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Hydrocarbon Degradation Potentials of Bacteria Isolated from Spent Lubricating Oil Contaminated Soil

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Abstract: Cleaning up petroleum hydrocarbon contaminated sites has been a major challenge. This has led to the exploration of many approaches to affect the cleanup of the polluted soils. The degradation of spent lubricating oil by bacteria species isolated from hydrocarbon contaminated soil was investigated in this study. A total of sixteen hydrocarbon degrading bacteria species were isolated from spent lubricating oil contaminated soil. The predominant species belonged to the genera *Pseudomonas* and *Enterobacter*. Three strains namely *Nocardia* sp., *Pseudomonas* sp and *Bacillus* sp showed the highest potential for hydrocarbon utilization. Their ability to degrade both the aliphatic (n-alkanes) and Polyaromatic Hydrocarbon (PAH) components of the spent lubricating oil in MSM was investigated after 21days of biodegradation studies using gas chromatographic (GC) techniques. Over 98% of the n-alkane and PAHs fraction of the spent lubricating oil supplied at 1.0% vv^{-1} concentration was degraded by the three strains. *Nocardia* sp showed the highest percentage of degradation of about 99%. This study has shown that resident bacteria strains in lubricating oil contaminated soils have potential application in the bioremediation of oil polluted sites and enhance the possibility of developing models and strategies for removing hydrocarbon pollutants from the environment.
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Keywords: Biodegradation, hydrocarbon, spent lubricating oil, gas chromatography

Introduction

Increasing exploration and production activities coupled with improper waste disposalpractices hasled to widespread contamination of both the aquatic and terrestrial ecologicalsystems (Odokuma and Ikpe, 2003). Many microorganisms have the ability to utilize hydrocarbons as sole sources of carbon as energy for metabolic activities .and these micro organisms are widely distributed in the nature. The microbial utilization of hydrocarbons depends on the chemical nature of the compounds within the petroleum mixture and on environmental determinant (Adeline *et al*., 2009). Lubricating oil is a common contaminant in water and soils. Generally, lubricating oil comprises 80% of hydrocarbon lubricant, with the remainder being additives, which consists partly of zinc diaryl, molybdenum disulfide, zinc dithiophosphate, metal soaps and other organometallic compounds (Lu and Kaplan, 2008). Large amounts of lubricating oil are liberated into the environment when the motor oil is changed and disposed into gutters, water drains, open vacant plots and farmlands, a common practice by motor mechanics and generator mechanics (Odjeda and Sadiq, 2002).

Spent lubricating oil is produced when new lubricating oil is subjected to high temperature and high mechanical strain (ATSDR, 1997). Despite efforts in some countries to recover and recycle used motor oils, significant amount of lubricants are input into the environment, particularly in environmentally sensitive applications such as forestry and mining, or through engine losses (Battersby, 2000). The rise in consumption of automotive lubricating oil is a worldwide problem and has increased, ending in large used oil volume and its waste (Koma *et al*., 2003). Contamination of soil by spent lubricating oil is prevalent in oil producing and industrialized countries of the world. The problem is more severe in the developing countries where there are no effective regulatory policies on the environment (Onuoha *et al*., 2011).

The presence of different types of automobile and machinery has resulted in an increase in the use of lubricating oil. Also, oil spills from industries, filling stations, loading and pumping stations, petroleum product depots during transportation and at auto mechanic workshops, all combine to contribute to soil contamination (Onuoha *et al*., 2011).

Hydrocarbon contamination of the air, soil, fresh water especially by polycyclic aromatic hydrocarbon (PAHs) attracts public attention because many PAHs are toxic, mutagenic, and carcinogenic (Clemente *et al*., 2001).

Prolong exposure of high oil concentration may cause the development of liver or kidney diseases, possible damage to the bone marrow and increased risk of cancer (Mishra *et al*., 2001; Lloyd and Cackette, 2011). The problems of pollution have led to the exploration of many remedial approaches to affect the cleanup of the polluted soils.

Pollution control strategies involving physico-chemical methods have often aggravated the problem rather than eliminate it. Mechanical methods to reduce hydrocarbon pollution are expensive and time consuming. Biodegradation is favored as a good option for the remediation of polluted sites mainly because it uses inexpensive equipment, environmentally friendly and simple. Environmental Biotechnologies has through decade of intensive study devised means of combating the problems of environmental pollution through a method known as bioremediation. The process of bioremediation involves the transformation and breakdown of complex organic molecules through biostimulation and bioaugmentation into simpler substances such as fatty acids, carbon-dioxide and water (Atlas, 1985).

