Nig J. Biotech. Vol. 20 (2009) 44 – 50
ISSN: 0189 17131
Available online at http://www.ajol.info/index.php/njb/index
and www.biotechsocietynigeria.org

NIGERIAN JOURNAL OF BIOTECHNOLOGY

Effects of the Consortium of *Pseudomonas, Bacillus* and *Micrococcus* spp on Polycyclic Aromatic Hydrocarbons in Crude Oil

Ifeanyi, V. O.

Microbiology Department, Anambra State University, Uli

Abstract

The effect of the consortium of *Pseudomonas, Bacillus* and *Micrococcus* spp on polycyclic aromatic hydrocarbons in crude oil was carried out using standard microbiological methods. Spectrophotometer, gas chromatography and viable count which determined the optical density, the polycyclic aromatic hydrocarbons and the total heterotrophic hydrocarbon degrading bacteria were used for the study and analysis was for a period three weeks at one week interval. The values of the polycyclic aromatic hydrocarbon decreased from 419mg/l to 0.06mg/l while the values of the optical density and viable counts, respectively, increased from 0.018 to 0.740 and from 9.80 x 10^5 cfu/ml to 2.74 x 10^6 cfu/ml. This research concluded that the consortium of these bacteria can be used for the decontamination of PAH-polluted sites.

Key words: *Pseudomonas, Bacillus, Micrococcus* spp, polycyclic aromatic hydrocarbons, PAH-polluted sites

Introduction

Crude oil occurs naturally in many parts of the world, particularly in the U.S.A, Russia, Mexico, Romania, Iran, Iraq, Kuwait, Saudi Arabia, Libya and Nigeria. The origin of crude oil, however, is uncertain but the most favoured theory is that it has been formed by microbial decomposition of plant and animal remains under pressure. Crude oil is a heterogeneous mixture of different liquids containing dissolved gases and solids. These components include: alkanes, alkenes, alkynes and aromatic hydrocarbon; crude oil may be broadly classified into two main types, namely: heavy and light crude oil.

The activities of living microorganisms, primarily bacteria, yeast, and filamentous fungi under certain conditions can alter and metabolize various classes of compounds present in oil. Biodegradation affects the oil fluid properties such as gravity, viscosity and other physical parameters of crude oil (Atlas, 1981). It is known that greater degradation of oil pollution is carried out in situ by consortia of microorganisms (Okpokwasili and Nnorom, 1990). Microbial degradation of oil has been shown to occur by attack on the aliphatic or light aromatic fraction of oil. Studies carried out by various researchers, (Watkinson, 1978; Atlas, 1977; Connan, 1986) have revealed that hydrocarbon degradation is an oxidative process. The saturated and aromatic hydrocarbons are transformed into oxygenated products by several metabolic processes; such products include fatty acids, alcohol; ketones, phenols, etc. These products are further broken down by alpha-& beta-oxidation, ring cleavage etc (Connan, 1986). The metabolic steps involved in biodegradation entail formation of increasing smaller molecules resulting ultimately to carbon dioxide, water and biomass (Brock, 1974).The physical state of petroleum hydrocarbon has marked effect on their biodegradation; such physical state of oil pollutant includes the viscosity, molecular weight, and specific gravity. The viscosity in turn determines surface area of oil available for

microbial colonization (Walker and Colwell, 1977). The nutrient dependence of oil degradation has been documented. Crude oil is essentially a mixture of carbon and hydrogen. For bacteria to grow effectively, they require about 10 parts of carbon to 1 part of nitrogen (Jobson *et al.,* 1972*).* Oxygen is considered a limiting factor to the occurrence of petroleum degrading microorganisms in oil-polluted site (Atlas, 1981). Crude oil biodegradation can occur under both aerobic and anaerobic conditions (Zengler *et al.,* 1999). This research was aimed at investigating the effects of the consortium of *Pseudomonas, Bacillus* and *Micrococcus* spp on polycyclic aromatic hydrocarbons in crude oil

Materials and methods

Sampling Site: The oil polluted water sample used for this research work was collected from Shell Petroleum Development Corporation (SPDC) located at Mahu village in Ohaji-Egbema Local Government Area of Imo State. The flow station of the SPDC empties into a river called Utu and the water sample was collected from a distance of about 500metres from the in-let point of the flow station into the river.

Collection of Samples: By adopting the method of Okpokwasili and Amanchukwu (1988), water sample was collected in sterile 1 litre plastic can previously rinsed with 70% ethanol. The water sample was collected aseptically and then transported quickly to the laboratory.

