Indigenous Bacteria and Fungi Responsible For Bioremediation of Oil-Polluted Soils in Ondo State, Nigeria

✉[1]Olukunle, O. F., [2]Boboye, B. and [3]Ikuomola, O.T.

Department of Microbiology, Federal University of Technology, P. M. B 704, Akure, Ondo State, Nigeria.
✉*Corresponding Author:email: ofolukunle@gmail.com, Tel. No.: +2348036141743*

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Abstract
In order to reduce or eliminate the effect of oil spillage on the environment and living organisms using biological process, a study was carried out to isolate oil-degrading bacteria and fungi associated with oil-polluted lands in Ondo State, Nigeria. In this study, the indigenous bacteria and fungi, isolated from some oil-polluted sites in Ondo State, Nigeria and which were able to degrade oil were investigated by the use of classical selective enrichment technique. These organisms were further studied to determine their biodegrading activities on hydrocarbons containing 2% (v/v) (diesel, kerosene, petrol) using enrichment medium. The microbial growths were determined using spectrophotometer. The physicochemical properties of the environmental samples were analysed using standard procedures and compared with the unpolluted samples.The bacterial isolates were identified by morphological and biochemical characterization using the taxanomic scheme of Bergey's Manual of Determinative Bacteriology. The bacteria obtained from the oil-polluted sites were Bacillus firmus, Bacillus sphaericus, Staphylococcus aureus, Micrococcoccus sp., *Acinetobacter* sp., *Pseudomonas sutzeri and Bacillus pumilus. Fungi isolated from the various sites included Penicillium italicum, Aspergillus niger, Penicillium oxalicum, and Streptothrix atra, based on the microscopic and macroscopic features of the hyphal mass, nature of the fruiting bodies and the morphology of cells and spores. These results showed that all the microbes maximally utilized the oil substrates (petrol, diesel, and kerosene) when supplied as the sole source of carbon and energy. The test on the degrading activity of isolates on oils revealed that B firmus, and Penicillium oxalicum are best degraders of petrol while B. sphaericus and Aspergillus niger and B. sphaericus and Streptothrix atraare bestdegraders of diesel and kerosene respectively. The physicochemical properties of the polluted soils showed higher levels of Pb. Zn and Fe than the unpolluted soil samples. The pH values obtained in this study were not too low or high which supported biodegradation activity of bacteria and fungi in the soil. The bacteria and fungi obtained in the study areas coupled with the pH value, revealed microbial activities during degradation, although at very slow rate. Further research into enhancing the degradation process by altering environmental factors and manipulating the genetic make up of these bacteria and fungi for effective and efficient bioremediation process will be of great relevance in promoting a sustainable development of our environment.*

Keywords:*Hydrocarbon, enrichment medium,degrading activity, bioremediation, physicochemical properties, microbial growth.*

Introduction

The greatest environmental problem connected with crude oil exploration in Nigeria is oil spill both on-shore and off-shore (Mandri and Lin, 2007). Crude oil, because of its characteristics, is one of the most significant pollutants in the environment as it is capable of causing serious damages to humans and the ecosystem, resulting in the contamination of drinking water, killing of fishes and poisoning aquatic life, thereby, placing hardship on those who make their living by fishing. It produces ecological problems of great dimension. It affects soil fertility adversely, causes alterations in soil physicochemical and microbiological properties (Agbogidi *et al.*, 2005). Oil spills also cause epidemics of many diseases because spilled oils contain toxic substances which could be injurious to human health. (Nwachukwu *et al*, 1999), Heavy metals present in hydrocarbon contaminated soils can be concentrated in the food chain, which eventually results in more risk to humans. The indirect effects of oil spill in soil include oxygen deprivation of plant roots because of exhaustion of soil oxygen by oil-degrading microorganisms, thereby creating anaerobic conditions which may lead to the formation of hydrogen sulphide (Agbogidi *et al.*, 2005).

