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HYDROCARBON DEGRADATION POTENTIALS OF BACTERIAL SPECIES ISOLATED FROM BITUMEN CONTAMINATED WATER AND SEDIMENTS IN ILUBIRIN, TEMIDIRE CAMP,AND AGBABU COMMUNITIES OF ONDO STATE, SOUTH WEST NIGERIA

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ABSTRACT

The seepage of bitumen, a natural resource found in abundance in the Nigerian Dahomy belt is gradually becoming a source of concern due to contamination of surface waters and soil. Developing microbial strains that could be useful in restoring contaminated environment is therefore needful. This work was carried out to isolate and identify bacterial species from bitumen contaminated sites in Ondo State, Nigeria. Water and sediment samples were collected and analyzed using standard microbiological techniques. Species of bacteria isolated include: Bacillus, Citrobacter, Flavobacterium, Micrococcus, Pseudomonas, Acinetobacter and Staphylococcus. Hydrocarbon degradation was determined using an enrichment medium supplemented with diesel as the sole carbon source over a 14 day period. Growth was monitored by taking pH, optical density at 600 nm, and total viable count (TVC: Cfu/ml) on days one, five, ten and fourteen. Analysis of the results of the hydrocarbon degradation revealed that among the isolates from water sources, Pseudomonas aeruginosa had the greatest ability to degrade diesel while Staphylococccus aureus had the least capacity. Also, among the isolates from sediments, Pseudomonas fluorescens demonstrated the highest degradation ability while Flavobacterium had the least ability to degrade hydrocarbon. The results from this study reveal the potentials of some of the isolates to degrade hydrocarbon. Further genetic work can be done on the isolates in order to develop models that can be useful in effective bioremediation of hydrocarbon contaminated sites.

KEYWORDS: Agbabu, Ilubinrin, Temidire Camp, Bitumen, Sediment, Seepage, Bacteria

INTRODUCTION

Petroleum hydrocarbons can be introduced into the environment through leaks and accidental spills. It can also be introduced by natural seepages (UNEP, 2010). Petrochemical hydrocarbons can seep into soil and contaminate underlying ground water (Das and Chandran, 2011). Although exploration of the Nigerian bitumen deposit in Agbabu has not yet commenced, the environment is already battling with the problem of pollution arising from seepages of the natural resource into surface waters and soil in communities where the resource is found. Release of hydrocarbons into the environment, whether naturally or due to human activities has done extensive and incalculable damage to the environment; a clean-up measure is thus urgently required. Due to the negative consequences of the physicochemical approach, more attention is now given to the exploitation of biological alternatives (Okoh, 2006; Malik and Ahmed, 2012). Biodegradation of hydrocarbon contaminated sites, which exploits the ability of microorganisms to degrade and/or detoxify organic contamination, has been established as an efficient, economic, versatile and environmentally sound treatment (Hoff, 1993; Mehrasbiet al., 2003). Many microorganisms have the ability to utilize hydrocarbons as the sole source of carbon and energy, and that such microorganisms are widely distributed in nature (Okoh, 2006). Hydrocarbons in the environment are biodegraded primarily by bacteria, yeast, and fungi. Many indigenous microorganisms in water and soil are capable of degrading hydrocarbon contaminants (Brinton and Warren, 1976). Bacteria are the most active agents in petroleum degradation, and they work as primary degraders of spilled oil in the environment. Several bacteria are even known to feed exclusively on hydrocarbons. Bartha and Bossert (1984) had listed 22 genera of bacteria and 31 genera of such fungi. Among heterotrophic microorganisms found in the soil are naturally occurring populations that have the ability to degrade petroleum products. Species of Pseudomonas, Arthrobacter, Alcaligenes, Corynebacterium, Flavobacterium, Achromobacter, Micrococcus, Nocardiaand Mycobacterium are the most consistently isolated hydrocarbon degrading bacteria from the soil (Malik and Ahmed, 2012). Throne-Holst et al. (2007) reported that Acinetobactersp. was capable of utilizing n-alkanes of chain length C_{10}–C_{40} as a sole source of carbon. Daugulis and McCracken (2003) reported the degradation of poly-aromatic hydrocarbons by Sphingomonas.

