, most plants species suffer depression in growth due to poor soil condition, dehydration and impaired nutrient uptake by the roots created by the presence of crude oil [8].

However, microorganisms are widely distributed in nature and can easily grow in a wide range of environmental conditions and still maintain their metabolic ability [8,9]. Their nutritional versatility can also be exploited by biodegradation of pollutants. Microbes such as bacteria, archaea and fungi are constantly employed due to their ability to convert, modify and utilize toxic pollutants to obtain energy and increase biomass production in the process [8]. Bioremediation is a food procedural activity to break down or transform contaminants to less toxic or non-toxic elemental forms. There are basically two main approaches to oil bioremediation [7,9]. Bioaugmentation which involves the addition of oil degrading bacteria to supplement the existing microbial population and biostimulation is the addition of nutrients or growth enhancing co-substrates and/or improvement in habitat quality to stimulate growth of indigenous bacteria. Microorganisms restore the natural surroundings and also prevent further pollution by eliminating the spilled petroleum from the environment [3].

Over the decades, the after effect of oil spillage has left our land desolate, bleak and barren. This have also left man with the only option of searching for possible methods to remediate the situation and one of these methods is the use of microorganisms isolated from oil contaminated sites to degrade the oil spills [8]. Physical and

chemical methods have been employed in remediating the effects of oil spillage but these methods have not proven to be cost effective and do not remediate the environment back to its original state [10].

Although recent research findings by scientists has shown that microorganisms isolated from crude oil contaminated sites possess the ability to biodegrade crude oil and remediate oil spillage within the environment [1]. It is important to investigate the ability of microorganisms isolated from other environment to biodegrade crude oil and also the possibility of utilizing these organisms as possible agents of bioremediation. There is therefore need to investigate on new frontiers in this pressing area of research which will be cost effective, safer, environmental friendly and reliable.

Hence, this study is aimed at isolation and molecularly identification of bacteria and fungi associated in the bioremediation of crude oil contaminated soils or sites.

## **2. MATERIALS AND METHODS**

### **2.1 Description of Study Area**

The location was chosen at the Rivers State University Research farmland in Nkpolu-Oroworukwo, Mile 3, Diobu area of Port Harcourt situated behind Agip Oil Company and shares boundary with the Nkpolu-Oroworukwo community. The experimental setup was cited on the portion of land situated at Longitude 4°48'18.50"N and Latitude 6°58'39.12"E. The annual rainfall is bimodal, begins from March and ends in November with peaks in June and September and a short period of lower precipitation in August. Annual temperature ranges between an average minimum temperature of 21°C and an average maximum temperature of 31°C [11,12].

### **2.2 Sample Collection**

Soil samples were collected according to the procedures stated by the Food and Agricultural Organization [13]. Random sampling technique was used in order to ensure that each soil bacterium within the location of the study area was given a chance to be represented.

Soil samples were collected using sterilized spatula at a tillage depth of 15 cm randomly from 10 core points and homogenized to obtain

composite soil samples. Soil samples were formed, mapped out separately to form ridges and left undisturbed for six days after which one of the ridges was contaminated with 500 g of crude oil [9] and left fallow for 3 weeks in order to mimic natural crude oil spill site. The soil samples were mixed after which a sub-soil was collected into sterile re-sealable bags and transferred to the Microbiology Laboratory within 30 minutes for analyses [14].

## **2.3 Microbiological Analyses**

### **2.3.1 Serial dilution**

One gram of the samples was separately added to 9 ml of 0.1% peptone water diluents. After shaking thoroughly a 10- fold (v/v) serial dilutions were made by transferring 1 ml of the original solution into freshly prepared peptone water diluents to a range of  $10^{-7}$  dilutions [14].

### **2.3.2 Enumeration of total heterotrophic microorganisms**

The counts of total heterotrophic bacteria in the soil samples were determined by the spread plate techniques as described by [14] using nutrient agar (NA). The NA medium was amended with nystatin in order to prevent the growth of fungal contaminants. An aliquot (0.1 ml) of the dilution of  $10^{-7}$  dilution was aseptically transferred onto properly dried NA plates in duplicates and spread evenly using bent glass rod. The total heterotrophic fungi counts were determined by spread plate techniques as described by [14] using Sabouroud dextrose agar (SDA) supplemented with streptomycin to inhibit the growth of bacterial contaminants [15,16]. An aliquot (0.1 ml) of the dilution of  $10^{-3}$  dilution was aseptically transferred onto properly dried SDA plates in duplicates and spread evenly using bent glass rod. Inoculated NA plates were incubated at 37°C for 24 to 48 hours while the SDA plates were incubated at room temperature for 3 to 5 days before enumeration of microbial colonies.

### **2.3.3 Enumeration of hydrocarbon utilizing microorganisms**

The vapour phase transfer method by [17] as modified by [18,19] was adopted in estimating the population of hydrocarbon utilizing microorganisms using spread plate techniques on mineral salt medium (MSM). The method employed the use of sterile filter paper discs saturated or soaked with 2 ml of sterilized crude oil and aseptically placed onto the inside cover of the inverted Petri dishes before incubating. One

milliliter (1 ml) of the composite soil samples was diluted serially from dilutions  $10^{-1}$  to  $10^{-7}$ . Aliquots of 0.1 milliliter from dilutions of  $10^{-2}$  were inoculated onto the media in duplicates. For enumeration of the hydrocarbon degrading bacteria, the medium was supplemented with fungusol Miconazole Nitrate to prevent the growth of fungal contaminants. On the other hand, the mineral salts medium supplemented with streptomycin to inhibit the growth of bacterial contaminants was used to ensure the enumeration of oil degrading fungi. The plates were incubated at room temperature for 5 days before enumeration. Mineral salt agar was constituted as adopted by [18] with little modifications is shown in Table 1 for the enumeration of hydrocarbon utilizing microorganisms.