Bioremediation is one of the forms of biodegradation which involves the use of microorganisms to detoxify or remove organic and inorganic xenobiotic compounds from the environment. The process relied upon microbial enzymatic activities to transform or degrade the contaminants from the environment (Philip *et al*., 2005). Hydrocarbon-degrading bacteria and fungi are widely distributed in marine, freshwater and soil habitats (Atlas and Bartha, 1973). The ability to isolate high numbers of certain oil-degrading microorganisms from oil-polluted environment is commonly taken as evidence that these microorganisms are the active degraders of that environment (Okerentugba and Ezeronye, 2003). Lack of essential nutrients such as nitrogen and phosphorus is one of the major factors affecting biodegradation of hydrocarbon by microorganisms in soil and water environment. In this study, we attempt to identify bacteria which can utilize spent lubricating oil as carbon and energy source from spent lubricating oil polluted soil.

MATERIALS & METHODS

Sample collection: Soil samples were collected from soil contaminated with spent lubricating oil in Southwest Nigeria. The soil samples were taken to the laboratory immediately for analysis.

Isolation of Hydrocarbon Utilizing Bacteria: Hydrocarbon utilizing bacteria in the soil sample were enumerated using modified mineral salt medium of Mills *et al*. (1978). The vapour phases transfer method (Amanchukwu *et al*., 1989) was used. A filter paper (Whatman No.1) saturated with sterile spent oil was aseptically placed on the inside of the inverted petri dishes and the culture plates were incubated at (28 ± 2ºC) for 7 days (Odokuma and Okpokwasili, 1993; Odokuma and Ibor, 2002). Colonies of different hydrocarbon utilizing bacteria were randomly picked and pure isolates were obtained by repeated subculturing on Nutrient agar (oxoid). The bacteria isolates were characterized using microscopic techniques and biochemical tests. The identities of the isolates were determined by comparing their characteristics with those of known taxa as described by Bergey's manual of Determinative Bacteriology (Holt *et al*., 1994) and Cheesbrough (2006). The cultures used for biochemical test were between 18 to 24 hours old.

Preparation of Standard Cultures for Axenic Bacterial Isolates: Standard cultures were prepared for the isolates adapting the methods of Gerhardt *et al*. (1994) and Seely and Van-Denmark (1981). One hundred (100) ml of mineral salt broth was dispensed into each of three different conical flasks and inoculated with each purified isolate from each stock culture and incubated at 28ºC for 24hr. after incubation, the cultures were serially diluted up to 10^{-2} and 0.1ml of each was added into sterile plates. Cool molten nutrient agar was added to the inoculated plates and incubated at 37ºC for 24hr. The plate counts were recorded and the values obtained were expressed as standard number of cells present in 0.1ml of the broth. This was used as the standardized culture.

Screening for the Ability of Microbial Isolates to Utilize Spent Lubricating Oil as Sole Carbon Source: The microbial isolates were screened for the ability to utilize spent lubricating oil as sole carbon source using mineral salt medium as described by Mills *et al*. (1978). The method employed was adapted from Okpokwasili and Okorie (1988). The medium (9.0ml) was dispensed into test tubes. Into each of the test tubes, 1.0ml of the spent lubricating oil added. After capping, all the test tubes were sterilized at 121ºC for 15 minutes and allowed to cool. On cooling, the first set of the test tubes were inoculated with 0.1ml of standardized bacterial cell suspension of the respective bacterial isolates. The test tubes which served as control were not inoculated. All the test tubes were incubated in Shaker Incubator (G24 Environmental Shaker, New Brunswick Scientific Co., Inc.) at 150rpm at 30ºC for 14 days after which each tube was scored for optical density(OD) using Jenway 6405 uv/vis. The optical density of the culture was measured at 600 nm.

Biodegradation Studies of Selected Isolates: Isolates that showed good utilization potentials of spent lubricating oil during the screening test were selected for biodegradation studies. The ability of the bacterial isolates to degrade spent lubricating oil was confirmed by inoculating each isolate into 250ml Erlenmeyer

flasks containing 100ml mineral salt medium as described by Mills *et al*. (1978) and modified by Okpokwasili and Okorie (1988). The medium was supplemented with 1.0% (v/v) spent lubricating oil as sole carbon and energy source. The isolates were previously grown in a mineral salt medium with 1.0% (v/v) spent lubricating oil to a cell density of 10^8 ml^{-1} and were inoculated into the medium with 5.0% (v/v) as inoculum. This was cultivated at room temperature in a rotary shaker at 150rpm for 21 days. The optical density (OD), total viable count and pH of the culture media were monitored every 3 days during the period of the studies as biodegradation indices and the residual hydrocarbon concentration (TPH) was determined using gas chromatography flame ionization detector (GC-FID).

Extraction of Samples for TPH Determination: Liquid-liquid extraction procedure was used. A 100 ml of sample was extracted in a glass separating funnel fitted with a glass stopper using 3 ml of hexane as extractant. The separating funnel was shaken vigorously for at least 3 minutes and the organic layer was allowed to separate clearly from the aqueous phase for a minimum of 5 minutes, after which, the organic layer was collected into a separate glass bottle. The extraction was repeated thrice for each sample. Water residues were expelled from the organic layer by passing extracts through funnels containing anhydrous sodium sulphate. Clean-up of the extracts was achieved with a glass syringe loaded with silica gel, glass wool and anhydrous sodium sulphate and then eluted with about 10 ml of hexane. The syringe was first conditioned by soaking in hexane before and after packing. Extracts were concentrated using rotary evaporators.