Jones et al. (1983) studied the extensive biodegradation of alkyl aromatics in marine sediments and microorganisms. Arthrobacter, Burkholderia, Mycobacterium, Pseudomonas, Sphingomonas, and Rhodococcus were found to be involved in alkylaromatic degradation. Adebusoye et al. (2007) reported the microbial degradation of petroleum hydrocarbons in a polluted tropical stream in Lagos, Nigeria from which nine bacterial strains, namely,Pseudomonas fluorescens, P. aeruginosa, Bacillus subtilis, Bacillus sp., Alcaligenessp.,Acinetobacterlwoffi, Flavobacterium sp., Micrococcus roseus, and Corynebacteriumsp. were isolated which all showed hydrocarbon degrading potentials. In a study done by Adebayo et al. (2009), the biodegradability of bitumen was found to be highly influenced by incubation temperature, pH and inoculum size using five strains of bacteria (Pseudomonas fragii, Streptococcus zymogenes, P. aeruginosa, P. fluorescens and Bacillus macerans) isolated from water samples collected from Agbabu in Ondo state. They reported that bitumen degradation was optimum at pH 7.0, temperature of 37ºC and with two plates of each bacterium in suspension using a two litre-fermenter.

The objective of this work was to isolate bacterial species from bitumen contaminated sites, and thereafter use the organisms to degrade hydrocarbon. Further work will then be done on bacterial species which show high degradation potentials.

MATERIALS AND METHODS

2.1 Collection of samples

Bitumen contaminated water and sediments were collected aseptically from four differentsampling points and two water wells from Ilubirin, Temidire camp, and Agbabu, all in Odigbo local government area of Ondo state. The first samples were taken from Odo-blaki in Ilubirin designated P1 and S1, (GPS: N06º38.553, E004º49.741). The second and third samples were taken from Temidire Camp designated points 2 and point 3 (GPS: N06º36.929, E004º49.825)and GPS: N06º.38.822, E004º49.831) respectively. The fourth sample was taken from Agbabu designated point 4 (GPS: N06º36.203, E004º49.784. The first well is located at Ilubirin (GPS N06º38.300, E004º49.772). The second well is located at GPS N06º36.127, E004º51.488. Samples were collected in sterile specimen bottles. The samples were labeled P1, P2, P3, P4, W1 and W2 respectively, and S1, S2, S3, and S4 respectively. Samples were transported to the laboratory the Redeemer's University, and analyzed immediately.

SCREENING FOR BIODEGRADERS

Screening for isolates with the ability to degrade hydrocarbon was carried out by inoculating pure cultures of isolates previously cultivated in peptone water for 24 hours in 99ml minimal salt medium (MSM) containing NaCl (5.0g/L); KCl (0.2g/L); NaH2PO4 (2.8g/L); MgSO4.7H2O (0.1g/L); $Na2HPO_4$ (6.0g/L). The pH was adjusted to 5.85 and supplemented with 1ml of diesel to serve as the sole carbon source. All experiments were performed in triplicate. The medium was incubated at 37^0C for 14 days. Bacterial growth was monitored on days 1, 5, 10 and 14 by measuring the optical density (OD)at 600nm, pH, and total viable count (TVC).

GROWTH OF BACTERIAL ISOLATES ON HYDROCARBON SUBSTRATE OVER A 14 DAY PERIOD

Time-course degradation of diesel was performed in minimal salts medium. The organisms Bacillus subtilis, Citrobacterfreundii, Flavobacteriumsp., Micrococcus sp., Pseudomonas aeruginosa and Staphylococcus aureus isolated from sampling points P_1, P_2 and W_1, S1, and S2were used for the degradation experiments.

The optical density at 600nm (OD_{600nm}), total viable count (TVC) and pH of the culture fluids were monitored at determined time intervals (Days 1, 5, 10 and 14) as biodegradation indices. Figure 1 represents the optical density reading for a period of 14 days. Tables 5 and 6 show the total bacterial counts and the pH of the culture media for 14 days.

RESULTS

Table 1 shows the total bacterial count/ml in the water samples collected from the six sites, both polluted and non-polluted (control) sites. The results show that the control site P4 (uncontaminated site) had the highest bacterial count, while point 2(most contaminated point) had the least count.

Table 1: Bacterial count of Bitumen polluted surface and well water

Sample	Bacterial count(Cfu/ml)
P_1	1.1×10^5
P_2	2.0×10^4
P_3	4.0×10^4
P_4	1.6×10^5
W_1	7.0×10^4
W_2	TNTC

Key: P_1 – Sample point 1, P_2 – Sample point 2, P_3 – Sample point, P_4 – Sample point 4, W_1 – Sample well 1, W_2 – Sample well 2

Table 2 shows the total bacterial count/ml in the sediment samples collected from the four sediments. S2 had the highest bacterial count while S3 had the lowest bacterial count. A total of 18bacterial species were obtained from the samples used for this study. Species of Bacillus, Citrobacter, Flavobacterium, Micrococcus, Pseudomonas, Staphylococcus and Veillonella, Flavobacterium, Acinetobacter, were confirmed as shown in tables 3 and 4.