**Table 1. Composition of the mineral salt agar**

Salt	Quantity (Gram)
K <sub>2</sub> HPO <sub>4</sub>	0.5
NaCl <sub>2</sub>	0.3
FeSO <sub>4</sub> .6H <sub>2</sub> O	0.02
ZnCl <sub>2</sub>	0.3
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.3
NaNO <sub>3</sub>	0.03
MnSO <sub>4</sub> .H <sub>2</sub> O	0.2
Agar	15
Distilled water	1000 ml

(Source: Obire et al. 2008)

### 2.3.4 Enumeration and estimation of the colony forming units per gram of soil samples

The numbers of colonies in each plate after incubation were counted and the average was taken and expressed as colony forming units per gram (cfu/g) of sample using the formula as adopted by [14].

$$\text{cfu/g} = \frac{N}{D \times V}$$

where;

N = Number of Colonies

D = Dilution

V = Volume plated [14]

### 2.3.5 Isolation, purification and maintenance of pure microbial isolates

Distinct representative Bacterial colonies were repeatedly transferred onto freshly prepared nutrient agar plates by the streak-plate method and allowed to grow for 24 hours for purification

of bacterial isolates. Similarly, distinct fungal colonies were subcultured repeatedly on freshly prepared Sabouroud Dextrose Agar plates for 72 hours for purification of fungal isolates [14]. Discrete colonies on the Nutrient Agar plates were aseptically transferred into 10% (v/v) glycerol suspension, well labeled and stored as stock cultures for maintenance of the bacterial isolates. While pure cultures of fungal isolates were subcultured onto Sabouroud Dextrose Agar slant in bijoux bottle for preservation of the fungal isolates [14].

## 2.4 Identification of Microbial Isolates

Identification of the bacterial isolates followed Bergy's Manual of Determinative Bacteriology [20] based on their microscopic examination, cellular morphology, colonial morphology, biochemical tests including: Gram staining reactions, coagulase test, oxidase test, spore test, motility test, indole test, methyl red test, Vogues Proskauer test, citrate utilization test and sugar fermentation test (sucrose, lactose and glucose) [20]. Identification of pure cultures of fungi species was done using macroscopically and microscopically techniques accordingly: The morphology of the fungal growth on plates was studied including their colors. Small portions of the fungal pure culture were teased and mounted in lactophenol cotton blue stain on a clean grease-free glass slide, covered with a clean cover slip and observed under the microscope. Referencing was made to the manual of fungi atlas by [16].

## 2.5 Molecular Characterization of the Isolates

### 2.5.1 Molecular identification for bacteria

#### 2.5.1.1 Bacterial genomic DNA extraction

Five milliliters of an overnight broth culture of the bacterial isolate in Luria Bertani (LB) was spun at 14000 rpm for 3 min. The cells were re-suspended in 500 ul of normal saline and heated at 95°C for 20 min. The heated bacterial suspension was cooled on ice and spun for 3 min at 14000 rpm. The supernatant containing the DNA was transferred to a 1.5 ml micro-centrifuge tube and stored at -20°C for other downstream reactions [21].

#### 2.5.1.2 DNA quantification

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The

software of the equipment was launched by double clicking on the Nanodrop icon. The equipment was initialized with 2 µl of sterile distilled water and blanked using normal saline. Two microlitre of the extracted DNA was loaded onto the lower pedestal, the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the “measure” button [21].

### 2.5.1.3 16S rRNA Amplification

The 16s rRNA region of the rRNA genes of the isolates were amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTACGACTT-3' primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 microlitres for 35 cycles. The PCR mix included: The X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95°C for 5 minutes; denaturation, 95°C for 30 seconds; annealing, 52°C for 30 seconds; extension, 72°C for 30 seconds for 35 cycles and final extension, 72°C for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 15 minutes and visualized on a UV transilluminator [22].

### 2.5.1.4 Sequencing

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10 µl, the components included 0.25 µl BigDye® terminator v1.1/v3.1, 2.25 µl of 5 x BigDye sequencing buffer, 10 µM Primer PCR primer, and 2-10 ng PCR template per 100 bp. The sequencing conditions were as follows: 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4 min [23].

### 2.5.1.5 Phylogenetic analysis

Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using ClustalX. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 500 replicates [22], is taken to represent the

evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method [23].

## 2.5.2 Molecular identification for fungi

### 2.5.2.1 Fungal genomic DNA extraction

Extraction was done using a ZR fungal/bacterial DNA mini prep extraction kit supplied by Inqaba South Africa. A heavy growth of the pure culture of the fungal isolate was suspended in 200 microlitre of isotonic buffer into a ZR Bashing BeadLysis tubes, 750 microlitre of lysis solution was added to the tube. The tubes were secured in a bead beater fitted with a 2ml tube holder assembly and processed maximum speed for 5 minutes. The ZR bashing beadlysis tube were centrifuged at 10,000xg for 1 minute [21].

Four hundred (400) microlitres of supernatant was transferred to a Zymo-Spin IV spin Filter (orange top) in a collection tube and centrifuged at 7000 xg for 1 minute. One thousand two hundred (1200) microlitres of fungal/bacterial DNA binding buffer was added to the filtrate in the collection tubes bringing the final volume to 1600 microlitres, 800 microlitres was then transferred to a Zymo-Spin IIC column in a collection tube and centrifuged at 10,000 xg for 1 minute, the flow through was discarded from the collection tube. The remaining volume was transferred to the same Zymo-spin and spun. Two hundred (200 µl) microlitres of the DNA Pre-Wash buffer was added to the Zymo-spin IIC in a new collection tube and spun at 10,000xg for 1 minute followed by the addition of 500 microlitres of fungal/bacterial DNA Wash Buffer and centrifuged at 10,000 xg for 1 minute. The Zymo-spin IIC column was transferred to a clean 1.5 microlitre centrifuge tube, 100 microlitres of DNA elution buffer was added to the column matrix and centrifuged at 10,000 xg microlitre for 30 seconds to elute the DNA. The ultra-pure DNA was then stored at -20 degree for other downstream reaction [21].

### 2.5.2.2 DNA quantification

The extracted genomic DNA was quantified using the Nano drop 1000 spectrophotometer. The software of the equipment was launched by double clicking on the Nano drop icon. The equipment was initialized with 2 µl of sterile distilled water and blanked using normal saline. Two microlitre of the extracted DNA was loaded onto the lower pedestal, pedestal was brought down to contact the extracted DNA on the

pedestal. The DNA concentration was measured by clicking on the “measure” button [21].

