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Comparative Studies on the Biodegradation of Crude Oil-polluted Soil by *Pseudomonas aeruginosa* and *Alternaria* Species Isolated from Unpolluted Soil

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Authors' contributions

This work is a collaborative effort of all authors. Authors OOL and OS designed the study and wrote the protocol and the first draft of the manuscript. Author GHJ managed the analyses of the study. Authors GHJ and SE managed the literature search and editorial work. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To examine the efficiency and compare the ability of *Pseudomonas aeruginosa* and *Alternaria* sp to degrade hydrocarbons found in crude oil-polluted soil.
Study Design: The study made use of a purposive sampling method.
Place and Duration: Department of Microbiology, University of Abuja and Environmental Biotechnology Laboratory, Sheda Science and Technology Complex (SHESTCO), Abuja between May and November, 2015.
Methodology: Soil samples were collected from non-contaminated garden in Gwagwalada, Abuja and were screened for the presence of these organisms using spread plate technique. These microorganisms were confirmed via morphological characteristics, biochemical tests and molecular techniques involving primer specific polymerase chain reaction. Degradation of crude oil by the

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bacterium and fungus was done by inoculating fresh cultures of these organisms in conical flasks containing Bushnell and Haas (1941) media supplemented with 5% v/v crude oil and incubated appropriately. The level of hydrocarbon degradation was determined using gas chromatography-mass spectroscopy (GCMS) analysis.

Results: *Alternaria* sp was almost twice as effective in degrading octane and decane (57.48%, 78.28%) in the sample as *Pseudomonas aeruginosa* (32.20%, 35.62%) respectively. However, the *P. aeruginosa* was more effective than *Alternaria* sp. in degrading dodecane and tetradecane by 63.33% and 100%, in comparison to the 45.70% and 54.27% by the fungus. The highest percentage of residual oil in the reaction vessel containing *Pseudomonas aeruginosa* was octane and decane at 67.8% and 64.38%. In the case of *Alternaria* sp., dodecane and pentadecane had the highest residual oil fractions at 54.10% and 48.71% after 21 days.

Conclusion: The result of this study indicates that organisms have the potential for bioremediation of crude oil and could be employed in large scale to remediate crude oil contaminated sites in consortium with known microorganisms possessing the same ability.

Keywords: Biodegradation; crude oil; P. aeruginosa; Alternaria sp.; soil microorganisms.

1. INTRODUCTION

Environmental contaminants represent a major challenge to both the ecology and diversity of our natural habitat. Such contaminating compounds can be traced to either anthropogenic or biogenic sources, the latter of which are referred to as either synthetic or natural occurring biochemical explored to sustain mankind. These compounds are deemed as contaminants when they agglomerate in the environment at concentrations that is beyond an acceptable limit which would otherwise be harmful to all life forms. Among these contaminants are petroleum products majorly hydrocarbons. The hydrocarbons and petroleum constitute one of the main environmental pollutants and their incidence, abundance and persistent in several polluted environments have been reported [1] to possess a serious environmental challenge. Crude oil is a complex polymeric mixture composed of several thousand saturated hydrocarbons, aromatics, asphaltene, resin compounds etc, all of which possess different chemical properties that accounts for the unique features of the oil [2]. Apart from the obvious environmental unsightliness associated with oil spills, such an event constitutes a potential threat to humans, animals, soils and the vegetation [3]. Crude oil spill contributes a major part in deteriorating the local environment due to persistent accumulation simple and branch-chained hydrocarbon compounds in the environment which are toxic and accumulate in the food chains in addition to the undesirable changes in the physical, chemical and biological characteristics of all the components of an environment. One of the best approaches to restoring these contaminated soils is to make use of microorganisms that are able to biodegrade those toxic compounds in a bioremediation process.

Crude oil bioremediation in soil can be enhanced by biostimulation which is the stimulation of indigenous microorganisms, by introducing nutrients and oxygen into the soil [4] or through bioaugmentation, the inoculation of an enriched microbial consortium into soil [5,6].

