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Biodegradation Potentials of Hydrocarbon Degraders from Waste-lubricating Oil-spilled Soils in Ebonyi State, Nigeria

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ABSTRACT

The potential of bacterial isolates as hydrocarbon degraders was investigated. A total of 27 bacterial isolates were able to grow on mineral salt medium by enrichment procedure. The result of the screening tests of the isolates for hydrocarbon degradation showed different degree of degradation in mineral salt medium using spent oil as sole source of carbon were characterized and identified using standard methods. The bacterial isolates identified as *Bacillus, Pseudomonas* and *Corynebacterium* species showed different growth as measured by the optical density, total viable count, and different range of pH values throughout the period of incubation. Hydrocarbon degradation was demonstrated by gas chromatography and the result showed that C*orynebacterium* sp. has the highest ability to degrade spent motor oil (71.85%) followed by *Pseudomonas* sp. (63.46%), while *Bacillus* sp. (2.76%) found it difficult to degrade the hydrocarbon. In addition, emulsification test was carried out for the three organisms, and the results showed that *Corynebacterium* sp. has the highest emulsification ability at 1% spent oil. In conclusion, this study suggests the potential use of the isolate for bioremediation of contaminated environments. © 2011 Friends Science Publishers

Key Words: Hydrocarbon; Degradation; Emulsification; Gas chromatography; Waste-lubricating oil

INTRODUCTION

The quality of life on earth is linked inextricably to the overall quality of the environment. Releases of persistent bioaccumulative and toxic chemicals have a detrimental impact on human health and the environment. Petroleum hydrocarbon is one common example of chemicals, which enters the environment frequently and in large volumes through numerous routes. The problem is worldwide, but more severe in the developing countries, where there were no effective regulatory policies on the environment.

The growth of the petroleum industries in Nigeria and the marketing of petroleum products have made oil pollution a serious environmental concern. Also, oil spill from industries, filling stations, loading and pumping stations, petroleum product depots, during transportation and at auto mechanic workshops all contribute to soil contamination, and actually make up a larger percentage of polluted ground in the world versus those contaminated by catastrophic spills. In Nigeria, oil spills at auto mechanic workshops have been left uncared for over the years and its continuous accumulation is of serious environmental concern, because of the hazard associated with it. For instance the spent motor oil disposed off improperly contains potentially toxic substances such as benzene (carcinogens), lead, arsenic, zinc and cadmium, which can seep into the water tables and contaminate ground water (Igwe e*t al.*, 2008; Shah *et al*., 2009). It consequently results in serious health hazard such as anemia and tremor, which can cause death.

Contamination of soil by petroleum hydrocarbon stimulates indigenous microbial populations, which are capable of utilizing the petroleum hydrocarbons as their carbon and energy source thereby degrading the contaminants. The ability to degrade hydrocarbon substrates is exhibited by a wide v*ariety* of bacteria genera *(*Dally *et al.*, 1997; Bogan *et al*., 2003; Malakootian *et al*., 2009; Abdulsalam & Omale, 2009; Abdulsalam *et al.*, 2011) using culture dependent and independent isolation techniques different bacterial genera have been characterized from hydrocarbon polluted soils in different geographical and ecological contexts (Van Hamme *et al.*, 2003; Maila *et al*., 2004; Maila *et al*., 2006; Refaat, 2010).

Although experimental and climatic conditions differed considerably in each study some general trends have indicated that Gram negative *Proteobacteria and Cytophaga Flavobacterium-Bacteriodes* group dominate during bioremediation and density shift with time to this group (Kaplan & Kitts, 2004). These groups are usually associated with the fast degradation phase and their abundance was positively correlated to hydrocarbon attenuation. Gram positive bacteria if detected are never

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diverse and dominant during bioremediation (Kaplan *et al.*, 2004). However, reports have shown that Gram positive bacteria mainly *Actinobacteria* can actually dominate during bioremediation of petroleum hydrocarbon owing to their metabolic versatility and their widespread occurrences both in pristine and hydrocarbon polluted soil (Bell *et al.*, 1998; Larkin *et al*., 2005; Hamamuro *et al*., 2006; Quatrini *et al*., 2008; Yousefi *et al*., 2009; Abdulsalam *et al*., 2011).

In this study, the bacterial diversity of hydrocarbon degraders from hydrocarbon stressed soils of some automobile workshops in Afikpo, Ebonyi State, Nigeria was evaluated. Some of the aims of the research include: (a). isolation of hydrocarbon degraders from automobile workshops. (b). determination of the ability of these isolates to degrade petroleum products (c) assessment of the impact of spent oil on the microorganisms. (d). Comparison of the rate of microbial degradation of the hydrocarbon.