#### 2.5.2.3 Internal transcribed spacer (ITS) amplification

The ITS region of the isolates were amplified using the ITS1F: 5CTTGGTCATTTAGAGGAAGTAA-3' and ITS4: 5'- TCCTCCGCTTATTGATATGC-3, Primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 microlitres for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4 M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95°C for 5 minutes; denaturation, 95°C for 30 seconds; annealing, 53°C for 30 seconds; extension, 72°C for 30 seconds for 35 cycles and final extension, 72°C for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 15 minutes and visualized on a blue light transilluminator [22].

#### 2.5.2.4 Sequencing

Sequencing was done using the Big Dye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10 µl, the components included 0.25 µl Big Dye terminator v1.1/v3.1, 2.25 ul of 5 x Big Dye sequencing buffer, 10 uM Primer PCR primer, and 2-10 ng PCR template per 100 bp. The sequencing condition were as follows 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4 min [23].

#### 2.5.2.5 Phylogenetic analysis

Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using ClustalX. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 500 replicates [22] is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method [23].

### 2.6 Determination of Total Petroleum Hydrocarbon the Soil Samples

Soil samples were analyzed for the total petroleum hydrocarbon (TPH) content before and

after crude oil contamination using gas chromatography (GC) according to [24]. The soil samples were extracted using a gas chromatograph (HP 5890, Hewlett Packard, and Avondale, PA, USA) equipped with a flame ionization detector (FID). The column with helium carrier gas, hydrogen gas and air flow at flow rates of 2 ml/min, 30 ml/ min and 300 ml/min respectively. The oven temperature was programmed from 50°C for 10 min then 20 min to 340°C. Retaining this temperature for 10 min, the residual total petroleum hydrocarbon (TPH) in the different treatment setups were extracted with 40 µl of n-pentane (HPLC grade) by sonicating the sample 5min at each extraction for 3 times. The pentane extract was centrifuged at 3000 g for 5 min. The extractable TPH was identified and quantified by comparison with a sample chromatogram with standard calibration [24,25].

## 3. RESULTS AND DISCUSSION

### 3.1 Microbial Counts

The results of the microbial counts are shown in Table 2. The results revealed that the total heterotrophic bacterial counts for uncontaminated soil ( $2.58 \times 10^8$  cfu/g) was higher than that of the contaminated soil ( $2.1 \times 10^9$  cfu/g) although there were no significant difference ( $P > 0.05$ ). However, total heterotrophic fungi counts was significantly higher ( $P < 0.05$ ) in the uncontaminated soil ( $2.0 \times 10^5$  cfu/g) than the contaminated soil samples ( $1.6 \times 10^5$  cfu/g). The increase in the microbial load of heterotrophic microorganisms in the uncontaminated soil can be linked to the presence of the favourable conditions created in the soil by the already existing nutrients which served as a platform for microorganisms to synthesize and utilize the nutrients as sole source of carbon and energy while the contaminated soils could be affected as a result of the toxicity of crude oil and impaired availability of nutrients to the microbes in the contaminated soil sample which may contribute to poor soil conditions [26]. Albert and Tane, [27] stated clearly that the presence of hydrocarbons in the soil causes pollution and therefore reduces the nutrient level in soil and also creates nutrient deficient conditions of such environment which may eventually not be favorable for the microorganisms to proliferate. This result corroborates with study of [28] who reported similar higher counts for total heterotrophic bacteria and fungi in uncontaminated soil than in contaminated soil.

The hydrocarbon utilizing microorganisms of the contaminated soils were significantly higher with  $5.0 \times 10^4$  cfu/g at  $P < 0.05$  in the contaminated soil than the uncontaminated soil that had  $8.0 \times 10^3$  cfu/g whereas the hydrocarbon utilizing fungi counts recorded  $9.0 \times 10^3$  cfu/g and  $7.0 \times 10^4$  cfu/g for uncontaminated and contaminated soil samples respectively. However, fungi are more tolerant under acidic conditions while bacteria have limited tolerance for acidic conditions. Furthermore, the reason for higher counts of hydrocarbon utilizing bacteria and fungi in crude oil contaminated soil than the uncontaminated soil could also be due to the presence of residual crude oil in the contaminated soil which boosts its carbon supply and also the presence of some toxic components in the pollutants that favor the growth of the hydrocarbon utilizing bacteria and fungi in crude oil contaminated soils as compared with the uncontaminated soils [7,29]. This also indicates that crude oil contamination shifts the dynamics of microbial population towards crude oil degrading microbes [30]. It has been established that oil pollution of soil leads to its degradation which has been previously reported other by researchers [9,27] to cause decrease in agricultural productivity and alterations in the number and types of environmental microbes.

This results concurs with research by [29,31] who reported significant difference between the

heterotrophic microorganisms of crude oil contaminated soils and pristine soils and also significant difference between the crude oil-degrading microorganisms of crude oil contaminated soil and pristine soil.

However, the increased counts of the hydrocarbon utilizers suggestively represents an immediate response to the added organic carbon present in the petroleum hydrocarbon which must have acted as additional carbon substrate for microbial growth, activity and multiplication [32].

The Rivers State University Research farmland is recognized as a significant area of high microbial diversity based on previous studies carried out on soil obtained from University Research farmland. In this study area, Six (6) genera of bacteria and four (4) Genera of fungi were isolated and identified from uncontaminated and contaminated soil samples as shown in Table 3. The bacterial genera were *Nitrosomonas*, *Pseudomonas*, *Staphylococcus*, *Bacillus*, *Proteus* and *Citrobacter*. While the fungi genera include *Penicillium*, *Mucor*, *Rhizopus* and *Fusarium*. The diversity of the microorganisms isolated could be as result of seasonal variation such as rainy season which is capable of introducing different microbes into the environment through erosion. Similar bacteria and fungi have been isolated and reported elsewhere by other workers [1,10,31,33].