The release of oil into the environment causes environmental concern and attracts the public attention (Roling *et al.*, 2002). Microorganisms in the environment attack and digest the oil and this is the basis for the emergent field of bioremediation**.** Bioremediation is defined as the use of microorganisms or their enzymes to provide an effective alternative (Singh *et al*., 2001) or to return the environment altered by contaminants to its original condition (Okon and Trejo–Hernandez, 2006). Bioremediation is a most widelyaccepted technique (Singh *et al.*, 2001) because it issafe, less disruptive to the environment, cost effective, the technology does not involve sophisticated equipment, and the method is accepted by the public because the organisms used are environmental friendly.

Oil-degrading microorganisms are ubiquitous in the environment, particularly in the hydrocarbon-polluted sites. They are capable of using organic substances, natural or synthetic, as sources of nutrients and energy hence, exhibiting remarkable range of degradative capabilities (Dua *et al*., (2002). Many microorganisms capable of degrading petroleum components have been isolated. However, few of them seem to be important for hydrocarbon biodegradation in natural environments (Harayama *et al.*, 1999). Certain indigenous microorganisms have shown to have the capability to degrade hydrocarbons in polluted soils thus leading to *in situ* bioremediation, thereby providing an effective, economical, versatile and environmental friendly means of reclaiming polluted lands and water bodies (Tesar*et al*., 2002). Both fungi and bacteria have been found to be useful in bioremediation process, even though many researches have been on bacteria in the recent times. *Pseudomonas* species and closely related organisms have been the most extensively studied owing to their ability to degrade so many different contaminants (Wackett, 2003).

The objectives of this study are to determine microbial and physicochemical properties of the oil-polluted soils; identify the microorganisms responsible for the degradation of the oil in the area; and investigate the degrading activity of the microorganisms.

Methodology

Source and collection of samples

Top soil samples were collected from three different communities in the Niger Delta area of Ondo State into sterile cellophane bags. The communities (Mese, Oluwa and Awoye) were reported to have been polluted with hydrocarbon in the past. The controlsample was collected

from an unpolluted site in the same vicinity. These samples were taken to the laboratory of the Federal University of Technology, Akure for microbiological and physicochemical analyses.

Microbial counts

Pour plate technique was used for the microbiological analysis of samples collected from oil polluted soils (Song and Bartha, 1990). One gram of the moist soil samples was used to make ten fold dilution series in triplicates; one milliliter each from dilution 10^{-4} was seeded into nutrient agar plates and incubated at 37ºC for 24 hours. The bacterial counts were thereafter, enumerated on nutrient agar. Potato dextrose agar (PDA) was used for the enumeration of fungal counts, incubated at room temperature for 72 hours based on the standard methods of Amund and Igiri, (1990).

Enumeration of oil-degrading bacteria

Minimal salt medium (MSM), an enrichment medium for the growth of oil-degraders was used to enumerate oil-degrading bacteria and fungi from the soil samples collected from the oil-polluted sites in Ondo state. The composition of the MSM was as follows: K_2HPO4 (1.8 g/L); NH_4Cl (4 g/L); $MgSO_4.7H_2O$ (0.2 g/L); NaCl (0.1g/L); $Na_2SO4.7H_2O$ (0.01 g/L); agar (20 g/L); carbon source (1%); and distilled water (1L) with pH 7.2.The medium was sterilized by autoclaving at 121ºC for 15 min. The samples were serially diluted and 1ml from 10^{-4} dilutions was seeded in the MSM. The medium was supplemented with 1% (v/v) filter sterilized hydrocarbons (kerosene, petrol and diesel) to serve as the only source of carbon and energy (Ijah and Abioye, (2003). The medium was incubated at room temperature for 7 days.

Isolation and Characterization of Isolates

Pure isolates were obtained using streak techniques and stored at 4ºC in agar slants. Individual colonies were identified by morphological and biochemical techniques using the taxanomic scheme of Beygey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974) for bacteria and Barnette and Hunter, (1972) for fungi.