MATERIALS AND METHODS

Sample collection: Soil samples were collected at 0-20 cm depth from different automobile repair workshops using sterile bottles and immediately transferred to the laboratory for analysis.

Isolation of hydrocarbon degrading bacteria: The isolation of hydrocarbon degrading Bacteria species was done by enrichment of mineral salt medium with petroleum products (Amund *et al.*, 1987; Okpokwasili *et al.*, 1990). The mineral salt medium was autoclaved at 121°C for 15 min. The petroleum products were autoclaved separately at the same temperature before it was added to the mineral salt medium (2 drops in 10 mL of mineral salt medium).One gram of each soil sample was added to 10 mL of the medium and incubated with test tube shaker at 30°C for 7 days. Pure culture was isolated by plating out the enrichment broth on nutrient agar.

Screening test for hydrocarbon utilization: The medium used in assessing the ability of the bacterial isolate to utilize hydrocarbon fraction (spent oil) was mineral salt medium containing 4% (v/v) (0.2 mL in 5 mL of mineral salt) of the hydrocarbon fraction. The medium was made out in 30 mL test tube containing 5 mL of the mineral salt medium and sterilized as stated before. The incubation was done at 30°C for two weeks in a test tube shaker. The optical density of the culture was measured at 550 nm to determine organism with high utilization.

Inoculum development: Three organisms with high utilization ability were chosen for the degradation test. Mineral salt broth containing 0.3 mL of spent motor oil was dispensed in 30 mL quantities into three 250 mL Erlenmeyer flask with 5 loopfuls of the isolate. Incubation was done at 120 rpm in a shaker at 30°C for 24 h. After incubation the pH, total viable count (TVC) and optical density (OD) at 550 nm were measured.

Degradation of spent motor oil: To do this, 1.5 mL of culture from inoculum development was added each into three 500 mL conical flask containing 1.2 mL of spent oil in 100 mL of mineral salt broth with the fourth flask serving as a control. Incubation was done at 30°C for 15days at 180 rpm. At 5 days interval during the incubation samples were drawn from the flask for measurement of pH, total viable count and optical density at 550 nm wavelength (Okpokwasili *et al.*, 1990).

Extraction and gas chromatography: To determine the extent of biodegradation of spent motor oil by the three isolates, the residual oil in the liquid medium at the end of the incubation period were extracted using carbon tetra chloride including the un-inoculated control (Amund *et al.*, 1987). Extraction was performed by adding 60 mL of the liquid culture containing the residue of spent motor to a separating funnel. To this was added 100 mL of carbon tetra chloride. The funnel was vigorously shaken after which the content was allowed to settle for the phase to separate. Upon separation the solvent layer containing the oil was drawn off into a clean plastic container and stored in the refrigerator until ready for analysis. These steps were repeated for all the samples including the control (Okpokwasili *et al.*, 1990).

Emulsification test: The emulsification test for the cell free filtrate was carried out by growing the bacterial isolates in mineral salt medium supplemented with 0.10, 0.25, 0.50, 0.75 and 1.00% v/v of spent motor oil as sole carbon source and incubation for 24 h at 30°C in a rotatory shaker at 200 rpm. A 100 mL culture contained in 250 mL Erlenmeyer's flask was centrifuged at 1000 rpm for 20 min. The cell free supernatant was carefully pipette out, while the cell was discarded. The supernatant was then filtered through a filter paper to extract the suspended hydrocarbons. To 5 mL of the filtrate was added 1.5 mL of the hydrocarbon and shaken to mix and allowed to settle for about 15-20 min. The bottom aqueous phase was carefully pipetted out into curvettes and the absorbance of aqueous phase was measured at 420 nm. The emulsification ability was expressed as a percentage increase in optical absorbance of the lower aqueous phase compared with the control.

$$\% \text{ Emulsification Ability} = \frac{\text{Increase absorbance}}{\text{Emulsification absorbance of control}} \times 100$$

Identification of isolates: A total of three isolates were subjected to a testing regime involving cell morphology, Gram stain, spore formation, production of oxidase, catalase, indole, fermentation of glucose, galactose, sucrose, maltose, mannitol, fructose, xylose, lactose, methyl red, Voges proskauer, motility test, urease utilization as well as growth and appearance on Simmon citrate agar and starch utilization. Identification of the isolate to the generic level followed the scheme in Bergey's Manual of Systematic Bacteriology (Buchanan & Gibbons, 1976).