Processing of samples: It was processed and analysed by making ten fold serial dilutions as stated in Prescott *et al.* (2005) within 48 hours of collection. Sterilization and disinfection of materials were as stated in Prescott *et al.* (2005). The media; nutrient agar (Oxoid), MacConkey agar (Oxoid) and mineral salts medium as described by Ekpo and Ekpo (2006) with composition of K_2HPO_4, 1.8g; NH_4Cl, 0.2g; $MgSO_4$ $7HO_2$, 0.1g; NaCl, 0.1g; $FeSO_4$ $7H_2O$, 0.1g; distilled water, 1 litre and agar agar 15g were used. These media were prepared as stated in Cheesbrough (2005) and according to the manufacturer's specification. Each medium was aseptically dispensed into sterile Petri dishes and used for the isolation of bacteria.

Isolation and characterization of organisms: Pure cultures of the isolates were obtained and stock cultures made from them were stored in a refrigerator at 4^0C and sub-cultured at intervals. The total heterotrophic bacterial count of the samples was performed in duplicates using the spread plate technique. Plates were incubated at room temperature and counts made after 24 hours of incubation. Characterization and identification of isolates were according to Buchanan and Gibbons (1974).

Enumeration of Hydrocarbon Utilizing Bacteria: For hydrocarbon utilizing bacteria enumeration, vapor phase transfer method described by Okpokwasili and Amanchukwu (1988) was adopted. Filter paper soaked in distilled water served as a control. The plates were incubated at room temperature for about 24-48 hours.

Crude Oil Utilization Adaptation Test: The Isolates selected were adapted for hydrocarbon utilization in mineral salts medium containing 1ml/L of crude oil as the carbon source. Incubation was at room temperature (30^0C) and aerated at 100 strokes per minute by manual shaking as described by (Chikere and Okpokwasili, 2003) for 30 minutes per day for 10 days.

Crude Oil Biodegradation: Mineral salts broth was dispensed into a collection of thirty biodegradation bottles with each containing 69ml of mineral salts medium and then sterilized. Each experimental set-up was added lml of filter sterilized bonny-light crude oil (Ekpo and Ekpo, 2006) and a consortium of the three isolates obtained by dispensing 10ml of each adapted isolate into each biodegradation bottle. A control was made without inoculation. The bottles were incubated at ambient temperature (30^0C) with manual shaking at 100 strokes per minute as described by Wang *et al.* (1990); Chikere and Okpokwasili, (2003) for 1hour each day for 3 weeks. Viable counts by spread plate, optical density by spectrophotometer and PAHs were monitored at one week interval for three weeks.

The polycyclic aromatic hydrocarbons (PAH) were determined using Gas Chromatography (HP6890, Capillary Column (HP-5) and the analysis was as described by Chikere and Okpokwasili (2003). Ten milliliters of each sample was extracted using 10ml of pentane solvent. The sample was poured into a separatory funnel and the bottle rinsed with 10ml of the solvent. The sample and solvent mixture were vigorously shaken for 2 minutes

and then allowed to separate for about 10-15 minutes. The extract (supernatant) was collected and evaporated to concentrate to about 1ml in an oven set at 60^{o}C after eluting in activated silica gel in 10ml methylene chloride. One milliliter of the concentrated sample was injected by means of hypodermic syringe through a rubber septum into the column. The sample was automatically detected as it emerges from the column at a constant flow rate by the FID detector.

Results

Three bacterial species of different genera, namely *Pseudomonas, Bacillus* and *Micrococcus* spp. were isolated from oil-polluted water, characterized and identified. The results of the isolation of *Pseudomonas, Bacillus* and *Micrococcus,* their colonial morphology and biochemical reactions are presented in Table 1.

Table1 Morphological, Cultural and Biochemical Characteristic of Isolates

Cell morphology	Rod	Rod	Cocci
Gram reaction	-	+	+
Colour	Fluorescent green	Milky	Yellow
Shape	Rod	Rod	Cocci
Margin	Entire	Entire	entire
Colony size (mm)	2	1-2.5	1-2
Elevation	Raised	raised	convex
Catalase	-	+	+
Coagulase	-	-	-
Oxidase	+	-	-
Spore	-	+	-
H_2S	-	-	-
Mortility	+	+	-
Citrate	+	+	+
Indole	-	-	-
Glucose	A	A	A
Sucrose	A	A	A
Lactose	A/G	A	A
MR	-	-	-
VP	-	-	-
Suggested isolate	*Pseudomonas sp.*	*Bacillus sp.*	*Micrococcus sp.*

Key + = Positive result. - = Negative result, A = Acid production, A/G = Acid and gas production

Table 2 Shows the values of the optical density of the medium. The increase was due to the multiplication of the cells as they utilize the hydrocarbon components of the crude oil as sole source of carbon. The low value in the optical density on the day zero may be due to the fact that the organisms had not started utilizing the hydrocarbon for their cell multiplication and thus were adjusting to new environmental conditions.