In general, either with evolutionary-adapted microorganisms or with native organisms of the same species, bioremediation is a relatively slow, extremely heterogeneous and complex process that sometimes requires a few weeks to several months to effectively clean up an oil spill [7]. A unique relationship exists between hydrocarbons and microorganisms in the environment; the latter thrives on these pollutants thereby leading to the remediation of the affected environment. The use of microorganisms to remove or detoxify environmental contaminants is driven by the need for a more environmentally friendly, cheaper and sustainable *in situ* alternative to engineering based remediation technologies. Microbial species especially bacteria possesses enzyme cascade and corresponding functional genes that are involved in alkane (a major component of petroleum) degradation. These enzymes are still poorly characterized [8-10]. Anaerobic degradation of hydrocarbon compounds is possible in a number of fungi and yeasts like *Candida sp.,* and *Pichia ohmen* through metabolic processes that utilises a range of compounds as terminal electron acceptors [11]. The specific type and number of microorganisms present in any environment are affected by the type of hydrocarbon present in

that same environment influenced usually by the prevalent environmental conditions.

Due to the complexity of oil products, a combination of bacterial strains with broad enzymatic capabilities will be required to achieve extensive degradation. However, most of the crude oil degradation studies reported in the literature have been carried out with single or mixed bacterial strains isolated because of their ability to grow in mineral media with crude oil as only carbon source [12]. *Pseudomonas aeruginosa* is an opportunistic pathogen responsible of nosocomial infections in immunodeficiency patients found ubiquitously in different ecosystems. *Alternaria* spp are major plant pathogens and common allergens in humans and capable of causing opportunistic infections in immunocompromised conditions [13]. In this study, an in vitro study on the crude oil-polluted soil biodegradation ability of by Pseudomonas *aeruginosa* and *Alternaria* species isolated from unpolluted soil.

2. MATERIALS AND METHODS

2.1 Sample Collection

Soil samples were collected from vegetable gardens in Phase 3, Gwagwalada metropolis, Abuja, Nigeria and stored in a sterile container and transported to the Environmental Biotechnology Laboratory, Sheda Science and Technology Complex (SHESTCO), Abuja. Light crude oil (Bonny Light) was collected from Nigerian AGIP Oil Company, Rivers state, Nigeria.

2.2 Purification and Characterisation of Microorganisms

Nutrient agar supplemented with 0.3% fluconazole to inhibit fungal growth while the PDA was supplemented with 3% chloramphenicol to inhibit bacteria.

Under the laminar flow hood, 1 g of soil sample was added to 9 ml of sterile (0.5%) peptone water and shaken vigorously. Serial dilutions were made, and 0.1 ml aliquots from these dilutions were inoculated unto both and using the spread plate method [14] in triplicates. These were then incubated appropriately and morphologically distinct bacterial colonies were purified and characterised [15] while fungal colonies were characterized according to [16]. Absorbance measurements were taken at 24 hourly intervals over 4 days at different temperatures with a wavelength of 400-500 nm using a UV-Vis spectrophotometer while monitoring the growth profile of the two isolates. Further identification was achieved via species genomics (molecular characterization).

2.2.1 Genomic DNA extraction

DNA of microbial cells was extracted using a ZR fungal/Bacterial DNA Miniprep ^{Tm} kit. 50-100 mg of each cell isolate was scooped from the agar plate and added to 750 µl of lysis buffer, vortexed for 5 minutes, and centrifuged at 12000 rpm for 2 minutes. The supernatant was transferred into a Zymo-spin^{TM} Spin filter then centrifuged at 10000 rpm for 2 minutes. 1200 µl of Bacterial DNA binding buffer was added to the filtrate. This mixture was then transferred to a Zymo-Spin^{TM} IIC column and centrifuged at 12000 rpm for 2 minutes. This step was repeated and the resulting filtrate was discarded. DNA pre-wash buffer was introduced to the column followed by centrifugation at 12000 rpm for 2 minutes. The fungal-bacterial DNA wash buffer was added then the mixture was centrifuged at 12000 rpm for 2 mins. A clean 1.5 ml micro tube was used to transfer 100 ul of the DNA elution buffer to the mixture. This mixture was centrifuged at 12000 rpm for 1 min to elute the DNA.