RESULTS

Isolation and screening for hydrocarbon degraders: A total of 27 bacterial isolates were able to grow on mineral salt medium by enrichment procedure. The result of the

Table I: Screening test result for hydrocarbon utilization (550 nm)

Gram +ve rod		Gram –ve rod		Gram +ve cocci		Gram –ve cocci	
MB2	0.932	KB9	0.612	CrB	0.583	MB2	0.786
A	0.902	KA6	0.532	Motor C	0.544	K^2B	0.552
KB2	0.879	KB7	0.473	B-1	0.523	MB4	0.538
P_2-1	0.761	MC2	0.293	MC	0.501	KB6	0.532
MC4	0.706	KB5	0.215	KB	0.499	MA	0.478
MA5	0.650			C^2B	0.494	C^2M	0.410
P_1-1	0.582					MB	0.400
CC	0.501						
MB6	0.446						

Table II: Properties of hydrocarbon degrading bacteria

	Isolate A	Isolate MB2	Isolate KB9
Cultural characteristics	**Cream, entire, convex, circular**	**White, entire, raised, circular**	**Cream, raised, entire, circular, produce green pigment**
Gram stain	**+ ve rod**	**+ve rod**	**-ve rod**
Glucose	+	+	+
Lactose	+	-	+
Maltose	+	-	+
Galactose	+	-	-
Sucrose	+	+	+
Xylose	+	-	+
Mannitol	+	-	+
Fructose	+	+	+
Motility	-	+	+
Methyl red	-	+	-
Indole	-	-	-
Catalase	+	+	+
Citrate Utilization	+	+	+
VP	+	+	-
Oxidase	+	-	+
Urease	-	-	-
Starch Hydrolysis	-	+	-
Spore Stain	-	+	-
Nitrate Reduction	+	-	-
Probable organisms	***Corynebacterium* sp**	***Bacillus* sp**	***Pseudomonas* sp**

Table III: Growth of Isolate KB9 in mineral salt medium containing spent oil as the sole carbon source

Days	Optical Density	Total Viable Count	pH
0	0.093 ± 0.002	0	6.66 ± 0.010
5	0.142 ± 0.002	1.49×10^8	7.32 ± 0.015
10	0.166 ± 0.003	2.09×10^8	7.32 ± 0.021
15	0.190 ± 0.002	0.62×10^8	6.57 ± 0.010

Data presented are averages of triplicate determinations and their standard deviation of optical density and pH

screening test of the isolates for hydrocarbon degradation shows that all the bacterial isolates showed different degree of degradation (Table I), but 3 out of all the isolates that have the highest degree of degradation were chosen for further studies and were characterized.

Characterization of the isolates: The three isolates designated as, A, MB2 and KB9, which showed the highest degree of degradation in mineral salt medium using spent oil as sole source of carbon were characterized and identified to the genus level on the basis of colony morphology, cultural, physiological and biochemical characteristics. They were identified as *Corynebacterium, Pseudomonas,* and *Bacillus* species (Table II) (Buchanan & Gibbons, 1976).

Growth potential of hydrocarbon utilizing bacteria: The growth potential of hydrocarbon utilizing bacteria in mineral salt medium using spent oil as the sole carbon source are shown in Table III, IV and V. From the Tables, the 3 isolates identified as *Bacillus, Pseudomonas,* and *Corynebacterium* species showed different growth as measured by the optical density, total viable count, and different range of pH values throughout the period of incubation.

Extraction and gas chromatography: Gas chromatographic tracing of the inoculated and uninoculated hydrocarbons was carried out. At the end of the incubation period it was observed that there was a reduction in the peak values of the inoculated hydrocarbons as compared to the un-inoculated control. Also, the total hydrocarbon degraded by the three isolates expressed in percentage showed that *Corynebacterium* sp. has the highest ability to degrade spent oil (71.85%), followed by *Pseudomonas* sp (63.46%), while *Bacillus* sp. was not able to degrade the hydrocarbon (2.76%).

Emulsification test: The result of the emulsification test shows that *Corynebacterium* has the highest emulsification (77.40%) at 1% spent oil, while Bacillus has the highest emulsification (96.30%) at 0.25% spent oil, while the least is *Pseudomonas,* which is (40.0) at 0.25% spent oil (Table VI).

DISCUSSION

The result of this investigation have shown the occurrence of high numbers of certain oil degrading microorganisms from oil polluted environment an evidence that these microorganisms are the active degraders of such environment. A total of 27 bacteria were isolated and identified to the genus level. Majority of these organisms isolated were *Pseudomonas, Corynebacterium, Bacillus, Flavobacterium, Chromobacterium* and *Alkaligenes*. The dominance of these organisms has been reported by different researchers as crude oil degraders (Leahy & Colwell, 1990; Bogan *et al.,* 2003). The population of culturable hydrocarbon degraders from the soil samples investigated showed that majority of the bacteria were Gram positive belonging to the Actinobacteria group.