**Table 2. Microbial counts of uncontaminated and contaminated soil samples with crude oil**

Parameter	Uncontaminated soil	Contaminated soil	p-value
THBC (cfu/g)	$2.58 \times 10^8 \pm 0.07$	$2.10 \times 10^8 \pm 0.50$	0.1375
TFC (cfu/g)	$2.0 \times 10^5 \pm 0.05$	$1.6 \times 10^5 \pm 0.08$	<0.0001
HUBC (cfu/g)	$8.0 \times 10^3 \pm 0.50$	$5.0 \times 10^4 \pm 0.50$	0.0045
HUFC (cfu/g)	$9.0 \times 10^3 \pm 0.50$	$7.0 \times 10^4 \pm 0.50$	0.0086

Key: Total heterotrophic bacterial count (THBC); Total fungal count (TFC); Hydrocarbon utilizing bacterial count (HUBC); Hydrocarbon Utilizing fungal count (HUFC)  
Statistical significance is considered at  $P < 0.05$

**Table 3. Bacteria and fungi isolated from the uncontaminated and contaminated soil samples**

Microorganism	Uncontaminated soil	Contaminated soil
Bacteria	<i>Proteus sp.</i> <i>Bacillus sp.</i> <i>Citrobacter sp.</i> <i>Pseudomonas sp.</i> <i>Nitrosomonas sp.</i> <i>Staphylococcus sp.</i>	<i>Pseudomonas sp.</i> <i>Citrobacter sp.</i> <i>Bacillus sp.</i> <i>Staphylococcus sp.</i> <i>Nitrosomonas sp.</i>
Fungi	<i>Mucor sp.</i> <i>Rhizopus sp.</i> <i>Penicillium sp.</i> <i>Fusarium sp.</i>	<i>Mucor sp.</i> <i>Rhizopus sp.</i> <i>Penicillium sp.</i> <i>Fusarium sp.</i>

Tables 4 and 5 presents the frequency and percentage of occurrence of both bacterial and fungal isolates obtained from the uncontaminated and contaminated soil samples in this study. The Table 4 and Fig. 1 shows that *Bacillus* species had the highest frequency of occurrence to a tune of 34.8% compared to other bacterial species which includes; *Pseudomonas* sp 22.9%, *Nitrosomonas* sp 16.9%, *Staphylococcus* sp 13.7%, *Citrobacter* sp 10.0%, *Proteus* sp 1.7%. However, *Proteus* sp was the least in the uncontaminated soil but was absent in the contaminated soil. Table 5 and Fig. 2 shows the result of the frequency of occurrence for fungi isolates with *Penicillium* species having the highest percentage of occurrence of 36.1% followed by *Mucor* 25%, *Rhizopus* sp 25% while *Fusarium* sp. had the least with 13.9%. For the bacteria isolates, *Bacillus* occurred more frequently in both the contaminated and uncontaminated soils (Table 4) while the fungal isolates, *Penicillium* was predominant in both the contaminated and uncontaminated soil (Tables 5). Although climatic conditions may differ considerably in such circumstance, which has the ability to alter the frequency and percentage occurrence of microorganisms in the study sites.

Furthermore, recent reports by Chikere et al. [29] have shown that gram positive bacteria can dominate during bioremediation of petroleum hydrocarbon spills owing to their metabolic versatility, their widespread occurrences both in pristine and hydrocarbon polluted soils and the presence of multiple hydrocarbon catabolic genes in these groups of bacteria. Gene composition of microorganism varies from place to place due to environmental factors which also has the capacity to influence the frequency of occurrence of bacteria and fungi isolates in a study area.

This observation is consistent with the results of Hamamura et al. [34] Quatrini et al. [35] who reported similar bacterial and fungal species associated with hydrocarbon contaminated soil. Gram positive hydrocarbon degraders have also been detected to have high frequency of occurrence in pristine soils [36,37,38]. It has been hypothesized that Gram Positive bacteria generally adapt to nutrient limited conditions and consequently do not fluctuate in response to hydrocarbon enrichment environment [35], which normally causes nutrient deficiencies in soil [39].

**Table 4. Distribution of bacterial isolates in the uncontaminated and contaminated soil**

Bacterial isolate	Uncontaminated soil		Contaminated soil		Total Freq.	%of Occurrence
	Occurrence	Frequency	Occurrence	Frequency		
<i>Proteus</i> sp.	+	8	-	0	8	1.7
<i>Bacillus</i> sp.	+	90	+	73	163	34.8
<i>Citrobacter</i> sp.	+	26	+	21	47	10.0
<i>Pseudomonas</i> sp.	+	40	+	67	107	22.9
<i>Nitrosomonas</i> sp.	+	30	+	49	79	16.9
<i>Staphylococcus</i> sp.	+	64	-	0	64	13.7
Total		258		210	468	100

Key: += present (growth), - = Absent (No growth)

**Table 5. Distribution of fungal isolates in the uncontaminated and contaminated soil**

Fungal isolate	Uncontaminated soil		Contaminated soil		Total Freq.	% of Occurrence
	Occurrence	Frequency	Occurrence	Frequency		
<i>Mucor</i> sp.	+	6	+	3	9	25
<i>Rhizopus</i> sp.	+	4	+	5	9	25
<i>Penicillium</i> sp.	+	7	+	6	13	36.1
<i>Fusarium</i> sp.	+	3	+	2	5	13.9
Total		20		16	36	100

Key: += present (growth), - = Absent (No growth)

