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OIL DEGRADATION ASSESSMENT OF MICROBES ISOLATED FROM USED ENGINE OIL CONTAMINATED SOIL IN OGBOMOSO, OYO STATE NIGERIA

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ABSTRACT

The indiscriminate disposal of used engine oil from vehicles by motor mechanics has been of great concern and a major source of oil pollution. This study is therefore designed to assess the microbiological and physicochemical characteristics of soils contaminated with used engine oil and investigate the biodegrading potentials of microorganisms isolated from contaminated soil. Oil-contaminated soil samples were collected from six different auto-mechanic workshops in Ogbomoso, Nigeria, uncontaminated soil samples were also collected to serve as control. The total heterotrophic bacterial counts for contaminated and uncontaminated soil samples ranged from $8.0x10^7$ to $2.1x10^7$ CFU/g and $22.0x10^7$ to $30.0x10^7$ CFU/g while the total colony counts for bacterial oil degraders ranged between $1.3x10^7$ and $2.0x10^7$ CFU/g in contaminated soil samples. The bacterial isolates were *Bacillus polymyxa*, *B. megaterium*, *B. alvei*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Escherichia coli*. Residual oil for contaminated soil samples was found to be between 12.02±0.20 mg/kg and 19.40±0.50 mg/kg. Engine oil contaminated soil samples showed the highest nitrate, sulphate and phosphate concentration compared with the uncontaminated samples. *E. coli* showed the highest degradability potentials for both aliphatic and aromatic functional groups because it had the lowest intensity of 19.25cm^{-1} and 18.22 cm^{-1} respectively after 30 days as compared with the intensity of the control (146.05$cm^{-1}$for aliphatic and 315.16 $cm^{-1}$for aromatic). Bacteria capable of metabolizing engine oil in the studied sites was observed with *E. coli* more effective for degradation of both aliphatic and aromatic functional groups which is an indication that the isolates could be exploited for natural attenuation and clean-up of engine oil in case of spillage or indiscrimate disposal.

INTRODUCTION

Engine oil is a complex mixture of hydrocarbons and other organic compounds including some organo-metabolic constituents (Butler and Mason, 1997) that is used to lubricate the parts of an automobile engine in order to avoid excessive wearing out (Hagwell *et al*., 1992).The presence of different substrates and metabolites in hydrocarbon contaminated soils has no doubt provided an environment for the development of a quite complicated microbial community (Butler and Mason, 1997; Udeani *et al*., 2008). Used motor oil is a common environmental contaminant which is defined by the United State environmental protection agency as any oil that has been refined from crude oil or any synthetic oil that has been used, and as a result of such use is contaminated by physical or chemical impurities (USEPA, 2001).

Used (also called spent or waste) motor engine oil contains metals and heavy polycyclic aromatic hydrocarbons (PAHs) and these could contribute to chronic hazards including mutagenicity and carcinogenicity (Hagwell *et al*., 1992; Boonchan *et al*., 2000). Additionally, prolonged exposure to oil as well as high concentration of oil could cause the development of liver or kidney disease, possible damage to the bone marrow and an increased risk of cancer (Deni and Penninck, 1999; Lloyd and Cackette, 2001; Mishra *et al*., 2001;Igwo- Ezikpe *et al*., 2009).As it is inevitable for the efficient and effective functioning of the automobile engines, soil contamination with used engine oil is becoming one of the major environmental problems (Mandri and Lin, 2006), mainly due to uncontrollable disposal, particularly in developing countries. A wide range of Hydrocarbon utilizers (HCUs) found to be useful in the soil include the following species: *Pseudomonas, Rhodococcus, Mycobacterium, Bacillus, Acinetobacter, Providecin, Flavobacter, Corynebacterium, Streptococcus spp* (Bhattacharya *et al*., 2002), other organisms such as fungi which include, *Trichoderma, mortierella, Aspergillus* and *Penicillium spp* are also capable of degrading the hydrocarbons in engine oil to a certain extent, but they take longer periods

of time to grow when compared to their bacterial counterparts (Prenafeta-Boldu *et al.*, 2001).