Although some studies have shown that, oil-polluted soils are dominated by Gram negative bacteria (Macnaughton *et al.,* 1999; Kapplan & Kitts, 2004), the dominant culturable hydrocarbon utilizing bacteria from the soil investigated were made up of Gram positives Actinobacteria of the genera *Corynebacterium, Mycobacterium and Arthrobacter.* This corroborates the finding of Quatrini *et al.* (2008) who isolated *2 Rhodococcus, 2 Gordonia* and 1 *Nocardia* strains as the dominant hydrocarbon degraders from a hydrocarbon contaminated mediterranean shoreline.

Table IV: Growth of isolate MB2 in mineral salt medium containing spent oil as sole carbon source

Days	Optical Density	Total Viable Count	pH
0	0.162 ± 0.005	0	6.67 ± 0.001
5	0.335 ± 0.001	2.15×10^8	7.30 ± 0.252
10	0.296 ± 0.006	0.79×10^8	7.31 ± 0.026
15	0.303 ± 0.001	0.14×10^8	6.57 ± 0.015

Table V: Growth of isolate A in mineral salt medium containing spent oil as sole carbon source

Days	Optical Density	Total Viable count	pH
0	0.156 ± 0.001	0	6.71 ± 0.010
5	0.207 ± 0.002	6.20×10^8	7.24 ± 0.040
10	0.305 ± 0.002	0.11×10^8	7.30 ± 0.015
15	0.212 ± 0.003	0.18×10^8	6.57 ± 0.015

Table VI: Emulsification test result expressed in percentage

Percentage of spent motor oil	A%	KB9 (%)	MB2 (%)
1.00	77.40	21.5	8.76
0.75	50.20	23.40	5.00
0.50	3.36	12.23	95.10
0.25	36.80	40.00	96.30
0.10	44.90	28.30	9.40

Earlier studies have also demonstrated the prevalence of *Actinobacteria* in hydrocarbon polluted soils from different geographical locations (Baouchez-Naitali *et al.*, 2006) *Pseudomonas, Corynebacterium* and *Bacillus* were used for biodegradation of waste lubricating oil (Amund *et al*., 1987).

During the growth of the organism on mineral salt medium with spent oil as source of carbon, there was an increase and decrease of the total viable counts during the growth of the organism. The population of the bacterial achieved highest count on day 5 and witnessed a drop later. The decrease could be attributed to decline in the availability of readily metabolizable hydrocarbons.

The reason for the higher counts of bacteria during its earlier growth may be as a result of the presence of appreciable quantity of nitrogen and phosphorus in the mineral salt medium especially higher nitrogen content, which is necessary for bacterial biodegradative activities (Nakasaki *et al*., 1992; Ijah & Antai, 2003a; Joo *et al.*, 2007; Adesodun & Mbagwu, 2008). Also the decrease in the chromatographic profile of the hydrocarbon extracts (comparing with the control) after growth of the isolates indicated degradation though at different rates (figure not shown). The result showed that *Corynebacterium* sp. has the highest ability to degrade spent oil (71.83%) followed by *Pseudomonas* sp. (63.46%), while *Bacillus* sp. (2.76%) found it difficult to degrade the hydrocarbon. The differences in the rate of hydrocarbon degradation may be due to presence of difference of catabolic genes involved in hydrocarbon degradation in the bacterial species (Kyung-Hwa *et al*., 2006; Majid *et al.*, 2008).

The present studies also revealed that the organism exhibited varying ability to grow, utilize and emulsify the spent oil (Table VI). *Corynebacterium* has the highest emulsification at 1% spent motor oil followed by *Pseudomonas* and then *Bacillus*. These organisms produced surface active agents (biosurfactants) during oil degradation. These organisms have earlier been associated with the production of biosurfactants when grown on petroleum hydrocarbons (Rocha *et al*., 1992). Microbial biosurfactants are useful as soaps and detergents and thus, their application in simple cleaning, tertiary oil recovery and oil spill clean-up (Cooper, 1986). It therefore means that these organisms may be useful in treating oil spills in the environment.

In conclusion, the result of the present study revealed that Nigeria soil may harbor hydrocarbon degraders that have been exposed to hydrocarbons as a result of the increased multifarious activities of the oil industry especially in the Niger Delta region (Chikere & Okpokwasili, 2003, 2004; Ayotamuno *et al*., 2006; Okpokwasili, 2006). Studies have shown that some of the isolates in this study can habour multiple aliphatic and aromatic hydrocarbons degradative genes with overlapping hydrocarbon substrate ranges (Van Beilen & Funhoff, 2007) and it could be that the genera isolated in this study may have these catabolic capabilities as shown by the degradation of the hydrocarbons in the oil-contaminated soil.

However, further molecular studies are needed to decipher the catabolic genes resident in these tropical isolates that were isolated from hydrocarbon polluted soils and their hydrocarbon specificities. This will invariably assist in developing cost effective and efficient bioremediation protocol for Nigerian oil polluted soil.

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