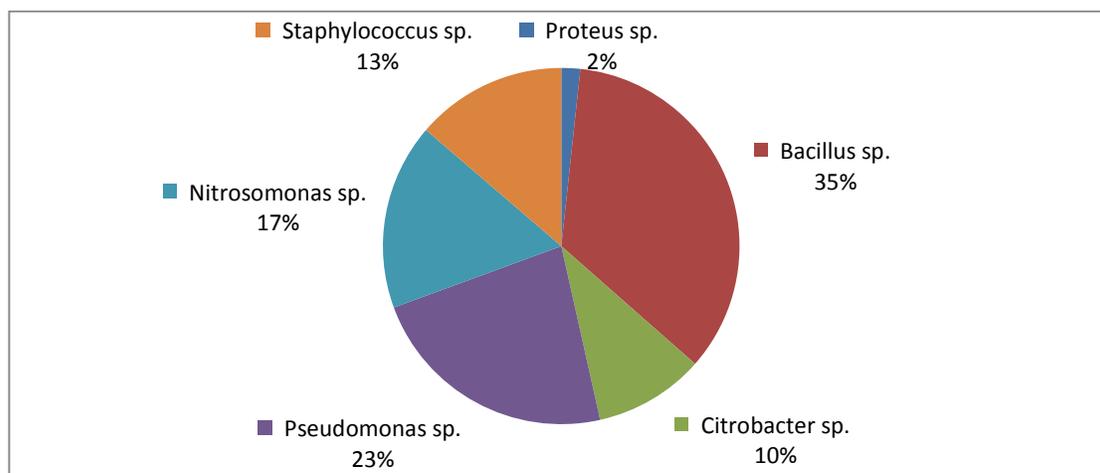


Fig. 1. Percentage occurrence of the bacterial isolates

Table 6. Phenotypic identification and molecular identification of bacteria isolates

Isolate code	Phenotypic identification	Molecular identification
MA1	<i>Nitrosomonas</i> sp	<i>Comamonas testosteroni</i>
MA2	<i>Staphylococcus</i> sp	<i>Staphylococcus saprophyticus</i>
MA3	<i>Citrobacter</i> sp	<i>Chryseobacterium cucumeris</i>
MA4	<i>Pseudomonas</i> sp	<i>Pseudomonas auriginosa</i>
MA5	<i>Bacillus</i> sp	<i>Bacillus amyloliquefaciens</i>

Table 7. Phenotypic identification and molecular identification of fungal isolates

Isolate code	Phenotypic identification	Molecular identification
MAF1	<i>Rhizopus</i> sp	<i>Kodamaea ohmeri</i>
MAF2	<i>Penicillium</i> sp	<i>Penicillium citrinum</i>
MAF3	<i>Penicillium</i> sp	<i>Penicillium brocae</i>
MAF4	<i>Mucor</i> sp	<i>Lentinus squarrosulus</i>
MAF5	<i>Fusarium</i> sp	<i>Fusarium solani</i>

### 3.2 Identification of the Bacterial and Fungal Isolates

In many distinct areas of microbiology, the ability to identify microorganisms has important application. For example, in Environmental Microbiology it is important to be able to accurately identify organisms that are responsible for degradation of specific materials such as crude oil in a contaminated environment. Generally microorganisms can be identified using conventional method such as morphological and biochemical methods but there are significant setbacks associated with the conventional method of identification, hence both the conventional and molecular techniques were used to identify microorganisms isolated in this study. The results of the bacteria and fungi associated with crude oil contaminated soils

identified in this study are presented in Tables 6 and 7 respectively. Phenotypic Identification shows that bacteria and fungi species identified employed morphological and biochemical methods while the Molecular Identification reveals the individual organisms identified to their respective strain levels by Polymerase chain reaction (PCR) method. The results obtained by molecular characterization revealed five bacterial isolates which include *Bacillus amyloliquefaciens*, *Pseudomonas auriginosa*, *Chryseobacterium cucumeris*, *Staphylococcus saprophyticus*, *Comamonas testosterone* (Table 6) while five fungi were identified as *Fusarium solani*, *Lentinus squarrosulus*, *Penicillium citrinum*, *Penicillium brocae* and *Kodamaea ohmeri* (Table 7). The predominant bacterial species were *Bacillus* sp., *Staphylococcus* sp., *Pseudomonas* sp. and *Comamonas* sp., while

the fungi species were *Penicillium* sp., *Fusarium* sp., *Lentinus* sp. and *Kodamaea* sp. However, *Chryseobacterium cucumeris*, *Comamonas testosteroni*, *Lentinus* sp. and *Kodamaea* sp. were previously identified as *Citrobacter* sp., *Nitrosomonas* sp., *Mucor* sp. and *Rhizopus* sp. as shown in Tables 6 and 7 for bacteria and fungi isolates respectively.

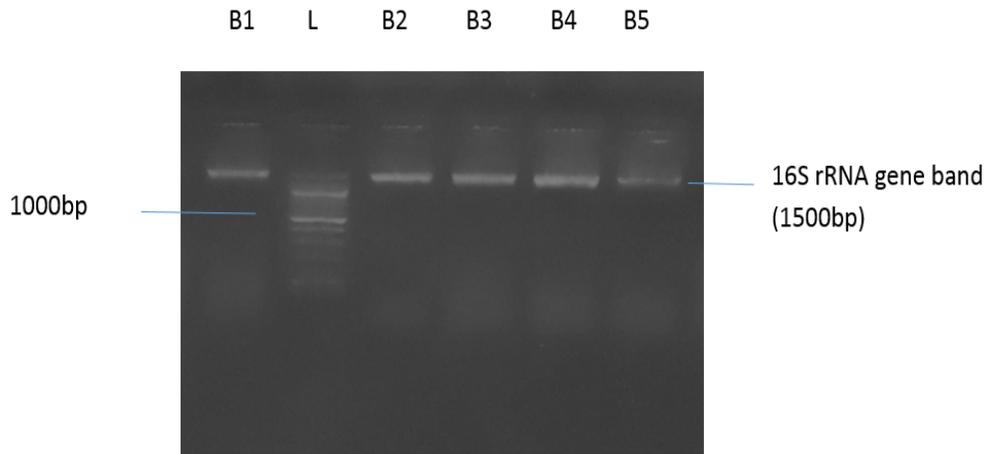
*Bacillus* and *Penicillium* species were more frequently isolated among the bacteria and fungi respectively. Several researchers have reported species of *Pseudomonas*, *Bacillus*, *Alcaligenes*, *Acinetobacter*, *Flavobacterium*, *Micrococcus* and *Corynebacterium* to have crude oil degradative capability and have been isolated from hydrocarbon polluted soils [1,10]. Similar bacteria genera including *Staphylococcus*, *Chryseobacterium*, *Enterobacter*, *Klebsiella*, *Proteus*, *Serratia* and *Escherichia* have also been reported to be associated with oil polluted sites [32]. *Comamonas testosteroni* has been routinely reported for the bioconversion of different steroids and heavy metal removal and have also shown to possess the capability of polycyclic aromatic hydrocarbons (PAHs) and total petroleum hydrocarbon (TPH) degradation [33]. Species of *Staphylococcus*, *Bacillus* and *Pseudomonas* have been found to show appreciable numerical increase in hydrocarbon polluted sites and have also been reported as a core petroleum hydrocarbon degrader [1,8,10,34]. This is because degradation of petroleum hydrocarbons by microorganisms is mainly caused by the catalysis of intracellular enzymes which these bacteria (*Staphylococcus*, *Bacillus* and *Pseudomonas*) possess. The process of microbial degradation of petroleum hydrocarbons has four main steps: First, petroleum pollutants are emulsified by surfactants secreted by microorganisms; Second, the emulsified petroleum hydrocarbon is absorbed by the surface of the microorganism; Then, the petroleum hydrocarbon adsorbed on the surface of the cell membrane enters the cell membrane through active transport or passive transport, endocytosis. Finally, the petroleum hydrocarbon entering the cell undergoes an enzymatic reaction with the corresponding enzyme to achieve the purpose of degrading the pollutant [40].