Biodegrading activities of bacteria on hydrocarbons

The degrading activities of each isolates were obtained by using MSM in which 1% of each hydrocarbon (petrol, kerosene and diesel) was added and incubated at room temperature for seven days. The optical density of each sample (OD_{600nm}) was read using spectrophotometer.

Physicochemical properties

The pH values of the samples were measured with the aid of electric pH meter (Jenway 3510) following calibration with buffer solutions of pH 4, 7 and 10. The moisture and total nitrogen in the samples were quantified by oven-drying and micro-Kjeldahl methods, respectively (Ijah and Abioye; 2003). Determination of available phosphorus was determined by standard titrimetric procedures. Exchangeable Mg and Ca were determined by EDTA titrimetric method while exchangeable Na and K in soils were determined using flame emission photometry. The presence and amounts of toxic metals (Pb, Zn, Fe) in the soil samples were investigated using Atomic Absorption Spectrophotometer (EPA, 1999). The organic carbon and organic matter were analysed by wet oxidation of soil using concentrated H_2SO_4 and aqueous $K_2Cr_2O_7$ (Walkey and Black, 1965).

Results

Microbial counts

The bacterial counts ($x10^4$) obtained from oil-polluted sites in Mese, Oluwa and Awoye, all in Ondo state ranged between 10.00 ± 1.00 and 16.00 ± 1.00 Cfu/g , while the fungal counts ($x10^4$) were between 8.67 ± 2.08 and 15.00 ± 1.00 Sfu/g (Fig. 1). The bacterial and fungal counts for the unpolluted soil were 7.33 ± 2.52 Cfu/g and 9.00 ± 1.00 Sfu/g, respectively.

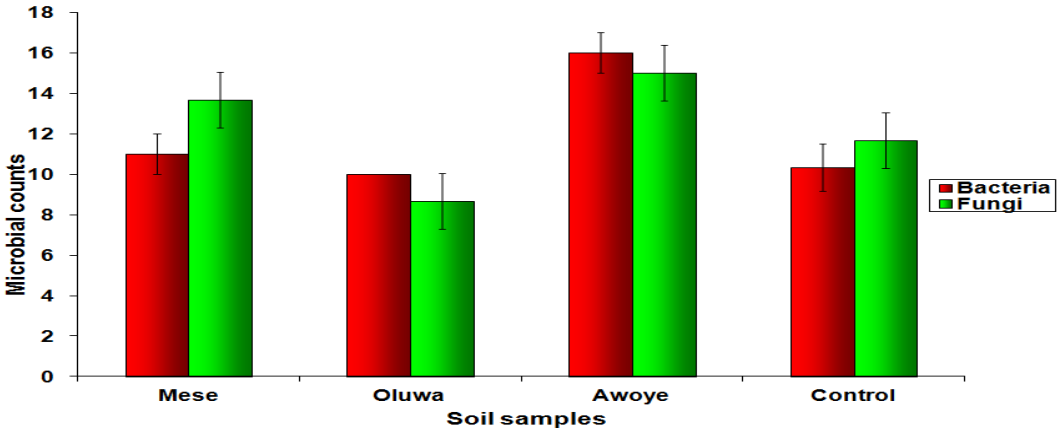

Figure 1: Microbial population of oil-polluted soilsUnits
Bacteria (Cfu/g); Fungi (Sfu/g)