2.2.2 PCR amplification

The genotypic identification of the microorganisms was performed using primers; 27F (5^1-GAGTTTGATCCTGGCTCAG-3^1) and 1492R (5^1-GGTTACCTTGTTACGACT-3^1), representing the forward and reverse primers for bacteria, while ITS-1 (5^1-TCCGTAGGTGAACCTGCGG-3^1) and ITS-4 (5^1-TCCTCCGCTTATTGATATGC-3^1) representing the forward and reverse primers for fungi. A second set of primers; PA-GS-F (5'-GACGGGTGAGTAATGCCTA-3') and PA-GS-R (5'-CACTGGTGTTCCTTCCTATA-3') for specific amplification of *Pseudomonas* species [17] and the AAF2 (5'-TGCAATCAGCGTCAGTAACAAAT-3')/ AAR3 (5'-ATGGATGCTAGACCTTTGCTGAT-3') primers specific for the amplification of *Alternaria spp* was used [18]. The PCR reaction was done in a 25 µl containing 2.5 µl of 10X buffer supplemented with 15 mM $MgCl_2$, 100 mM Tris-HCl, 500 mM KCl, 0.5 µl of each oligonucleotide primer (F+R), 2 µl of 0.04 mM each dNTPs, 2 µl of the extracted DNA, 0.25 µl of 0.25 units/25

µlTaq DNA polymerase and 7.25 µl of autoclaved distilled water. The reaction was run in a Thermal cycler (GeneAmp®PCR System 2400, Perkin Elmer, U.S.A.) by heating at 95℃ for 5 min, and then amplification was performed with 36 cycles at 94℃ for 2 min, an annealing step at 52℃ for 1 min as well as an elongation step at 72℃ for 2 min. Amplification ended with an elongation phase at 72℃ lasting 7 minutes and a final hold at 4℃ using Pettier based Thermo cycler. All PCR products were checked for length on 1.5% agarose gels. 5µl of PCR product was mixed with 2 µl of loading dye (Fermentas) and introduced into the wells in the gel. A standard DNA ladder (Fermentas-10,000 bp) was also loaded in the gel well.The amplification result was detected on 1.5% agarose gel electrophoresis staining with 10 mg/ml of ethidium bromide. The electrophoresis of the PCR products was carried out at 140 volts for 30 minutes. Gel pictures taken using the gel documentation system (Alphamager^{TM}). Gene specific primers were employed to serve as an alternate means of molecular identification.

2.3 Hydrocarbon Analysis

Pure test cultures of bacteria and fungi were grown to log phase at for 48 hours in Nutrient broth and Potato dextrose broth respectively in a shaker incubator at 120 rpm, 30℃. Microbial concentration was measured using a UV-VIS-spectrophotometer. The optical density was measured at 600 nm. 10 ml of this culture was used to inoculate different 250 ml flasks containing;

i. Bushnell and Hass media ($MgSO_4.7H_2O$ 0.5 g/L, $CaCl_2.2H_2O$ 0.1 g/L, KH_2PO_4 1 g/L, $(NH_4)_3PO_4$ 0.1 g/L and $FeCl_3$ 0.1 g/L, pH 6.2) supplemented with 5% (v/v) crude oil (Bonny light) and *Pseudomonas aeruginosa*
ii. Bushnell and Hass media ($MgSO_4.7H_2O$ 0.5 g/L, $CaCl_2.2H_2O$ 0.1 g/L, KH_2PO_4 1 g/L, $(NH_4)_3PO_4$ 0.1 g/L and $FeCl_3$ 0.1 g/L, pH 6.2) supplemented with 5% (v/v) crude oil (Bonny light) and *Alternaria* sp.
iii. Bushnell and Hass media supplemented with 5% (v/v) crude oil (Bonny light) but without isolates as control

All flasks were incubated shakers at 120 rpm, 27-37℃ for 21 days. Following this, the samples were centrifuged at 10000 rpm for 10 minutes and the top layer was recovered and transformed to a clear vial for GC-MS analysis.

1 µl of the treated crude was injected into the GC column using a 1:20 split ratio. Mass spectra as well the retention times of standard mixture of hydrocarbons were used to quantify each analyte. GC-MS analysis was carried out using a QP-2010 spectrometer coupled to a Hewlett-Packard Model 6890, GC equipped with a cool-on-column inlet and capillary direct interface. The instrument conditions were the following: capillary column HP-1MS, 60 m x 0.2 mm; helium column flow 1 ml/min; pressure 18.5 psi and split ratio 20:1. The initial temperature was 70℃ kept for 5 minutes with a temperature ramp of 14℃ per minute and final temperature of 280℃ kept for 10 minutes with total run time 30 minutes. A solvent delay was employed in order to prolong detector lifetime at 0-4.5 minutes. The solvent front reached the detector at 4.0 minutes and initial analyte retention time was approximately at 7 minutes.