Gas Chromatography Flame Ionization Detector (GC-FID) Analysis: Concentrated extracts was re-dissolved in dichloromethane (DCM) and the solution of extract was transferred to the bottle in which 10 ml of hexane had been added. Sample was injected by auto sampler into GC-FID.The peak areas for the residual hydrocarbon were measured, and the percentage decreases based on the areas of the control peaks were calculated. The values obtained were taken as quantitative measure of hydrocarbons.

RESULTS

The ability of the bacteria isolates to utilize spent lubricating oil as the sole source of carbon and energy is shown in Table 1 below. *Pseudomonas* sp., *Bacillus* spand *Nocardia* sp appeared to be the best with absorbance of 0.406, 0.327 and 0.310 respectively.

Table 1: Hydrocarbon utilization of bacterial isolates

Isolate code	Bacteria	Absorbance at 600nm
S102	*Micrococcus sp*	0.279
S103	*Enterobacter aerogenes*	0.183
S104	*Acinetobacter sp*	0.087
S105	*Enterobacter aerogenes*	0.168
S106	*Micrococcus sp*	0.265
S108	*Bacillus sp*	0.327
S109	*Pseudomonas sp*	0.406
S110	*Pseudomonas sp*	0.394
S111	*Staphylococcus sp*	0.283
S112	*Corynebacterium sp*	0.177
S115	*Proteus miabilis*	0.234
S116	*Staphylococcus sp*	0.306
S119	*Nocardia sp*	0.310
S124	*Acinetobacter sp*	0.164

Growth Profiles of Selected Isolates for Biodegradation Studies: The ability of the selected strains to grow onspent lubricating oil was tested in MSM. Increase in turbidity, total viable count (TVC), reduction in residual oil concentration determined gravimetrically as well as disappearance of individual HC peaks by GC analysis were measured. Changes in pH, turbidity and TVCare shown in Figures 1-3.Relatively high turbidity was observed in less than three days in all the cultures. The oil layers were slowly emulsified and eventually disappeared with incubation. The growth dynamics showed a consistent increase in TVC from day 0 to day 18 for *Bacillus sp* (4.5×10^6-1.28×10^8cfu/ml) before it declined. There was no decline in TVC for *Pseudomonas sp* (5.3×10^6-1.23×10^8) during the period of the study while *Nocardia sp* showed increase in TVC from day 0 to day 15 (4.8×10^6- 2.32×10^8) before decline.