Table 2: Bacterial count of the bitumen contaminated sediments

Samples	**Bacterial count (Cfu/ml)**
S_1	3.0×10^4
S_2	3.3×10^4
S_3	1.3×10^4
S_4	2.2×10^4

Key: S_{1-} sediment 1, S_{2-} sediment 2, S_{3-} sediment 3, S_{4-} sediment 4

Table 3: Occurrence of bacterial isolates in water

Site	Microorganisms isolated
P_1	Micrococcus, Bacillus subtilis, Pseudomonas aeruginosa
P_2	Pseudomonas aeruginosa, Bacillus subtilis, Bacillus licheniformis
P_3	Bacillus licheniformis, Pseudomonas aeruginosa,
P_4	Citrobacterfreundii, Bacillus sphaericus
W_1	Citrobacterfreundii, Flavobacterium, Bacillus subtilis, Staphylococcus aureus
W_2	Staphylococcus aureus, Citrobacterfreundii, Pseudomonas aeruginosa, Bacillus subtilis, Veillonella

Table 4: Occurrence of bacterial isolates in sediments

Site	**Microorganisms isolated**
S1	Pseudomonas fluorescens, Flavobacterium, Bacillus megaterium.
S2	Acinetobacter, Pseudomonas citronellolis, Bacillus megaterium, Penicillium spp.
S3	Staphylococcus, Acinetobacter, Bacillus thuringiensis
S4	Staphylococcus, Bacillus cereus

The optical density of the culture medium (isolates from water), supplemented with diesel inoculated with bacteria isolates for a 14 day period is displayed in Fig. 1.Bacillus megaterium had the highest optical density while Acinetobacter sp. had the lowest optical density at day 1.

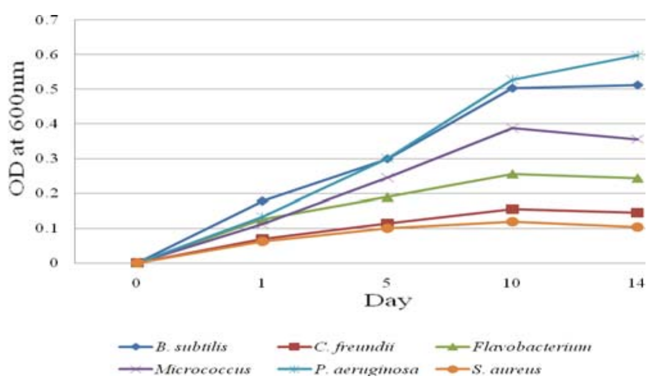

Fig 1: Graph of the Optical Density values of bacterial isolates from water in minimal salt medium supplemented with diesel for a 14 day incubation period

At day 5, Bacillus megaterium also had the highest optical density while Flavobacteriumspp. had the lowest optical density. At day 10, Pseudomonas fluorescens had the highest optical density while Flavobacterium spp. had the lowest optical density. At day 14, Pseudomonas fluorescens had the highest optical density, while Flavobacterium spp. had the lowest optical density.

Figure 2 shows a graph showing Optical density values of bacterial isolates from sediments in minimal salt medium supplemented with diesel for the 14 day incubation period, with Pseudomonas fluorescence having the highest value while Flavobacterium had the least.

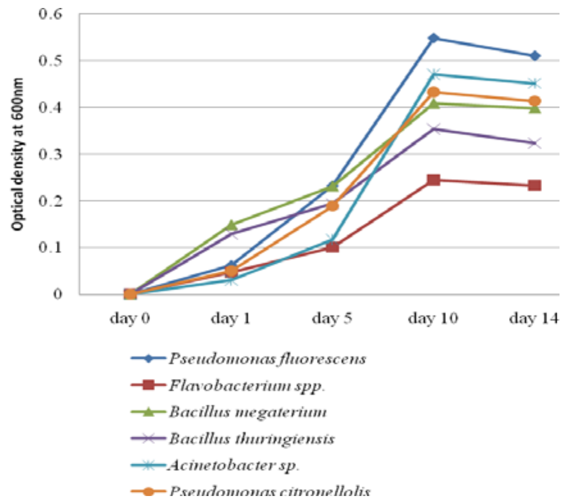

Fig 2: Graph of Optical Density values of bacterial isolates from sediments in minimal salt medium supplemented with diesel for a 14 day incubation period

Table 5 shows the total viable count of isolates from water during the 14 day course degradation experiment. It also confirms that Pseudomonas aeruginosa had the highest ability to utilize the hydrocarbon.