Oil-degrading organisms

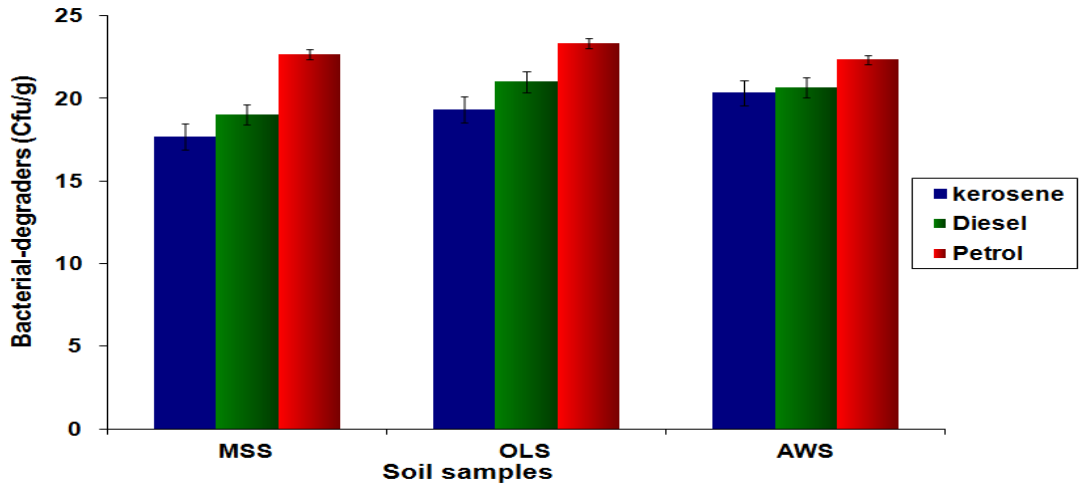

Figure 2: Population of oil-degrading soil bacteria on kerosene, diesel and petrol
Legend:

MSS	Mese
OLS	Oluwa
AWS	Awoye

Figure 2 shows the population of oil-degrading bacteria in the soil samples. The mean of oil-degrading bacteria on kerosene ($x10^4$) ranged from 17.67 ± 3.5 to 20.33 ± 3.06 Cfu/g, on diesel, it ranged from 17.00 ± 1.00 to 22.00 ± 1.00 Cfu/g; while on petrol, it ranged from 19.67 ± 2.81 to 21.33 ± 2.52 Cfu/g. Soil samples collected from Awoye, had the highest population of oil-degrading bacteria on kerosene; while samples collected from Oluwa had the highest population of oil-degrading bacteria on both diesel and petrol.

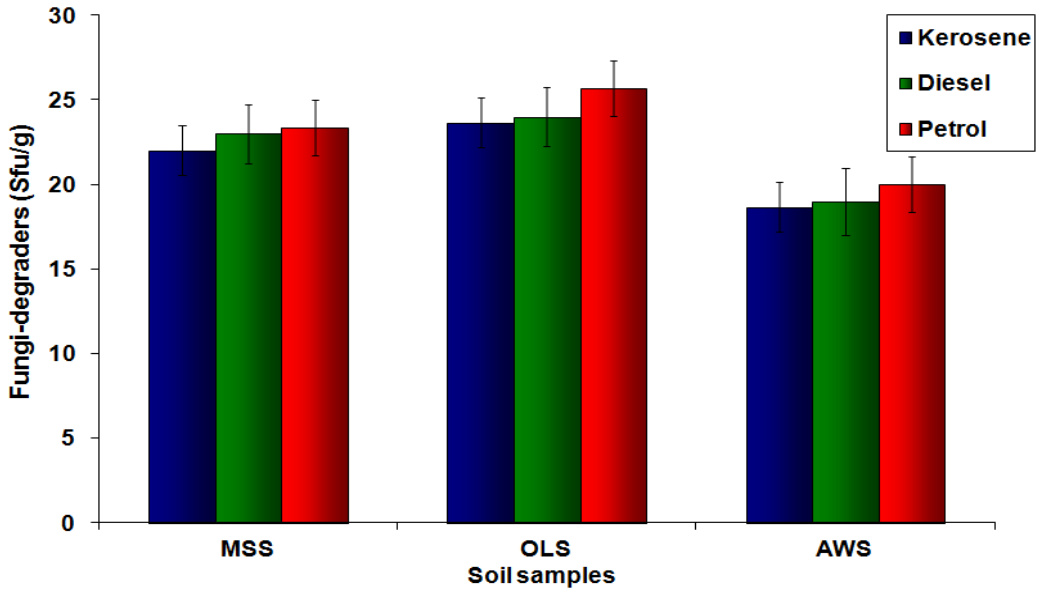

Figure 3: Population of oil-degrading soil fungi on kerosene, diesel and petrol

Legend:

MSS	Mese
OLS	Oluwa
AWS	Awoye

Figure 3 shows the population of oil-degrading fungi in the soil samples. The mean of oil-degrading fungi varied between 18.67 ± 2.51 and 23.67 ± 1.53 Sfu/g on kerosene; 19.00 ± 2.00 and 24.00 ± 1.73 Sfu/g on diesel; and 20.00 ± 1.00 and 25.67 ± 1.15 Sfu/g on petrol.

Identification of bacteria

Table 1: Morphological and biochemical characteristics of bacteria isolated from oil-`polluted soil samples collected from Ondo State

Characteristics	Isolate A	Isolate B	Isolate C	Isolate D	Isolate E	Isolate F	Isolate G
Cultural							
Colony shape	Circular	Irregular	Irregular	Circular	Oval	Circular	Irregular
Elevation	Raised	Flat	Flat	Raised	Raised	raised	Flat
Edge	Entire	Undulate	Entire	Entire	Entire	Entire	Undulate
Pigmentation	White	Yellow	Yellow	Cream	Cream	Yellow	White
Gram stain	+	+	+	+	-	-	+
Cell shape	Rod	Rod	Rod	Spherical	Rod	Rod	Rod
Spore stain	+	+	+	-	-	-	+
Catalase	+	-	+	+	+	+	+
Motility	+	+	-	-	-	+	+
Oxidase	-	-	-	-	-	-	-
Fermentation							
Glucose	+	-	+	+	+	+	+
Mannitol	+	-	+	+	-	-	+
Xylose	-	-	-	-	+	+	+
Sucrose	-	-	+	-	-	-	-
Lactose	+	-	+	+	-	-	+
Probable bacteria	***Bacillus firmus***	***Bacillus sphaericus***	***Staphylococcus aureus***	***Micrococcus* spp.**	***Acinetobacter* spp.**	***Pseudomonas stutzeri***	***Bacillus pumillus***

Legend:

+ = Present

- = Absent

Table 1 shows the morphological and biochemical characteristics of the seven bacterial isolates found in the soil samples. The bacteria obtained from the oil-polluted sites were *Bacillus firmus*, *Bacillus sphaericus*, *Staphylococcus aureus*, *Micrococcus* sp., *Acinetobacter* sp., *Pseudomonas sutzeri and Bacillus pumilus*.

Identification of fungi

Table 2:Cultural and morphological characteristics of fungi isolated from oil-pollutedsoil

Cultural traits		Tentative Identity
Black colonies	Conidiophores are upright, simple and terminating in a globose or clavate swelling bearing phialides at the apex	*Asperillus niger*
Yellowish green to dark green colouration. Yellowish base	Septate hypha, hyphae bears conidiophores, presence of primary and secondary stigmata.	*Penicillium oxalicum*
Brown colonies appearing creamy at the edges	Branched conidiophores branches with conidia. Dark mycelium growing loosely with spirally coiled, ail conidiophores	*Streptothrix atra*
Yellowish green to dark green colouration	Conidiophores arise from the mycelium singly near the apex mostly apex or globose	*Penicillium italicum*

Table 2 shows the identity of the isolated fungi based on the microscopic and macroscopic features of the hyphal mass, nature of the fruiting bodies and the morphology of the cells and spores. Fungi isolated from the various sites included *Penicillium italicum*, *Aspergillus niger*, *Penicillium oxalicum*, and *Streptothrix atra*.