The analysis of 16S ribosomal RNA genes sequences can be applied for measuring and determining the relationships among all bacterial isolates, therefore by using 16S rRNA sequences, numerous bacterial genera and

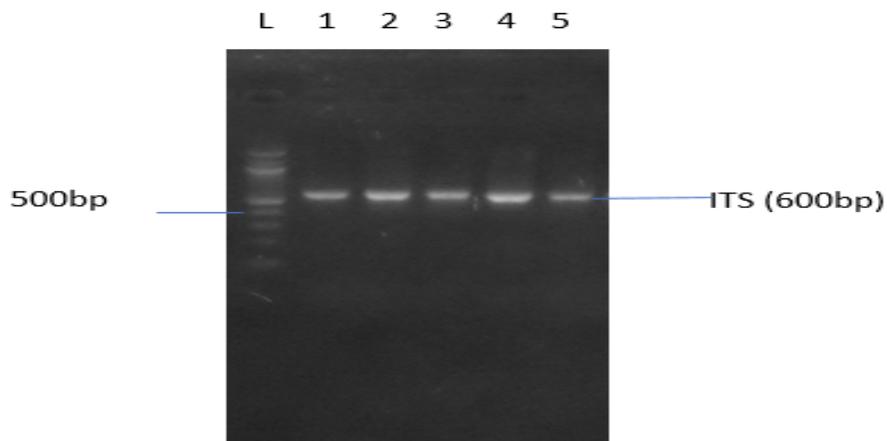
species have been reclassified and renamed [6]. Partial sequences of 16S rRNA gene is sufficient for the identification of species when compared with some longer, closely related sequences. In contrast, the 16S rRNA gene sequence is necessary and required for identification of new species [7,8]. The 16S rRNA gene sequence long is about 1,550 bp, including both variable and conserved regions. The length of 16S rRNA gene with sufficient interspecific polymorphisms was found enough to provide distinguishing and statistically valid measurements. The agarose gel electrophoresis of the 16S rRNA gene as seen in Plate 1 revealed the band pairs of individual bacteria analyzed. Lane L represents the 1000 base pairs molecular ladder and lanes B1-B5 represent the 16SrRNA gene bands 1500 base pairs while the agarose gel electrophoresis of the amplified ITS of the fungal isolates is shown in Plate 2. This shows the band pairs of individual fungus analyzed. Lane L represents the 500 base pairs molecular ladder and lanes 1-5 represent the amplified ITS bands 600 base pairs. The obtained 16SrRNA sequence from the isolate produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The 16SrRNA of the isolate MA2 showed a percentage similarity to other species at 100%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16SrRNA of the isolate MA2 (MN273754) within the *Staphylococcus* genus and revealed a close relatedness to *Staphylococcus saprophyticus* (KF322102) than other *Staphylococcus* sp. [23]. MN5 (MN273757) was closely related to *Bacillus amyloliquifaciens* (MK886655.1), MN4 (MN273756) was closely related to *Pseudomonas aeruginosa* (MK3637616.1), MA1 (MN273753) isolate was closely related to *Comamonas testosteroni* (MK534021.1) and MA3 (MN273755) was closely related to *Chryseobacterium cucumeris* (MK 212371.1) as shown in the phylogenetic tree in Fig. 1. Similarly, the internal transcribed space (ITS) of the isolates showed a percentage similarity to other species at 99-100%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of ITS of MAF2 (MN273760) within the *Penicillium* sp. and revealed a close relatedness to *Penicillium brocae* (MG827321.1); the other *Penicillium* sp., MF3 (MN273761) showed a close relatedness to *Penicillium citrinum* (MK290862.1), MAF5 (MN273763) was closely related to *Fusarium*

*solani* (KT184397.1); MAF1 (MN273759) was related closely to *Kodamaea ohmeri* (MG241526.1) and MAF4 (MN273762) was closely related to *Lentinus squarrosulus* (KT273380.1) as shown in the phylogenetic tree in Fig. 2. These results are indications that the DNA of these isolates were actually extracted [23]. Extracted isolates were submitted to GenBank (National Centre for Biotechnology Information, Maryland, USA), and were given accession numbers. Palys et al. [22] has suggested in their study that, a different rate of >0.5% between different sequences gene of 16S rRNA could be considered indicative of a new species within a known genus. Also results reported by Drancourt et al. [20] was showed that, 99% similarity is suitable cutoff percentage

similarity for identification at the same species level, while for the same aim, 97% similarity enough for identifying of genus level. The use of 16S rRNA gene sequencing for definitive microbial identification and for publication requires a harmonious set of guidelines for interpretation of sequence data that needs to be implemented, so that results from one study can be accurately compared to another [23]. The interspecific polymorphisms of universal primer of 16S rRNA gene between same species and different genus may be occurred. Hence the microorganisms isolated and identified in this study cannot be considered as new species within a known genus because their similarity rate is one hundred percent (100%) as shown in 1 and 2 respectively.