3. RESULTS

Viability counts of the colony forming units (CFU) averaged $7.1x10^8$ cfu/ml for bacteria and $5.7x10^8$ cfu/ml for fungi.

Table 1. Mean total viable count of *P. aeruginosa* and *Alternaria* sp. *(*cfu/ml)

Microorganism	Mean total viable count
Pseudomonas aeruginosa	$7.1 \times 10^8 \pm 1.70$.
Alternaria sp	$5.7 \times 10^8 \pm 1.42$

Values are means of triplicate results ± standard error

4. DISCUSSION

Microorganisms surviving in a vegetable garden environment are exposed to a number of organic and inorganic materials including biological and chemical fertilizers. This enhances their inability to metabolize a wide range of substances for their growth and survival and thus limiting the diversity of microbes in such environment. The bacterial isolates used in this study obtained from garden soil exhibited a smooth-surfaced greenish colour that changed the colour of the nutrient agar green. The isolate was gram negative and possessed a rod-like shape.

Morphological observations of the fungus revealed the profuse growth of mycelia on potato dextrose agar (PDA), creamy to ashy white with a circular shaped appearance. The mycelium grew as a singular concentric ring that seemed sparse and submerged. This observation tallies with a recent study that involved the

morphological characterisation of *Alternaria alternata* [19].

Table 2. Percentage of Hydrocarbons present in control before degradation

Formula	Name	Molecular weight
C_7H_8	Toluene	92
$C_6H_{12}O$	2-Pentanone	100
C_8H_{10}	o-Xylene	106
C_8H_{16}	Cyclohexane*	112
C_8H_{16}	Cyclopentane*	112
C_9H_{12}	Benzene	120
$C_8H_{16}O$	2-Hexanone	128
C_9H_{20}	Nonane	128
$C_{10}H_{22}$	Decane	142
$C_{11}H_{24}$	Octane	156
$C_{12}H_{26}$	Dodecane	170
$C_{13}H_{28}$	Tridecane	184
$C_{14}H_{30}$	Tetradecane	198
$C_{15}H_{32}$	Pentadecane	212
$C_{16}H_{34}$	Hexadecane	226
$C_{18}H_{38}$	Octadecane	254
$C_{16}H_{32}O_2$	n-Hexadecanoic acid	256
$C_{18}H_{34}O_2$	Oleic Acid	282
$C_{20}H_{42}$	Eicosane*	282
$C_{20}H_{42}$	Nonadecane*	282
$C_{18}H_{36}O_2$	Octadecanoic acid	284

**Steroisomers*

Biodegradation of crude oil in soil can occur by biostimulation or bioaugmentation. The degradation of hydrocarbon by any microorganism is influenced by the composition of the community and its adaptive response to the presence of hydrocarbons. The high rate of crude oil degradation shown in (Tables 3 and 4), agree with [20] who stated that bacteria and fungi are the key agents of degradation. In this study, the fungal species was averagely more effective in the biodegradation of the crude oil from the soil samples than the bacterial species, supporting what [20] said that bacteria assumes dominant role in marine ecosystems while fungi are more important in freshwater and terrestrial environments in terms of degradation.

In this study, there was no particular pattern of degradation by individual microorganism following the available parameters. However, there was generally an increase in retention times as the number of carbon atoms increased**.** *Alternaria* sp has been extensively studied among other fungi that play a major role in biodegradation process of petroleum products [21-23]. Previous study [24] reported a particular *Pseudomonas* sp. strain C450R and *Halomonas* sp. strain C2SS100 that degraded 93–96% of the aliphatic fraction of crude oil (C13–C29), while producing biosurfactants. Similarly, in this study