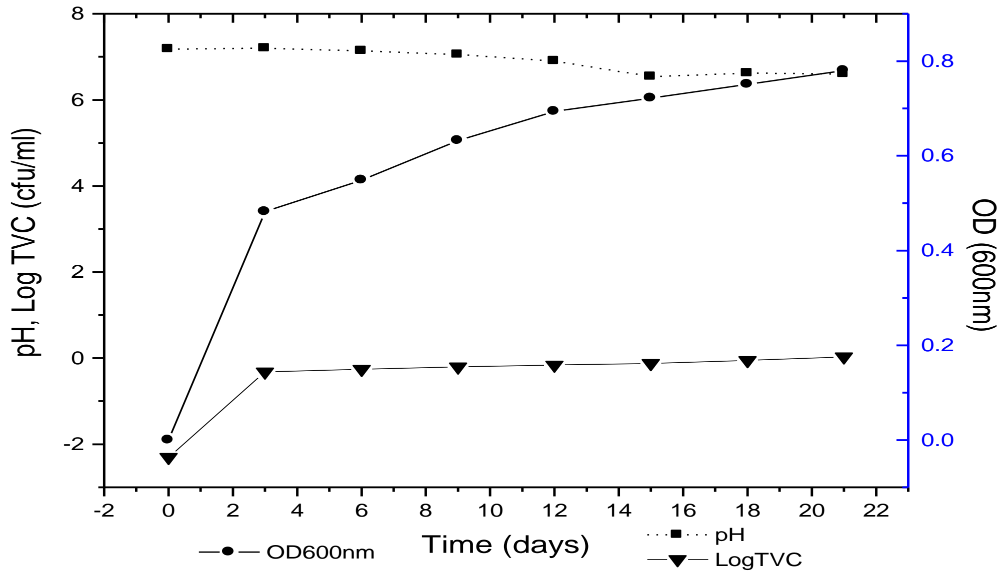

Figure 1: Growth profiles of *Bacillus sp* on spent lubricating oil

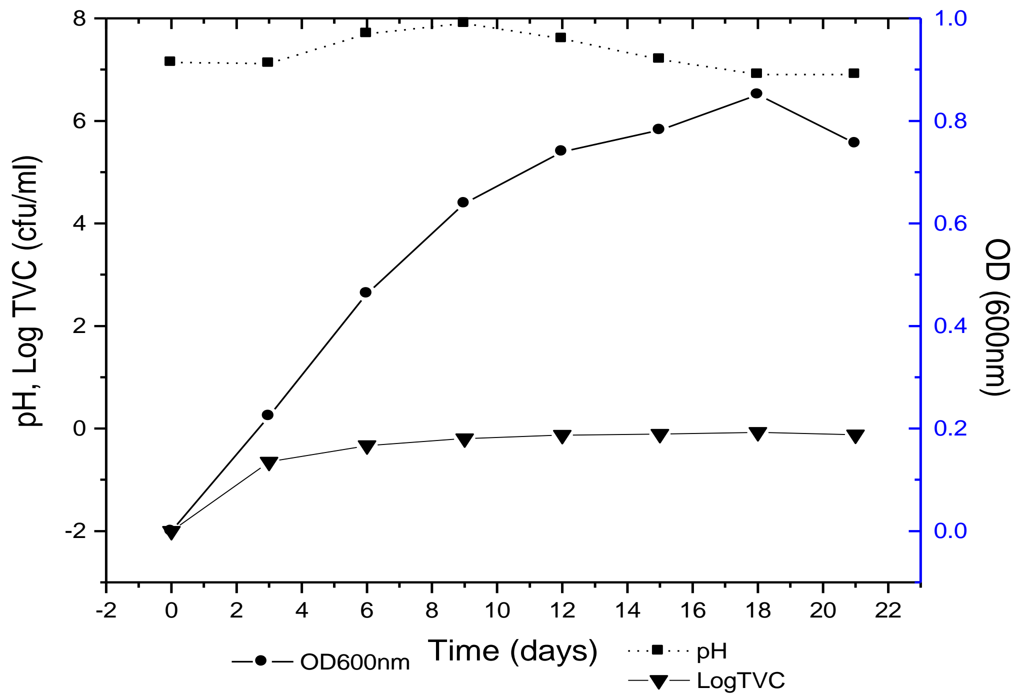

Figure 2: Growth profiles of *Pseudomonas sp* on spent lubricating oil

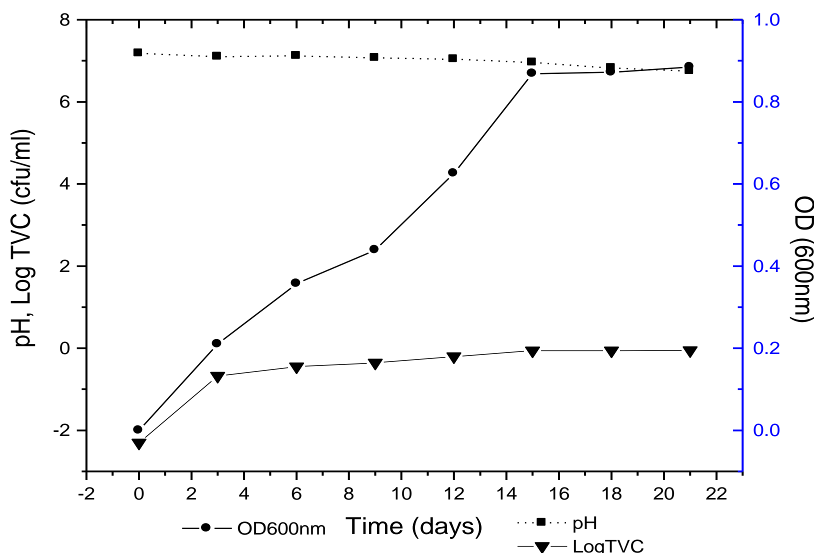

Figure 3: Growth profiles of *Nocardia sp* on spent lubricating oil

Biodegradation of Hydrocarbon Fractions by the Selected Isolates

Polycyclic Aromatic Hydrocarbons (PAHs) and a range of Aliphatic Hydrocarbons (C_8-C_{40}) present in the spent lubricating oil was degradedby *Bacillussp*, *Pseudomonassp* and *Nocardiasp*. The extent of biodegradation was determined after 21 days using GC/FID. Figures 4 and 5 show the concentration (µgml^{-1}) of different residual PAHs and Aliphatic Hydrocarbons present in the spent lubricating oil at the end of the biodegradation studies. Figures 6, 7 and 8 shows the percentage degradation of the Aliphatic, PAHs and Total Petroleum Hydrocarbon (TPH) component of the spent lubricating oil by *Bacillus sp*, *Pseudomonas sp* and *Nocardia sp* respectively.Figure 9 shows the chromatogram of the control sample for the aliphatic hydrocarbon containing no inoculated organism with 1% spent lubricating oil where C_8-C_{10} were below detection limits.The components of aliphatic and polycyclic aromatic hydrocarbons (PAHs) present in the spent lubricating oil show that *Nocardia sp* (S119) was the most effective degrader of both the aliphatic and PAHs components of the hydrocarbon. It degrades from 5055.82µg/ml and66.02µg/ml to 37.83µg/ml and 0.6µg/ml of aliphatic and PAHs respectively which corresponds to 99.3% and 99.1% degradation. Carbon chains between C_8 – C_{13} were degraded by all the isolates below detection limits. *Pseudomonas sp* (S109) degraded C_{14} –C_{16}, Phytane and C_{20} below detection limits while *Bacillus sp* (S108) and *Nocardia sp* (S119) degraded all the PAHs except Chrysene which none of the organisms was able to degrade below detection limits.