**Plate 1. Agarose gel electrophoresis of the 16S ribosomal Ribonucleic Acid (rRNA) genes of the bacterial isolate**



**Plate 2. Agarose gel electrophoresis of the amplified internal transcribed spacer (ITS) of the fungal isolates**

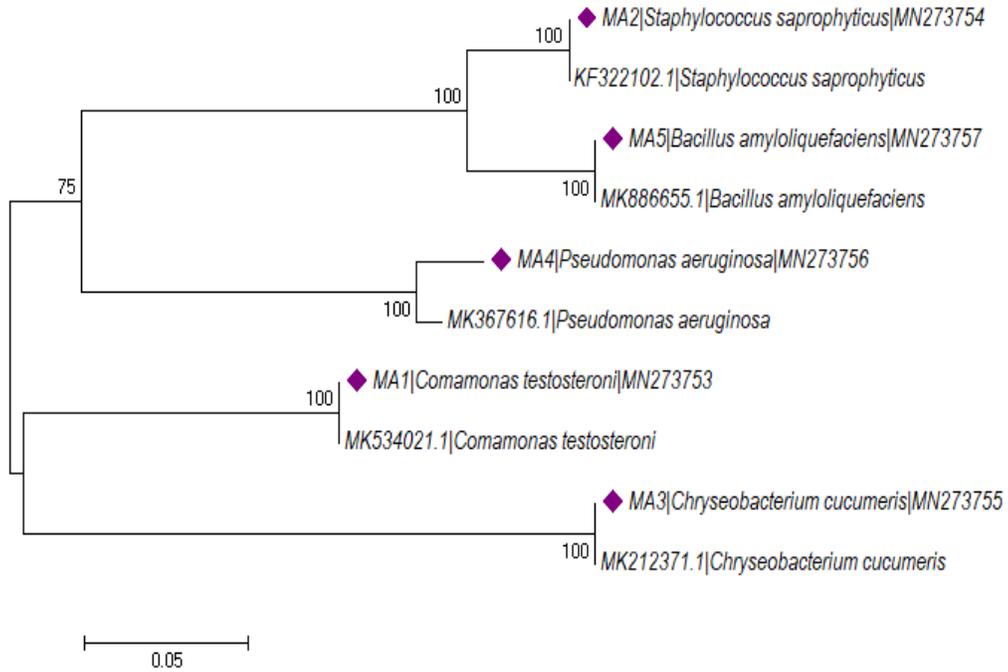


Fig. 2. Phylogenetic tree showing the evolutionary distance between the bacterial isolate

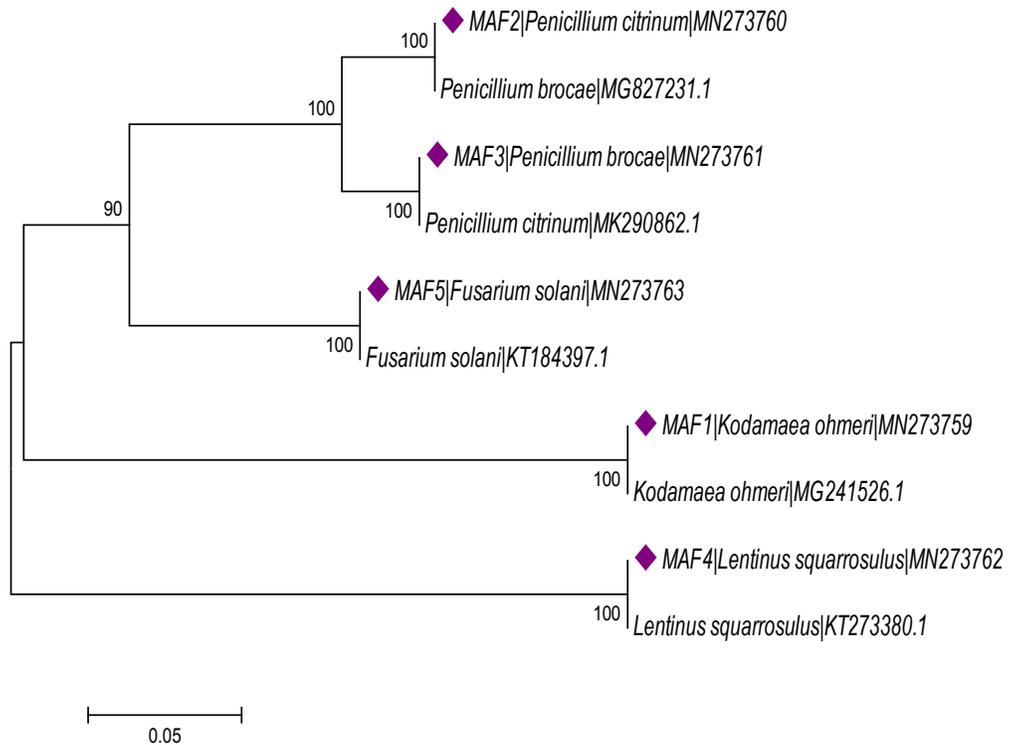


Fig. 3. Phylogenetic tree showing the evolutionary distance between the fungal isolates