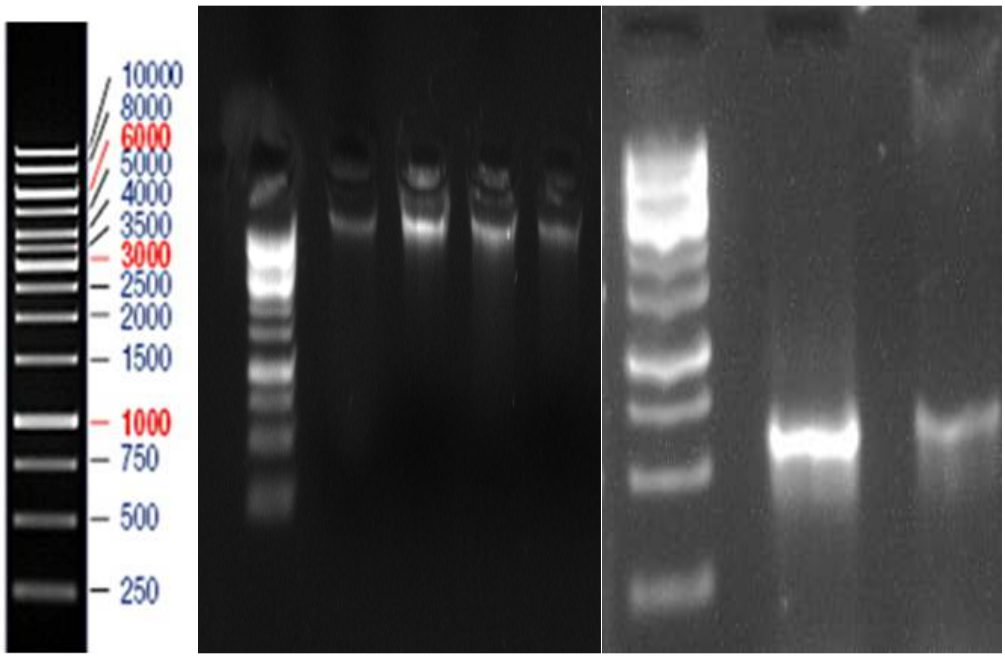

Fig. 1. Gel electrophoresis of extracted genomic DNA and amplified PCR products. Left to right: GeneRuler™ 1kb DNA ladder (Thermoscientific), gel electrophoresis micrograph of genomic DNA extracted from isolated microorganisms and gel electrophoresis micrograph of PCR products from the isolates

Pseudomonas aeruginosa was also observed to degrade higher hydrocarbon (Table 3) even more than the fungal species, converting crude oil into both cell mass and biosurfactants. This result varies with that of an earlier study where *P. aeruginosa* was unable to degrade oleic acid, hexadecanoic acid (palmitic acid) and octadecanoic acid in palm oil mill effluent (POME) [25]. Biomolecular methods have progressively been useful in the detection and quantification and identification of diverse kinds of microorganisms. Moreover, the need for a rapid and easy molecular detection and quantification technique is imperative [26]. These techniques of identification of microorganisms have offered a new frontier in diagnosis and thus providing a faster and robust mechanism that permits specie specific analysis of a tested organism. In this study, both *Pseudomonas aeruginosa* and *Alternaria* sp. were identified via PCR, employing primers that identify and anneal to sequence specific regions that only *Pseudomonas* and *Alternaria* possess respectively. The specificity of the primers was such that the amplification of any other microorganism would be unlikely as these primers (PA-GS-F 5'-GACGGGTGAGTAATGCCTA-3'/ PA-GS-R 5'-CACTGGTGTTCCTTCCTATA-3') for *Pseudomonas* targets the species-specific signature sequences in 16S rDNA variable regions 2 and 8 found only in this specie [27]. The amplification of the extracted DNA with these primers confirms that the isolated organism is of the *Pseudomonas* family (Fig. 2). For the fungal isolate, amplification by AAF2 (5'-TGCAATCAGCGTCAGTAACAAAT-3')/AAR3(5'-ATGGATGCTAGACCTTTGCTGAT-3') for *Alternaria* confirmed the molecular identity of this fungus (Fig. 2).