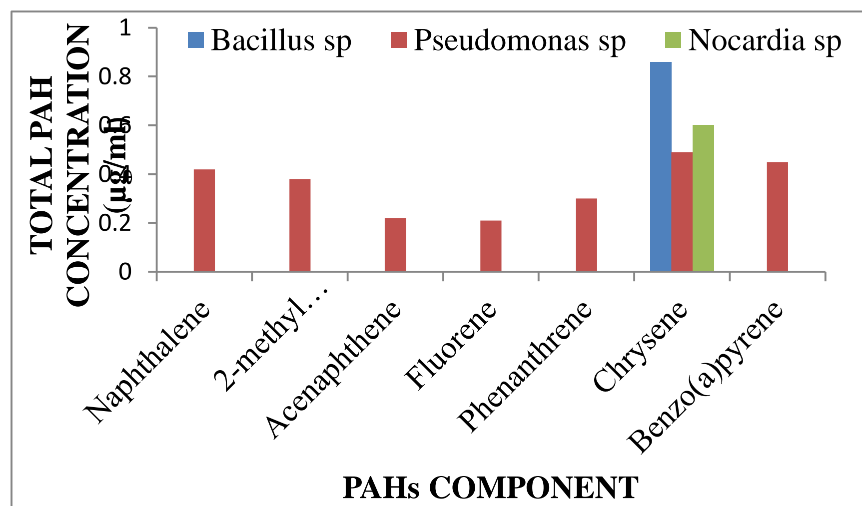

Figure 4: Total Concentration of PAHs detected after the biodegradation studies

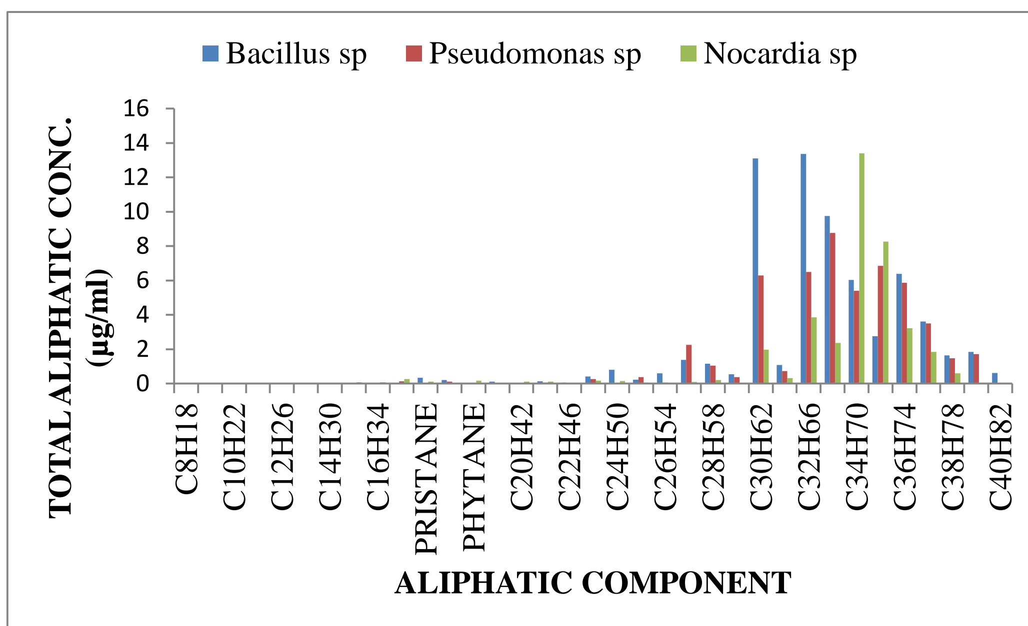

Figure 5: Total Concentration of Aliphatic Hydrocarbon detected after the biodegradation studies.