Basically, there are two different approaches to bioremediation technologies, depending on the pollution situation and type of micro-organisms being used, the first is the one which involves the activation of the indigenous microflora in the polluted area by addition of nutrients and forming the best conditions of other chemical, physical and biological factor, or known as biostimulation, the second (bioaugumentation) is the one which involves the addition of oil-oxidizing micro-organisms isolated from other sites, or addition of genetically engineered micro-organisms (Amund *et al.*, 1987).

Bacteria and fungi are the primary agent for degradation of organic contaminants in soil (Alexander, 1994). Increasing diversity of microbial populations and common structure can accelerate the degradation of the contaminants (Cole and Liu, 1994).

Hydrocarbon soil degrading microorganisms require an environmental habitat that has a sufficient and preferably sustainable source of nutrients, water, air, mild ambient temperature, and a moderate pH (Stegmann *et al*., 1991). At optimum levels, these environmental factors provide the energy and metabolic resources that create a widely diverse group of beneficial microorganisms that will suddenly reproduce on a very rapid scale (Rahman *et al.*, 2002). While these degrader microorganisms increase their populations, they also work rapidly and effectively to degrade petroleum hydrocarbons for food (from carbon) to sustain their growth pattern (Stevenson, 1994; USEPA, 2001). Hydrocarbon utilizing microorganisms are important in combating the problem of oil pollution in our environment (Atlas and Bartha, 1992). Although, hydrocarbon-utilizing microorganisms are ubiquitous, their proportion within the microbial community is thought to be a sensitive index of environmental exposure to hydrocarbons (Leahy and Colwell, 1990).

Used motor oil is the brown-to-black oily liquid removed from motor vehicle, when the oil is changed, it is similar to unused oil, except that it contains additional chemicals that are produced or build up in the oil, when it is used as an engine lubricant at high temperatures and pressures, inside an engine as it runs (Dorsey *et al.*, 1997). Therefore, this study was designed to investigate the biodegrading potentials of microorganisms isolated from engine oil contaminated soil.

MATERIALS AND METHODS

Study Site

Oil-contaminated soil samples were collected from six different auto-mechanic workshops in Ogbomoso, Nigeria at depths of 0-15cm and 15-45cm. Uncontaminated soil samples were also collected 100m away from the workshops to serve as control.

Sample Collection

The soil samples were aseptically collected using auger into a sterile polythene bag and then transported to the laboratory immediately for microbiological and physico-chemical analysis.

Physico-Chemical Analysis

Determination of Temperature

The temperature of each soil samples were taken at the site using mercury-in-glass thermometer by placing the thermometer bulb into the ground for 2 minutes at the depth of 10cm and 20cm below the surface respectively, it was then removed from the soil and readings taken on the scale of the thermometer and recorded.

Determination of pH

2 g of the soil samples were weighed with a weighing balance into a beaker and was mixed with 10 ml of distilled water and allowed to settle to obtain a clear solution. The pH meter was calibrated using buffer solution of pH 7.0 until the reading is stabilized at 7.0 and was then rinsed with distilled water. The pH of the samples was then determined by immersing the pH meter into the clear top solution of the mixture to obtain the readings.

Determination of Moisture content

10g of soil samples were weighed into previously dried and weighed beaker using weighing balance (W1) and then dried at 105^0C for 18 hours in an oven, the samples were allowed to cool and re-weighed (W2).The moisture content was then obtained by difference in weights.

Nitrate determination

The soil samples were crushed using mortar and pestle and then passed through a 2mm soil sieve.10grams of the sieved soil samples were then weighed with weighing balance into an extraction cup and dissolved with 20ml of distilled water, the mixture was shaken on a shaker for 2 minutes and allowed to settle for 3 minutes. 3 drops of 2M calcium chloride solution was added and allowed to stand for another 3minutes, the filtrate was then obtained by pouring the mixture through whatmann 1mm filter paper into a beaker and then allowed to settle for 3 minutes. The nitrate meter was put on and calibrated using 2000ppm nitrate standard, nitrate distilled water blank was measured and the nitrate value was then determined by immersing the nitrate meter in the filtrate and recorded.