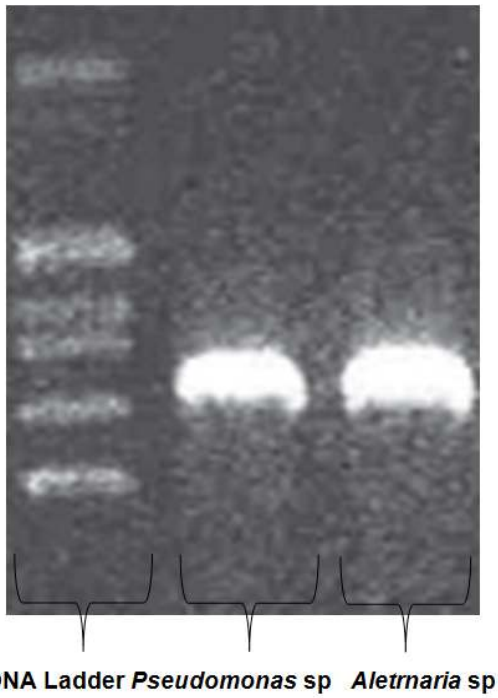

Fig. 2. PCR detection of DNA from different bacterial and fungal cultures. Lane 1 represents the DNA Ladder, Lane 2 represents the *Pseudomonas* isolate using PA-GS-F/PA-GS-R primers, Lane 3 represents *Alterrnaria* using AAF2/AAR3 primer pairs for amplification

Table 3. Percentage residual hydrocarbon (oil) after 21 days of degradation

Residual hydrocarbon	Retention time	*Pseudomonas aeruginosa* (%)	*Alternaria* Sp. (%)
Octane	11.717	67.80	42.52
Decane	10.167	64.38	21.72
Dodecane	19.450	36.67	54.10
Tridecane	12.258	41.67	42.19
Tetradecane	13.642	-	45.73
Pentadecane	17.567	60.39	48.71
Hexadecane	18.192	37.89	31.44
Octadecane	25.683	21.23	19.52

Formula for calculating percentage residual hydrocarbon from GC-MS data;

Biodegradation (%) = $(^{\text{Height of sample}}/_{\text{Height of control}})$ X 100

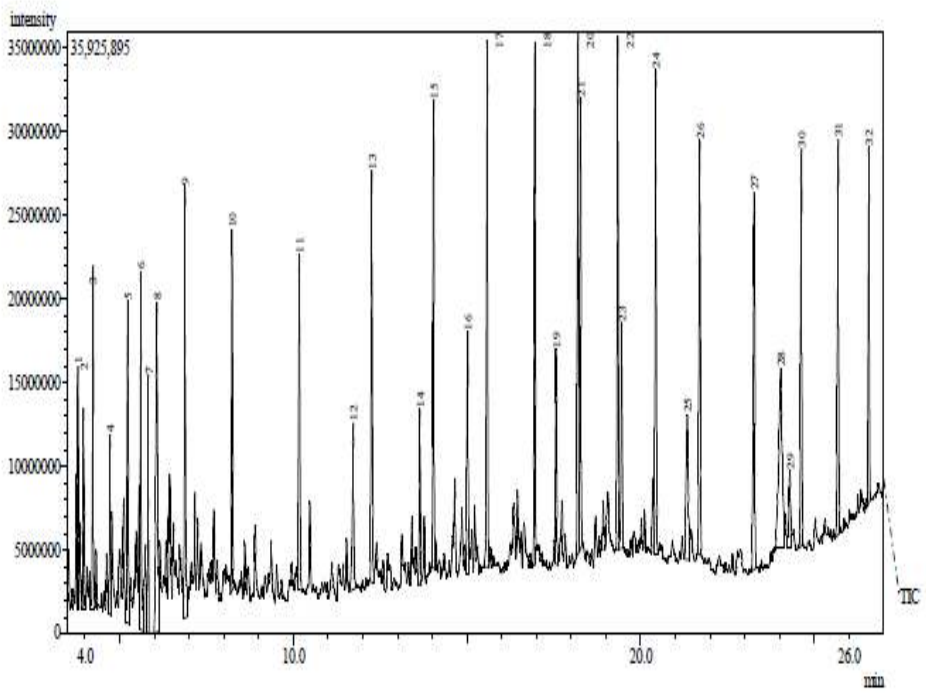

Fig. 3. GC-MS chromatogram of control sample. Major peak areas (1-32) correspond to the identified hydrocarbon compounds listed in Table 3. Certain hydrocarbons appear as repeats at different retention times