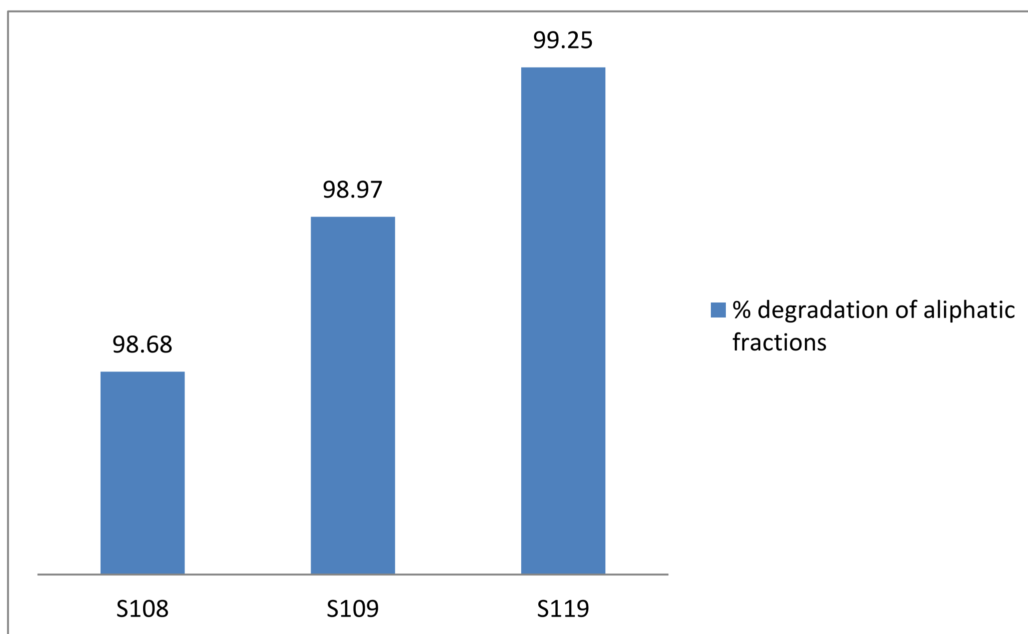

Figure 6: Percentage degradation of the Aliphatic components of spent lubricating oil by *Bacillus sp* (S108), *Pseudo monas sp* (S109) and *Nocardia sp* (S119).

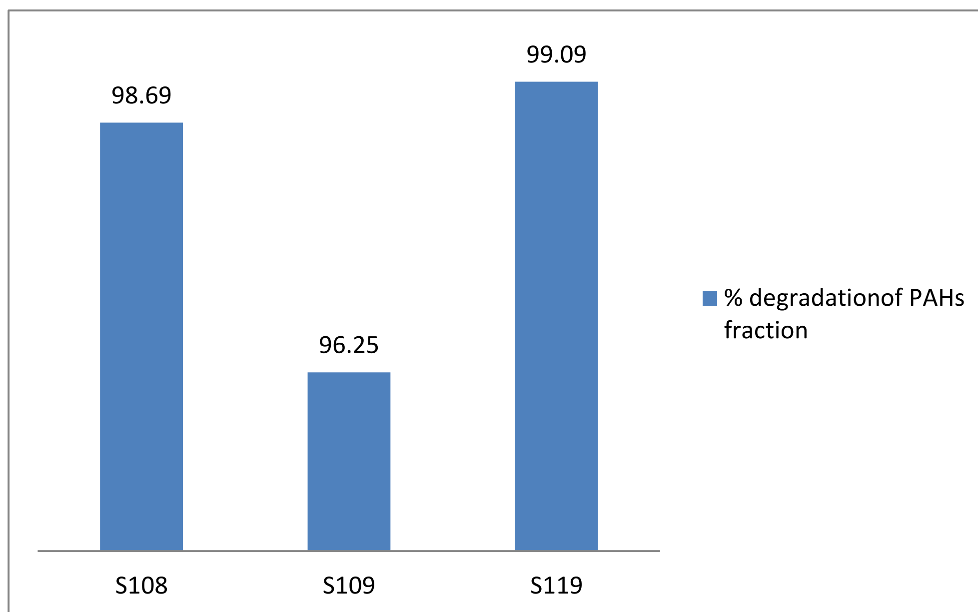

Figure 7: Percentage degradation of the PAHs component of the spent lubricating oil by *Bacillus sp* (S108), *Pseudo monas sp* (S109) and *Nocardia sp* (S119).