Table 5: Total viable count of isolates from water during the 14 day course degradation experiment

Microorganism	DAY 1	DAY 5	DAY 10	DAY 14
Bacillus subtilis	6.0×10^3	1.5×10^4	2.6×10^4	2.1×10^4
Citrobacterfreundii	2.0×10^3	7.0×10^3	1.3×10^4	9.0×10^3
Flavobacteriumsp.	3.0×10^3	9.0×10^3	1.7×10^4	1.3×10^4
Micrococcus sp.	4.0×10^3	1.3×10^4	2.1×10^4	1.9×10^4
Pseudomonas aeruginosa	5.0×10^3	1.6×10^4	TNTC	TNTC
Staphylococcus aureus	2.0×10^3	5.0×10^3	1.1×10^4	8.0×10^3

TNTC – Too numerous to count

The total viable count of isolates from sediments during the 14 day course degradation experiment is shown in Table 6 with Pseudomonas fluorescence having the highest count followed by Acinetobacter sp. and Pseudomonas citronellolis respectively.

Table 6: Total viable count of isolates from sedimentsduring the 14 day course degradation experiment

Microorganism	Day 1	Day 5	Day 10	Day 14
Pseudomonas fluorescens	5.0×10^3	1.2×10^4	2.4×10^4	1.9×10^4
Flavobacteriumsp.	3.0×10^3	7.0×10^3	1.0×10^4	8.0×10^3
Bacillus megaterium	4.0×10^3	1.9×10^4	1.5×10^4	1.1×10^4
Bacillus thuringiensis	2.0×10^3	8.0×10^3	1.2×10^4	9.0×10^3
Acinetobacter sp.	6.0×10^3	1.4×10^4	2.4×10^4	1.6×10^4
Pseudomonas citronellolis	6.0×10^3	1.1×10^4	1.9×10^4	1.4×10^4

Table 7 shows the different pH values of the inoculated medium of isolates from water, with hydrocarbon (diesel) over the 14 day period. The pH difference between day 5 and day 10 was at a high rate, this implies that the degradation probably occurred during these days while the rise in pH reduced between day 10 and 14.This is probably because the microorganisms had utilized the hydrocarbon substrate in the culture medium, hence reducing the acid produced.

Table 7: pH of culture fluids of isolates from water during the 14 day course degradation experiment

Microorganism	DAY 1	DAY 5	DAY 10	DAY 14
Bacillus subtilis	5.940	6.015	6.260	6.360
Citrobacterfreundii	6.040	6.100	6.475	6.555
Flavobacterium	5.975	6.175	6.550	6.670
Micrococcus sp.	6.075	6.175	6.440	6.570
Pseudomonas aeruginosa	6.060	6.145	6.475	6.520
Staphylococcus aureus	6.070	6.195	6.445	6.510

Figure 3 shows graph of pH of isolates from sediments during the 14 day course degradation. Pseudomonas fluorescence obviously having the highest value at the end of the growth period followed by Bacillus megaterium and Pseudomonas citronellosis respectively.

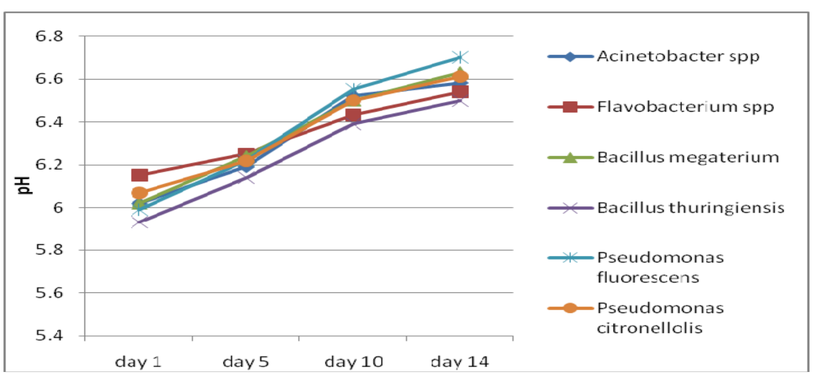

Fig. 3: Graph of pH of isolates from sediments through the 14 day course degradation