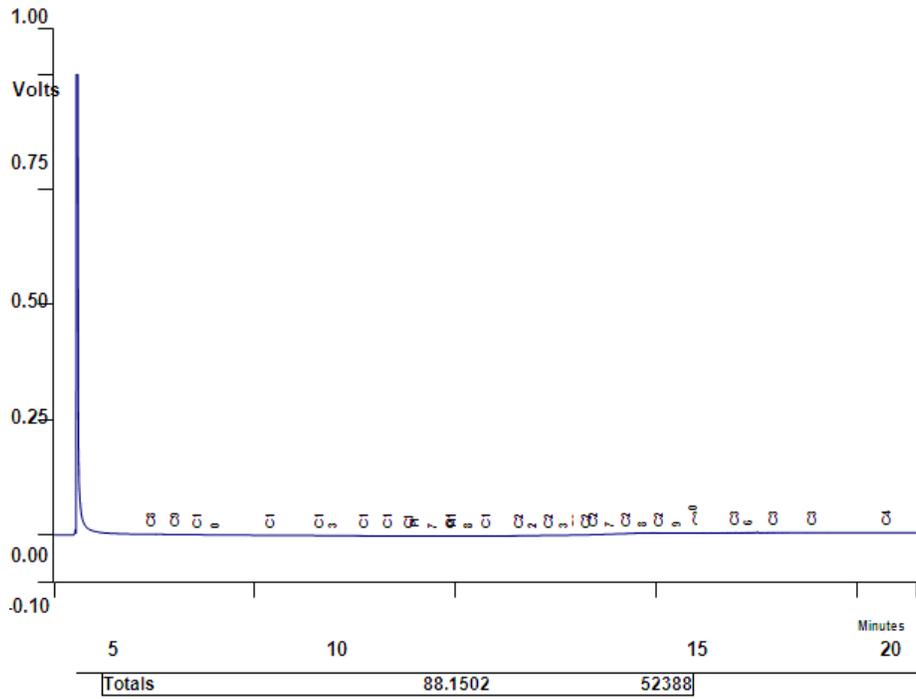


Fig. 4. Chromatogram showing the TPH content of the crude oil uncontaminated soil

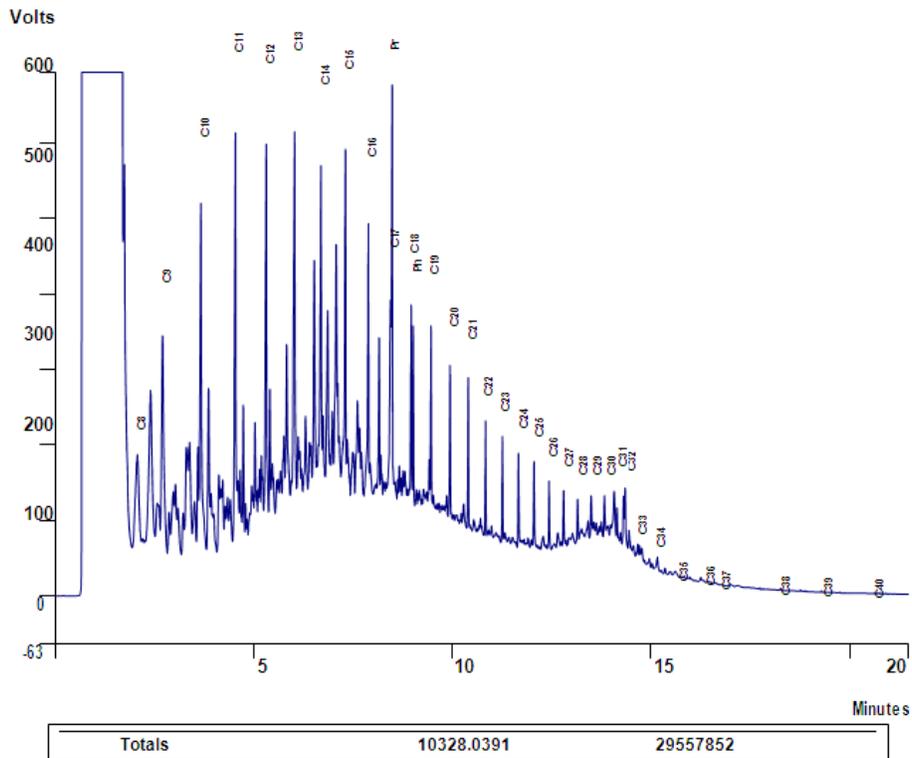


Fig. 5. Chromatogram showing the TPH content of the crude oil contaminated soil

In order to ascertain that microorganisms isolated in this study are actually hydrocarbon utilizing microorganisms, five kilograms (5 kg) of soil sample was contaminated with 500 ml of crude oil and allowed for six days to mimic natural oil spilled site, another set of five kilograms (5 kg) of soil sample which served as control sample was set-up without addition of crude oil. Total petroleum hydrocarbon content (tph) was analyzed in both set-ups to determine the crude oil content. The content of total petroleum hydrocarbon (tph) in the experimental soil before application crude oil was 88.55 mg/kg whereas, after application of 500 ml of crude oil, tph content was 10,328.03 mg/kg. Fig. 3 is the chromatogram showing the tph content of the uncontaminated soil sample which is the control sample (88.55 mg/kg). C8 to c40 represents the concentrations of the individual hydrocarbon components. Their concentrations were very small and could not be demonstrated using appreciable peaks whereas, Fig. 4 shows the chromatogram showing the tph content of the contaminated soil sample (10,328.03 mg/kg) illustrating appreciable peaks as a result of high concentrations of the individual hydrocarbon components. The differences observed in the peaks of the hydrocarbon components of the chromatograms of the uncontaminated soil and the contaminated soil sample in an indication that the contaminated soil sample is considered polluted and needs intervention/ remediation). The tph value is above the intervention value of 5000 mg/kg according to environment guidelines and standard for the petroleum industry in Nigeria (egaspin)/ department of petroleum resources (dpr), standard for crude oil spill value of the soil is 5000 mg/kg and above [41]. This indicates that microorganisms isolated from this contaminated soil sample that have tph value of 5000 mg/kg which according to department of petroleum resources (dpr), standard is very polluted, must have the ability to utilize crude oil as a sole carbon source by possessing the enzymes required for the utilization of the crude oil.

#### 4. CONCLUSION AND RECOMMENDATION

The hydrocarbon utilizing microorganisms isolated from the contaminated soils were species of *Staphylococcus saprophyticus*, *Bacillus amyloliquefaciens*, *Pseudomonas aeruginosa*, *Comamonas testosteroni*, *Chryseobacterium cucumeris* for bacteria and *Penicillium citrinum*, *Penicillium brocae*,

*Fusarium solani*, *Kodamaea ohmeri* and *Lentinus squarrosulus* for fungi. *Bacillus* and *Penicillium* Genera were predominant. This may be due to the ability of the organisms to produce spores, which may shield them from the toxic effects of the hydrocarbons. From the current study, the microorganisms isolated are able to utilize the crude oil as their sole carbon source. Hence, can be used for bioremediation of crude oil polluted sites or soils.

#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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