Biodegrading activity of each isolates on oils

Table 3: Bacteria with the highest and least oil-degrading activities

Oil	Least	Highest
Kerosene	*Acinetobacter sp.*	*Bacillus sphaericus*
Diesel	*Pseudomonas stutzeri*	*Bacillus sphaericus*
Petrol	*Staphylococcoccus. aureus*	*Bacillus firmus*

Table 3 shows the highest and least oil-degrading activities on oils (kerosene, diesel and petrol). *Bacillus sphaericus* demonstrated the greatest ability to degrade kerosene and diesel while*Bacillus firmus* demonstrated the greatest ability to degrade petrol. *Acinetobacter* sp., *Pseudomonas sutzeri* and *Staphylococcus aureus* had the least degrading activities on kerosene, diesel and petrol respectively.

Table 4: Fungi with the highest and least oil-degrading activities

Oil	Least	Highest
Kerosene	*Streptothrix atra*	*Asperillus niger*
Diesel	*Penicillium italicum*	*Streptothrix atra*
Petrol	*Penicillium italicum*	*Penicillium oxalicum*

Table 4 shows the highest and least oil-degrading activities of fungi on oils (kerosene, diesel and petrol). *Aspergillus niger*, *Streptothrix atra* and *Penicillium oxalicum* demonstrated the highest activities on kerosene, diesel and petrol respectively. Fungi that demonstrated the least activity incude *Streptothrix atra* on kerosene and *Penicillium italicum* on both diesel and petrol

Physicochemical properties

The physicochemical properties of both polluted and unpolluted soils are presented in Table 5.

Table 5: Physicochemical properties of oil-polluted soil

Parameters	Mese	Oluwa	Awoye	Control
Moisture content (%)	78.65 ± 1.2	77.89 ±1.5	77.95 ±1.3	79.34 ± 1.2
pH	6.7 ± 0.09	6.5 ± 0.03	6.4 ± 0.09	7.9 ± 0.09
Organic carbon (%)	2.76 ± 1.0	3.01 ± 0.8	3.41 ± 0.9	2.00 ± 1.0
Organic matter (%)	4.75 ± 1.4	4.97 ±1.6	5.88 ±1.8	5.96 ± 1.7
Nitrogen (%)	0.42 ± 1.4	0.48 ±2.2	0.56 ±1.1	0.65 ± 1.6
Phosphorus (mg/kg)	1.75 ± 1.1	2.34 ± 1.8	5.51 ±2.1	5.88 ± 2.1
Potassium (mg/kg)	0.81 ±1.3	1.02 ± 2.6	1.15 ±3.4	1.74 ± 0.8
Sodium (mg/kg)	0.73 ±1.2	0.82 ± 3.2	0.91 ±0.8	0.71 ± 1.4
Calcium (mg/kg)	2.3 ± 1.4	4.8 ± 2.2	5.8 ± 1.6	4.0 ± 1.1
Magnesium (mg/kg)	2.7 ± 1.3	2.4 ±1.6	2.0 ±2.3	2.8 ± 1.4
Lead (Pb) (mg/kg)	60 ± 0.9	62 ± 0.2	60 ± 0.7	30 ± 1.8
Zinc (Zn) (mg/kg)	0.162 ± 1.8	0.169 ±0.9	0.161 ± 1.1	0.005 ± 1.4
Iron (Fe) (mg/kg)	0.214 ±1.1	0.218 ± 1.0	0.212 ± 2.3	0.01 ± 1.2

The moisture contents (%) of the polluted soils were lower than the control and ranged between 78.65 ± 1.2 and 79.34 ±.1.3. The pH ranged between 6.4 ± 0.09 and 7.9 ± 0.09 with the control having the highest value. The values obtained for Nitrogen (%) were from 0.42 ± 1.4 to 0.65 ± 1.6; Phosphorous (mg/kg) ranged from 1.75 ± 1.1 to 5.88 ± 2.1; and Potassium (mg/kg) had values between 0.81 ± 1.3 and 1.15 ± 3.4. The values obtained for lead, zinc and iron showed that the control soil samples had the lowest with 30, 0.005 and 0.01 (mg/kg) respectively.