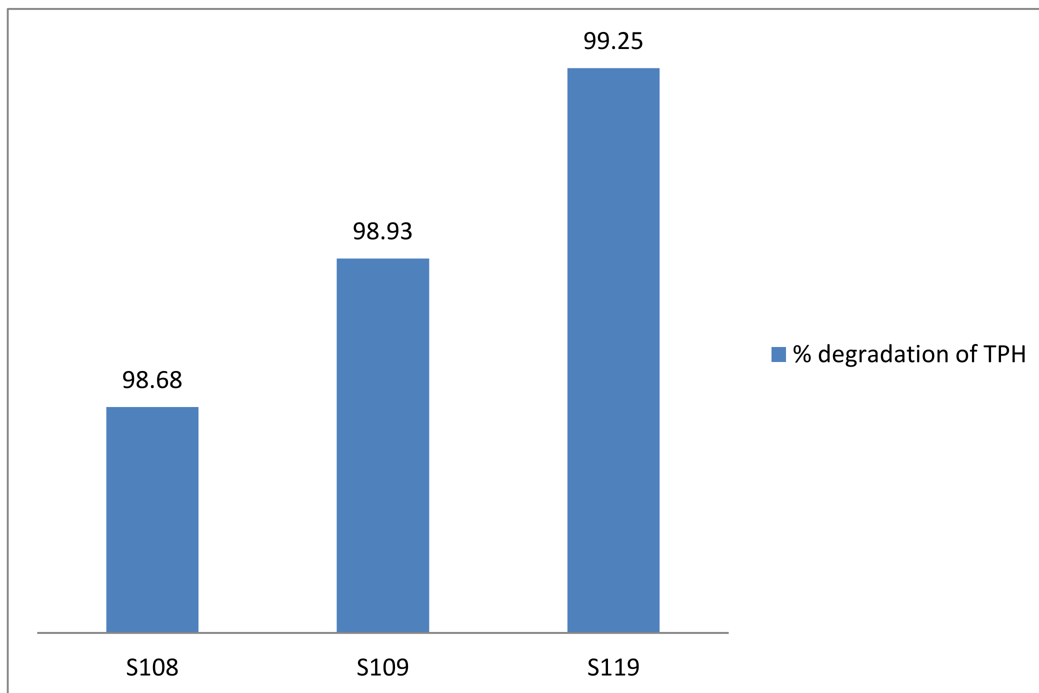

Figure 8: Percentage degradation TPH component of the spent lubricating oil by *Bacillus sp* (S108), *Pseudomonas sp* (S109) and *Nocardia sp* (S119).

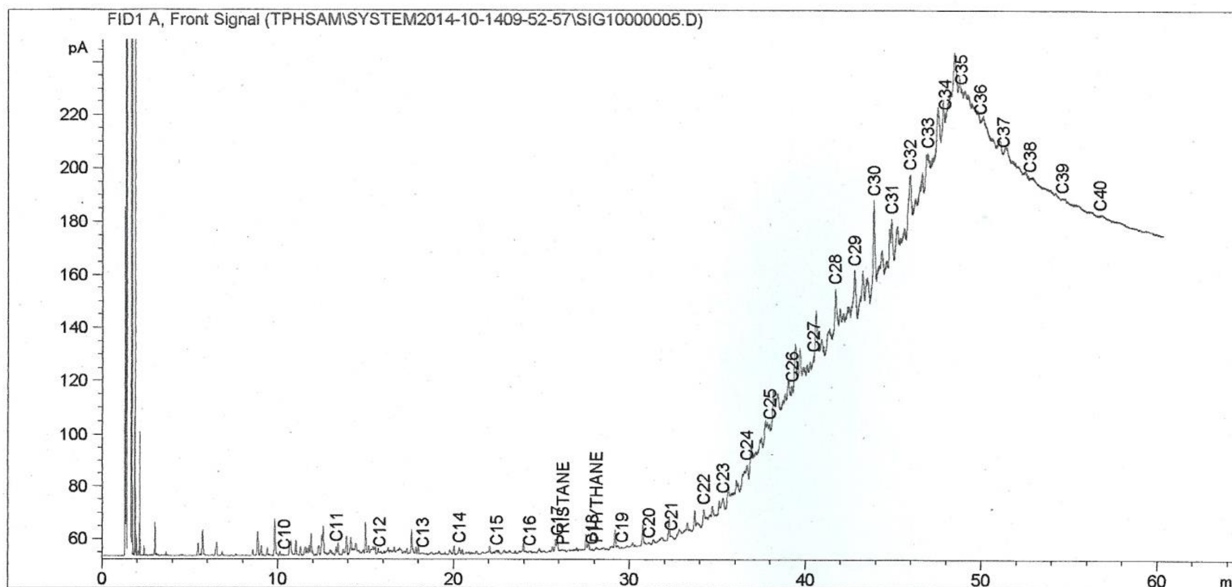

Figure 9: GC-FID Chromatogram of Aliphatic Hydrocarbons from extracted spent lubricating oil in the control sample.