Phosphate determination

The soil samples were crushed using mortar and pestle and then passed through a 2mm soil sieve.10grams of the sieved soil samples were then weighed with weighing balance into an extraction cup and dissolved with 20ml of distilled water, the mixture was shaken on a shaker for 2 minutes and allowed to settle for 3 minutes. 3 drops of 2M calcium chloride solution was added and allowed to stand for another 3minutes, the filtrate was then obtained by pouring the mixture into through Whatmann 1mm

filter paper into a beaker and then allowed to settle for 3 minutes. 8ml of 0.01M calcium chloride was measured into a vial; 2ml of the filtrate and 1 sachet of colour developer was also added and was allowed to stand for 30 seconds. After 30 seconds, the mixture was shaken until the colour developer has totally dissolved in the solution and was allowed to stand for 30 minutes for colour development; the phosphate value was then determined by immersing the phosphate checker meter in the mixture and recorded.

Sulphate determination

The soil samples were crushed using mortar and pestle and then passed through a 2mm soil sieve.10 grams of the sieved soil samples were then weighed with weighing balance into an extraction cup and dissolved with 20ml of distilled water, the mixture was shaken on a shaker for 2 minutes and allowed to settle for 3 minutes. 3 drops of 2M calcium chloride solution was added and allowed to stand for another 3minutes, the filtrate was then obtained by pouring the mixture into through Whatmann 1mm filter paper into a beaker and then allowed to settle for 3 minutes. 4ml of distilled water was measured into a vial, 4ml of the filtrate was added and 5 drops of 400ppm K_2SO_4 (potassium sulphate) was added. The vial was inverted 3 times, 2 level scoop of Barium chloride (BaCl) was added and shaken for 10 seconds, sulphate checker meter was then immersed in the mixture to obtain the sulphate value.

Extraction of residual engine oil

The residual engine oil was extracted from the soil sample using n-hexane: dichloromethane system (1:1) and quantified gravimetrically. To achieve this, 10grams of homogenized soil sample was weighed into a 75ml beaker and 50ml of n-hexane: dichloromethane was added to extract the residual engine oil in the soil sample. After shaking vigorously, the mixture was allowed to stand for 5 minutes and then filtered through whatmann No1 filter paper into 75ml beaker of known weight (W1) as residual oil extract (ROE). The residual oil extract was placed in an oven at 80 ^{0}C for 5-10 minutes to evaporate the solvent. The combined weight of the residual oil and the beaker was taken and recorded as W2. The residual oil content (ROC) was then obtained by difference in mass (W2 - W1 = ROC).

Total colony count

One gram of each sample was mixed with sterile de-ionized water and serially diluted. One milliliter of appropriate dilution was seeded on plate count agar using spread plate method, and then incubated for 24 hours. The plate count agar was examined and colonies present were counted and recorded after incubation, to get the total colony count in CFU/g (Colony forming unit/gram)

Culture preservation

Distinct colony of the organisms was picked using flame sterilized wire loop and inoculated on already prepared, sterilized and solidified agar slant. The slant was incubated at 28 ^{0}C for 24 hours, the slants with visible growth were then preserved in the refrigerator at -4 ^{0}C.

Isolation of oil degrading bacteria

To do this, the method of Umanu *et al.*, 2013 was used. Briefly, sterile minimal salt broth containing 10% V/V sterile motor oil was inoculated with one gram of thoroughly mixed soil sample and incubated at 28°C for 7 days. Two millilitres of this culture was aseptically transferred into a fresh enrichment culture medium and incubated for another 7 days. The process was repeated for the third time. Then 0.1ml from the third enrichment culture was plated onto nutrient agar and incubated. Serial sub-culture was done to obtain pure culture isolates. Microscopic and biochemical tests were carried out on the bacterial isolates to determine their probable identities. The result of each test was recorded and the probable identity of the bacteria was determined by the use of Bergey's Manual of Determinative Bacteriology.