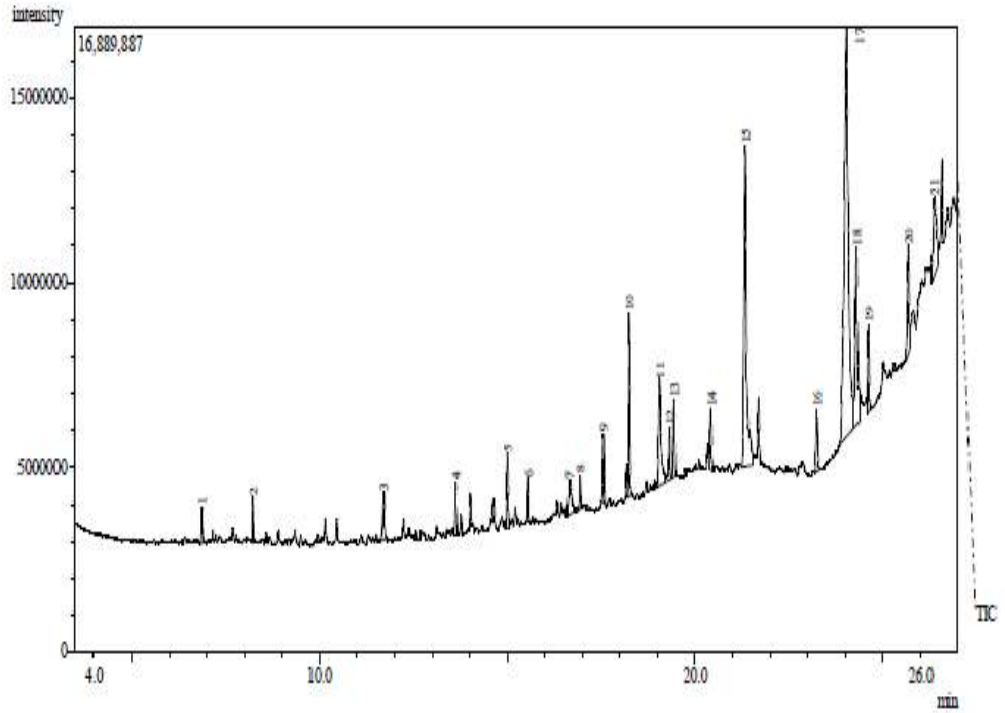

Fig. 4. GC-MS chromatogram after 21 days of degradation with *Pseudomonas aeruginosa.* Major peak areas (1-21) correspond to certain identified hydrocarbon compounds listed in Table 3. The observed numbers reduced in comparison to the control as a direct result of their degradation