Discussion

The microbial population obtained from this study shows that Oluwa and Awoye soils had higher bacterial counts (fig. 2) than fungi counts (fig. 3) in line with the work carried out by Onifade *et al.*, (2007). The nutrients status of these soils may be responsible for this phenomenon (Jobson *et al.*, 1979). The presence of bacteria and fungi in the oil-polluted soils buttresses the fact that microorganisms are ubiquitous. The loads for oil-degrading microbes were high indicating the presence of indigenous microbes in the polluted soils. The oil-degraders are found to possess enzymes which enable them break down the complex molecules of hydrocarbons (Sohail and Srivastava, 1994). In an oil spilled site, microorganisms are found to play a major role in the biodegradation process. The polluted sites are found to harbour a variety of species of microorganisms, which have the capacity to use the oil as source of carbon and energy (Das and Mukherjee (2006). This confirms that the metabolic activities of the indigenous microbes, hence utilization of the oil leading to the degradation of the oil when supplied as the sole source of carbon and energy. Ojo (2006) postulated that the activities of these microorganisms could be responsible for the bioremediation of the environment. These provide useful information in the biological activities going on, in the soil and the level at which these organisms are able to adapt to the harsh environment. The bacterial isolates were identified by morphological and biochemical characterization using the taxanomic scheme of Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974). The bacteria obtained from the oil-polluted sites were *Bacillus firmus, Bacillus sphaericus, Staphylococcus aureus, Micrococcus*sp.

Acinetobacter sp., *Pseudomonas sutzeri and Bacillus pumilus*.Fungi isolated from the various sites included *Penicillium italicum*, *Aspergillus niger, Penicillium oxalicum,* and *Streptothrix atra,* based on the microscopic and macroscopic features of the hyphal mass, nature of the fruiting bodies and the morphology of cells and spores (Barnette and Hunter, 1972).

The degradation activity showed that all the bacterial isolates have potentials to degrade the three types of oil used in this study, i. e. kerosene, diesel and petrol. This hypothesis suggests similarities in the gene that encodes degradation in their genetic make up (Boboye *et al.*, 2010). *Bacillus sphaericus* had the highest ability to degrade both kerosene and diesel while *Bacillus firmus* was able to degrade petrol best. In case of the fungi, *Aspergillus niger, Streptothrix atra and Penicillum oxalicum* were found as best degraders of kerosene, diesel and petrol respectively. Previous studies from literatures confirm that *Bacillus* has been reported as oil-degraders in different environments (Boboye *et al.*, 2010). *Bacillus* found in this study is in agreement with the researchers that postulated that *Bacillus* species are more tolerant to the high levels of hydrocarbons due to their resistant endospores (Ghazali *et al.*, 2004).

It has been demonstrated that hydrocarbons have negative effects on the biochemical and physicochemical characteristics of soils, as well as its microbiological properties (Wyszkowska *et al.* 2002). The moisture contents of the oil polluted soils were lower than that of the control in all the samples. This is in agreement with the work done by Onifade *et al.*, (2006). This may be probably due to the presence of oil coating the soil, thereby preventing the penetration of water into the soil. It has been reported that a moisture content range of 20 – 80% is generally optimum for hydrocarbon degradation (Bossert and Bartha, 1984). The pH values of polluted soil were lower than the control in all the samples. This also is in line with the work carried out by Ogaji *et al.* (2005). The ranges of pH obtained in this study were reported to support biodegradation activity of bacteria and fungi in the soil (DPR, 1991). The physicochemical properties of the polluted soils showed higher levels of Pb. Zn and Fe than the control soil samples. The nutrient status i.e. (nitrogen, phosphorus and potassium) of the polluted soils for Oluwa and Awoye were higher than those of Mese and the control which may be responsible for the higher population of bacteria. The bacteria and fungi obtained in the study areas coupled with the pH value, revealed microbial activities during degradation, although at very slow rate. Further research into enhancing the degradation process by altering environmental factors and manipulating the genetic make up of these bacteria and fungi for effective and efficient bioremediation process will be of great relevance in promoting a sustainable development of our environment.

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