Determination of Isolate's oil degrading potential

The isolated oil degrading bacteria (2.12 x 10^5 CFU/ml) were inoculated into minimal salt broth containing 10% V/V sterile engine oil as the sole carbon and energy source, the control tubes contain the minimal salt broth and sterile engine oil only. The tubes were then incubated at 28°C for 30 days and samples were withdrawn at 10 day intervals for analysis. The residual oil in the experimental and control bottles were extracted with the aid of chloroform using separating funnel. The funnel was allowed to stand for two hours, the layer containing the organic solvent and residual oil was emptied into a pre-cleaned container, the organic solvent was allowed to evaporate, after the evaporation the residual oil was collected and Infrared analysis was done.

RESULTS AND DISCUSSION

The total heterotrophic bacterial counts for contaminated and uncontaminated soil samples ranged from $8.0x10^7$ to $2.1x10^7$ CFU/g and $22.0x10^7$ to $30.0x10^7$ CFU/g respectively. Heterotrophic bacteria count was high in uncontaminated soil samples compared to engine oil contaminated soil samples which is in agreement with the previous report of Umanu *et al.* (2013) as shown in Table 1. The total colony counts for bacterial oil degraders ranged between $1.3x10^7$ and $2.0x10^7$ CFU/g in contaminated soil samples and $0.3x10^7$ and $1.2x10^7$ CFU/g for uncontaminated samples. Engine oil degrading bacteria were higher in contaminated soil samples which also supports the results obtained by Umanu *et al.* (2013), Hubert *et al.* (1999) and Michalcewicz (1995) in their earlier work (Table 2). Umanu *et al.* (2013) had also implicated that higher populations of oil degrading bacteria has been attributed to stimulatory effect of additional carbon and energy source in the form of lubricating oil. Table 3 shows the distribution of the isolated organisms in both engine oil contaminated and uncontaminated soil samples.

The following organisms were isolated from the soil samples; *Bacillus polymyxa*, *B. megaterium*, *B. alvei*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Escherichia coli*. The identities of the organisms were confirmed using biochemical tests according to Bergy's manual of determinative bacteriology (Buchannan and Gibbons, 1975). *Bacillus spp especially* capable of degrading hydrocarbon have been reported by Nwagwu *et al*. (2008).

The physico-chemical parameters of the engine oil contaminated and uncontaminated soil samples analyzed are shown in Table 4. Zero residual oil content was obtained in all the uncontaminated soil samples collected confirming that they were not polluted with oil. Residual oil for contaminated soil samples was found to be between 12.02±0.20 mg/kg and 19.40±0.50 mg/kg. Engine oil contaminated soil samples showed the highest nitrate, sulphate and phosphate concentration compared with the uncontaminated samples. Atlas (1984) opined that depending on the nature of the environment, nutrients such as nitrogen and phosphorous could be limiting thus affecting the biodegradation process. However, this is contrary to the findings of Lovely and Cackette (2001) that, significant increases in total nitrogen and organic carbon of soil have been observed as a result of oil contamination.

Figure 1 and 2 shows degradability potentials of the isolates. *E. coli* showed the highest degradability potentials for both aliphatic and aromatic functional groups because it had the lowest intensity of 19.25cm^{-1} and 18.22 cm^{-1} respectively after 30 days as compared with the intensity of the control (146.05cm^{-1}for aliphatic and 315.16 cm^{-1}for aromatic) and other isolates. Generally, all the isolates were able to reduce the hydrocarbons present in the used engine oil maximally. Since all the bacteria isolates used in this study were isolated from oil contaminated soil, the innate potential of degradation exhibited by these bacteria isolates is in conformity with the reports of Vidalii (2001) and Chaerun *et al*. (2004). Microorganisms have been reported to have enzymatic systems which empower them to degrade and utilize petroleum hydrocarbon as source of carbon and energy according to Antai and Mgbomo (1993); Ezeji *et al*. (2005)