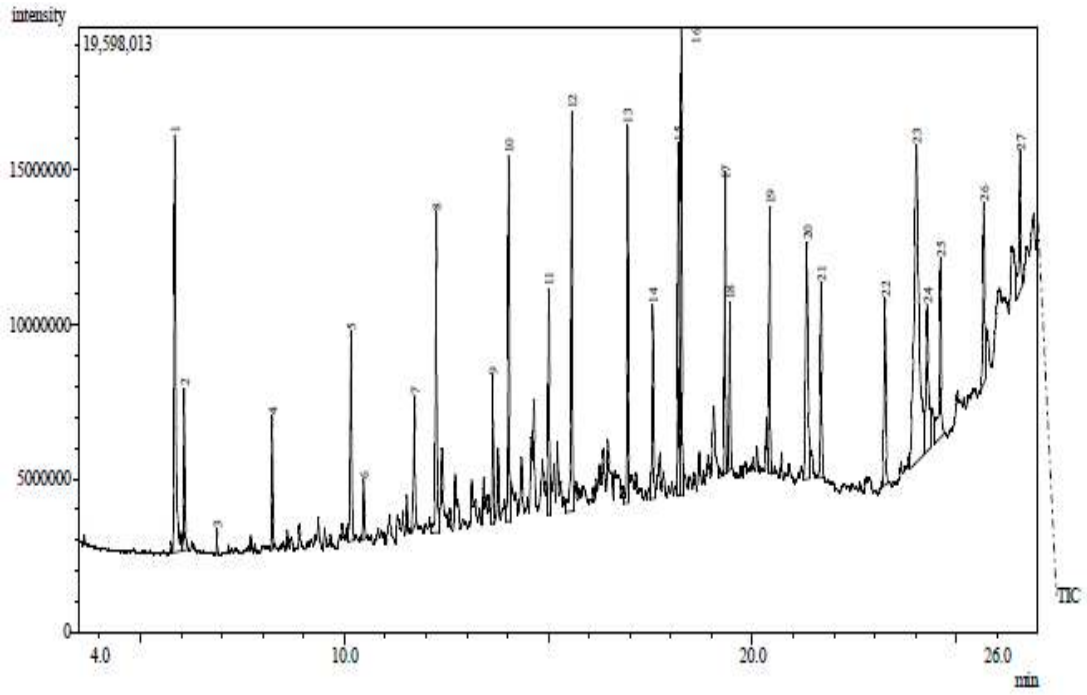

Fig. 5. GC-MS chromatogram after 21 days of degradation with *Alternaria sp.* Major peak areas (1-27) correspond to certain identified hydrocarbon compounds listed in Table 3. The observed numbers reduced in comparison to the control as a direct result of their degradation

Table 4. Percentage degradation of hydrocarbons by isolated microorganisms

Hydrocarbon	Retention time	*Pseudomonas aeruginosa* (%)	*Alternaria* Sp., (%)
Cyclohexane	3.958	100	100
Cyclopentane	4.708	100	100
O-xylene	5.208	100	100
Nonane	5.600	100	100
Octane	11.717	32.20	57.48
Decane	10.167	35.62	78.28
Dodecane	19.450	63.33	45.90
Tridecane	12.258	58.33	57.81
Tetradecane	13.642	100	54.27
Pentadecane	17.567	39.61	51.29
Hexadecane	18.192	62.11	68.56
Heptadecane	24.292	100	100
Octadecane	25.683	78.77	80.48
Nonadecane	26.567	100	100

Calculation of percentage degradation of hydrocarbon from GC-MS data;

Biodegradation (%) = 1 – ($\frac{Height\ of\ sample}{Height\ of\ control}$) x 100

Chromatograms obtained from the GC-MS analysis of both the control and studied samples are given (Figs. 1 and 2). The alkane compounds were the most targeted complexes for degradation or utilization by the isolated microorganisms. Peak height ratios of the respective alkanes from microbial activated oil samples were compare to that of the control.

Further analysis of the GC-MS data as indicated in Table 4, revealed that the lower chain hydrocarbons; cyclohexane, cyclopentane, O-xylene and nonane were all degraded as it was the first set of aromatics to be degraded. Interestingly, in the sample that contained only crude oil and the mineral medium, these compounds were degraded. It stands to reason that microorganisms are present even within crude oil samples and as such, the introduction of mineral nutrients promoted its growth and consumption of crude oil. This sample served as a control to compare the effectiveness of the two studied isolates. Between the studied bacteria and fungi, n-Dodecane ($C_{12}H_{26}$), n-Tridecane ($C_{13}H_{28}$) and n-Tetradecane ($C_{14}H_{30}$) were degraded to a much higher extent with the bacterium than with the fungus. However, data revealed that the n-Hexadecane ($C_{16}H_{34}$), n-Pentadecane ($C_{18}H_{38}$), n-Octadecane ($C_{19}H_{40}$) fractions were degraded comparatively higher with the fungal strain than with the bacterial strain. In this study, the relative biodegradability of a bacterium and fungus were examined. The data revealed that simple hydrocarbons and petroleum fuels were the first targets. Degradability decreased as the molecular weights and degree of branching/side chains increased.

5. CONCLUSION

From the discussion above, it can be seen that each of the microorganisms used in this work, *P. aeruginosa* and *Alternaria* sp demonstrated the ability to effectively degrade different hydrocarbons in the crude oil-polluted soil. The results of this study suggest that the isolated bacterium and fungus, *Pseudomonas aeruginosa* and *Alternaria* sp. may have the potential for bioremediation of crude oil (light) polluted soils especially in semi-dry regions indicating the possibility of their use either singly or in consortium for the bioremediation of oil-polluted sites.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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