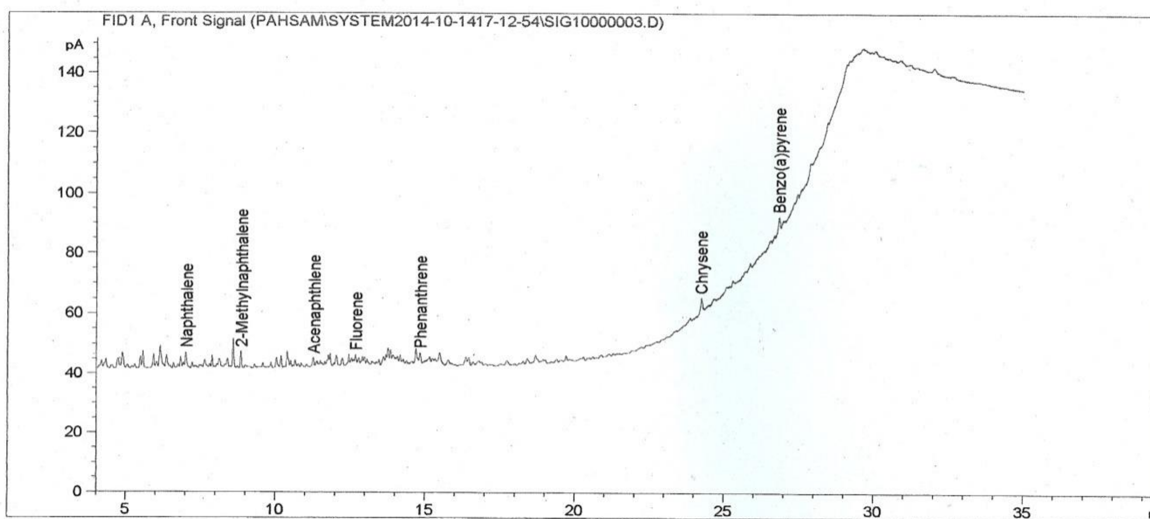

Figure 10: GC-FID Chromatogram of PAHs from extracted spent lubricating oil in the control sample.

DISCUSSION

Hydrocarbons are naturally occurring compounds and the ability to utilize it is widely distributed among diverse microbial populations. In this study, indigenous bacteria with the ability to utilize hydrocarbon were employed to degrade the aliphatic and PAHs components of spent lubricating oil. The growth profile of the bacteria strain was similar to previous reports (Okerentugba and Ezeronye, 2003; Oboh *et al*., 2006; Sebiomo *et al*., 2010). Their increasing growth

dynamics during degradation can be attributed to the constitutive expression of hydrocarbon assimilating capabilities or adaptation of the strains owing to previous exposure to exogenous hydrocarbons in spent lubricating oil, which may be followed by concomitant development of the ability to use the oil or its catabolic products as carbon and energy source (Adebusoye *et al*., 2007; Sebiomo *et al*., 2010; Omotayo *et al*., 2011). The reduction in the pH levels may have resulted from organic acids produced in the cultures. This was earlier reported by Okpokwasili and James (1995) and Oboh *et al*., 2006. The GC analysis shows that all the bacteria strains degraded the total petroleum hydrocarbon (TPH) content above 98%. Similar work has reported the degradation of hydrocarbons by these bacteria species (Oyetibo *et al*., 2012). Mandri and Lin (2007) and Shojaosadati *et al*. (2008) had also reported the reduction of engine oil hydrocarbons by *Pseudomona*s *sp*. Some earlier findings (Facundo *et al*., 2001; Kulwadee *et al*., 2001; Amund *et al*., 1994; Obire, 1988) had shown that microbial consortia are better degraders than pure isolates. It was noted that individual organisms often metabolize limited range of hydrocarbon substrates (Marin *et al*., 1996), notwithstanding the pure cultures in this study were able to degradation short and middle chain aliphatic compounds extensively. However, the degradation of the long chain hydrocarbons was relatively low. Several authors have reported excellent degradation of oil by pure cultures. Malij *et al*. (2013) showed that *Bacillus can* degradation up to 82.41% and 81.56% of aliphatic and aromatic hydrocarbons. The ability of *Nocardia sp* to utilize spent lubricating oil has been documented (Idemudia *et al*., 2014). It was observed that it could degrade high percentage of hydrocarbon in spent oil. Abioye *et al*. (2012) also reported that *Nocardia sp* demonstrated higher ability in utilizing hydrocarbon. These findings corroborate the data from this present study. The ability of *Nocardia sp* to utilize hydrocarbon may be due to the presence of hydrocarbon degrading enzyme systems and the presence of catabolic genes involved in hydrocarbon degradation in the bacteria species (Kyung-Hwa *et al*., 2006; Majid *et al*., 2008). This study has shown that the use of native bacteria strains with petroleum hydrocarbon utilizing capabilities as seed unto oil-impacted environment could prove a more efficient process which would on the long run enhance sustainable development rather than the use of exotic bacteria strains and chemicals.

CONCLUSION

The indigenous bacteria capable of metabolizing lubricating oil were observed in this study. However, the biodegradation of petroleum and other hydrocarbon in the environment is a complex process whose qualitative and quantitative aspects depend on the nature and amount of the oil, nutrients, ambient environmental condition and the composition of the microbial community. Therefore, further research should be directed towards understanding the interaction between individual bacterium and the soil microbial community in influencing the effectiveness of a microbial association in oil degradation.

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