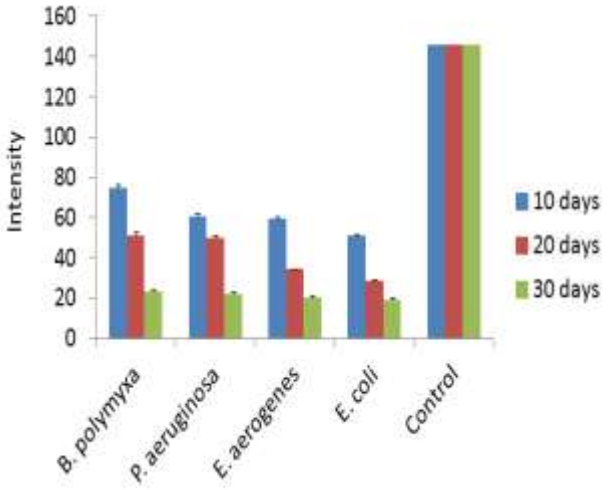

Figure 1: Organisms degradability potential of aliphatic functional group.

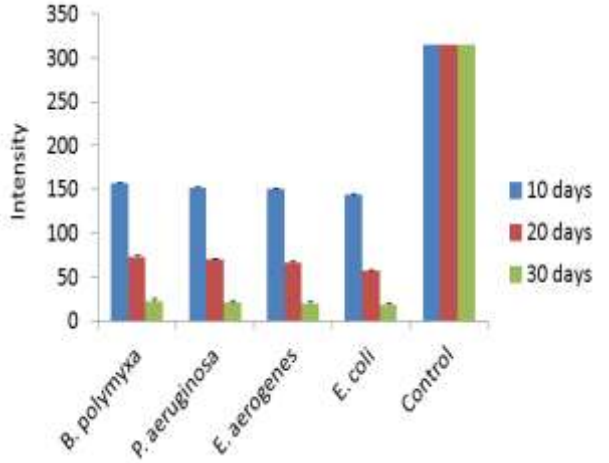

Figure 2: Organisms degradability potential of Aromatic functional group

Table 1: Heterotrophic bacterial counts of engine oil contaminated and uncontaminated soil samples.

Sample Location	Depth (cm)	Contaminated (CFU/g)	Uncontaminated (CFU/g)
A	0 - 15	15 X 10^7	25 X 10^7
	15 - 45	13 X 10^7	27 X 10^7
B	0 – 15	8 X 10^7	25 X 10^7
	15 – 45	11 X 10^7	28 X 10^7
C	0 – 15	14 X 10^7	27 X 10^7
	15 – 45	11 X 10^7	30 X 10^7
D	0 – 15	21 X 10^7	26 X 10^7
	15 – 45	20 X 10^7	30 X 10^7
E	0 – 15	18 X 10^7	22 X 10^7
	15 – 45	20 X 10^7	24 X 10^7
F	0 – 15	15 X 10^7	24 X 10^7
	15 - 45	12 X 10^7	27 X 10^7

Table 2: Total colony counts of bacterial oil degraders in engine oil contaminated and uncontaminated soil samples.

Sample Location	Depth (cm)	Contaminated (CFU/g)	Uncontaminated (CFU/g)
A	0 - 15	1.7 X 10^7	1.2 X 10^7
	15 - 45	1.5 X 10^7	1.0 X 10^7
B	0 – 15	1.6 X 10^7	1.0 X 10^7
	15 – 45	1.3 X 10^7	0.7 X 10^7
C	0 – 15	2.0 X 10^7	1.1 X 10^7
	15 – 45	1.8 X 10^7	1.0 X 10^7
D	0 – 15	2.0 X 10^7	1.1 X 10^7
	15 – 45	1.7 X 10^7	0.3 X 10^7
E	0 – 15	1.5 X 10^7	0.9 X 10^7
	15 – 45	1.3 X 10^7	0.6 X 10^7
F	0 – 15	1.7 X 10^7	1.0 X 10^7
	15 - 45	1.5 X 10^7	0.7 X 10^7

Table 3: Distribution of the isolated bacteria in engine oil contaminated and uncontaminated soil samples.