DISCUSSION

The organisms isolated from study sites 1 and 2 (contaminated by bitumen) in this study have been confirmed by other workers to have hydrocarbon degrading abilities. Whereas, organic materials serve as a source of nutrition for some bacterial species, it could be toxic to other species. This probably accounts for the microbial count as shown in table 1 in which well 2 which has no seepage of bitumen into it, had the highest count. Point 2 with the greatest seepage, had the least bacterial count of 1.1×10^4cfu/ml. This is because not all microorganisms can utilize hydrocarbon as substrate for growth. Furthermore, the presence of hydrocarbon can inhibit the growth of many microorganisms. The microbial species isolated from sampling points 1 and 2 as shown in table 2 are members of genera that have been reported by other researchers as hydrocarbon degraders in various polluted areas. Obohet al. (2006) also isolated bacterial species of Pseudomonas, Bacillus, Alcaligenes and Citrobacter from core and surface bitumen polluted areas. Boboyeetal. (2010) reported the isolation of Staphylococcus aureus, Micrococcus luteus, Lactobacillusacidophilus and Bacillus spp. from petroleum polluted area.

In this study, the predominant species were mainly Pseudomonas spp. and Bacillus spp. The ability to isolate high numbers of certain oil degrading microorganisms from an environment implies that these organisms are the active degraders in that environment (Okerentugba and Ezeronye, 2003; Jyothi, 2012)

The growth dynamics of the organisms isolated in this study was determined by the optical densities, total viable counts and the pH of the culture media. The utilization of the hydrocarbons resulted in increase in cell densities with a visual gradual reduction in the oil layer and complete disappearance of the oil with prolonged incubation. Interestingly, no lag phase was observed in the in the growth profiles of all the organisms used for the degradation experiments. This could be explained by the fact that the organisms have a previous exposure to hydrocarbon present in the bitumen and hence have developed enzymes capable of degrading hydrocarbon.

The optical density readings based on the turbidity of the minimal salt medium at regular intervals of 5 days show the degradation activities of the bacteria on hydrocarbons. The level of utilization differs between the bacterial species. The gradual increase in the concentration of the broth (turbidity) indicates bacterial growth hence the degradation of hydrocarbons, mostly between days 5and 10. The increase in cell densities as a result of hydrocarbon utilization showed a gradual and visible disappearance of oil layer and a complete disappearance of oil layer after prolonged period. A gradual decline in the turbidity of the broth suggests decrease in the bacterial population indicating that the hydrocarbon has been degraded between day ten and fourteen. The results demonstrated that Pseudomonas aeruginosaand Bacillus subtilis had the highest hydrocarbon degradation ability, while Staphylococcus aureus had the lowest degrading ability to degrade diesel used in this study which agrees with the work of Jyothiet al. (2012).Dussan and Numpaque (2012) had reported that Pseudomonas sp. is a useful candidate for diesel degradation. The organisms were able to utilize the available nutrients, and grew steadily from days 1 to 10. As from the day 10, there was a gradual decline in the total viable count which implied that the cells had ceased to divide. This could have resulted from exhaustion of nutrient. Growth may also have ceased due to the accumulation of toxic metabolic products produced by the organism. The presence of toxic waste products may cause changes in the culture medium and inhibit the growth of the microorganism. In addition, the pH of the culture media remained acidic, within the range of 5.90 – 6.60, as shown in the results. The difference between the pH values was highest between days 5 and 10, indicating that the rate of biodegradation was highest between these days. Microbial degradation of hydrocarbons often leads to production of organic acids and other metabolic products (Nwachukwu and Ugoji, 1995; Okpokwasili and James, 1995). The initial pH of the culture medium was adjusted to 5.85, which eventually increased above 6 for all the organisms. This indicated that batch cultures often results in production of weak acids. Thus, the acids produced accounted for the acidic pH in all the culture fluids, and confirmed biochemical changes as a result of enzymatic actions on the substrate.

CONCLUSION

The need to develop strains that could be useful in the bioremediation of hydrocarbon polluted sites cannot be overemphasized. This study reveals that bacterial species isolated from bitumen contaminated sites can be harnessed in an attempt at developing strains that will be useful in environmental bioremediation of contaminated sites. Many indigenous microorganisms that are capable of degrading hydrocarbon contaminant can be readily isolated from contaminated sites as shown in this study. Models and strategies which will enhance the removal of hydrocarbon contaminants from oil impacted sites need be promoted. Further studies are ongoing to determine the abilities of these isolates to effectively degrade hydrocarbons with long chains. From these studies, strains with high degradation capabilities can then be genetically developed.

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