Isolates	**Samples**			
	CSS	**CSS**	**USS**	**USS**
Depth	**0-15cm**	**15-45cm**	**0-15cm**	**15-45cm**
Bacillus megaterium	+	+	_	+
Bacillus polymyxa	+	+	+	+
Bacillus licheniformis	+	+	_	+
Pseudomonas aeruginosa	+	+	+	+
Enterobacter aerogenes	+	+	+	+
Escherichia coli	+	+	+	-

CSS = contaminated soil sample
USS = uncontaminated soil samples

Table 4: Physico-chemical Parameters of Contaminated and uncontaminated Soil Samples.

Sample Location	Depth (cm)	Moisture content (%)		Residual oil content (%)		NO_3 Concentration		PO_4 Concentration		SO^{-2}_4 Concentration	
		CSS	USS	CSS	USS	CSS	USS	CSS	USS	CSS	USS
A	0 - 15	7.20±0.5	4.10±0.3	19.40±0.5	0.00	12.63±1.5	6.31±0.9	61.26±1.1	25.05±1.7	15.09±1.6	14.41±1.5
	15 - 45	9.10±0.3	7.10±0.5	18.20±0.4	0.00	31.5±0.7	12.10±0.7	84.57±1.0	70.21±1.3	15.32±1.4	14.86±1.2
B	0 – 15	6.10±0.6	6.80±0.4	15.10±0.6	0.00	51.19±1.2	37.98±1.4	85.67±1.6	60.62±1.1	28.25±1.1	19.16±1.3
	15 – 45	8.80±0.5	7.50±0.1	12.30±0.5	0.00	44.02±0.9	24.09±1.2	67.43±1.4	37.12±1.0	26.38±1.5	17.35±1.0
C	0 – 15	5.20±0.2	5.10±0.3	19.40±0.4	0.00	12.63±1.0	2.10±1.1	84.21±1.5	61.05±1.6	19.09±1.2	14.41±1.4
	15 – 45	9.10±0.5	8.10±0.4	18.20±0.2	0.00	31.57±1.4	6.31±1.0	75.26±1.2	51.57±1.3	20.32±1.0	14.86±1.0
D	0 – 15	7.40±0.4	4.40±0.3	14.05±0.5	0.00	56.84±1.2	44.21±0.9	30.25±0.9	17.89±1.2	22.77±1.3	16.90±1.2
	15 – 45	9.80±0.2	7.60±0.2	12.05±0.2	0.00	67.36±1.0	23.15±1.1	87.89±1.0	58.42±1.0	49.84±1.9	14.64±1.5
E	0 – 15	5.30±0.1	8.10±0.3	15.20±0.3	0.00	37.10±1.1	38.18±1.3	32.17±1.5	14.53±1.5	59.18±1.4	30.68±1.3
	15 – 45	7.20±0.2	9.10±0.5	13.11±0.4	0.00	40.22±1.5	30.11±1.7	32.17±1.3	15.04±1.2	67.18±1.8	77.51±1.7
F	0 – 15	8.10±0.3	6.80±0.1	15.10±0.2	0.00	61.98±1.4	37.19±1.3	88.67±1.6	48.62±1.4	28.25±1.0	19.16±1.4
	15 - 45	9.80±0.5	7.50±0.5	12.30±0.5	0.00	54.02±1.5	40.09±0.8	41.43±1.0	26.12±1.1	26.38±1.5	17.35±1.8

Residual oil content (mg/kg), NO_3 Concentration (mg/kg), PO_4 Concentration (mg/kg), SO^{-2}_4 Concentration (mg/kg)

CONCLUSION

Bacteria capable of metabolizing engine oil in the studied sites was observed with *E. coli* more effective for degradation of both aliphatic and aromatic functional groups which is an indication that natural attenuation and clean-up of engine oil can take place in the studied sites given time. Also, the isolates obtained from this study could be exploited for bioremediation or clean-up of similar environments. The consciousness should be instilled into automobile mechanics to avoid indiscriminate disposal of used engine oil and researchers should work towards recycling of used engine oil.

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