Table 2 Optical Densities During Crude Oil Biodegradation.

Time/ Week	OD1	OD2	OD3	Mean value
0	0.017	0.019	0.018	0.018
1	0.287	0.0288	0.289	0.288
2	0.497	0.496	0.498	0.497
3	0.741	0.739	0.740	0.740

Key: OD = optical density

Table 3: shows the respective values of the total viable counts The high counts at week three was

due to the fact that the organisms were growing rapidly on their log phase as they utilized the nutrients in the medium.

Table 3 Total Viable Counts (TVC) During Crude Oil Biodegradation

Time/Week	Number of colonies	CFU/ML
0	16	-
1	98	9.80×10^6
2	198	1.98×10^6
3	274	2.74×10^6

Figures 1-4 show the changes in polycyclic aromatic hydrocarbons (PAHs) concentration during the biodegradation study. There was a continuous decrease in the crude oil polycyclic aromatic hydrocarbons (PAHs). The continuous decrease was due to the increase in the population of crude oil degrading bacteria as a result of their adaptation and utilization of the hydrocarbon components of the crude oil, which included PAHs. Also the organisms synthesized strong enzymes that had ability to mineralize crude oil.

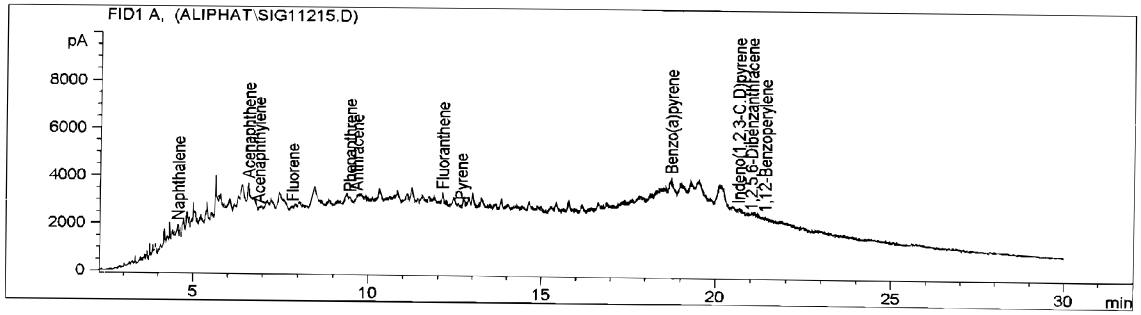

Figure 1 Polycyclic aromatic hydrocarbons before incubation

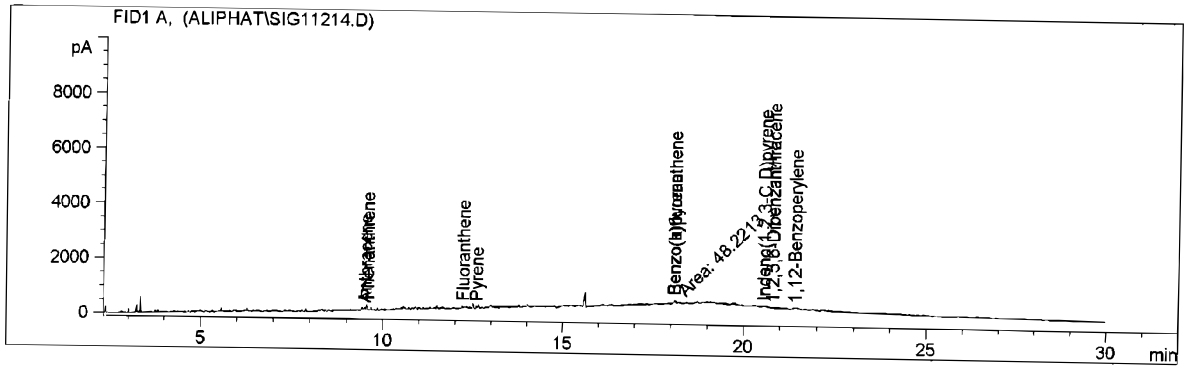

Figure 2 Polycyclic aromatic hydrocarbons after one week incubation

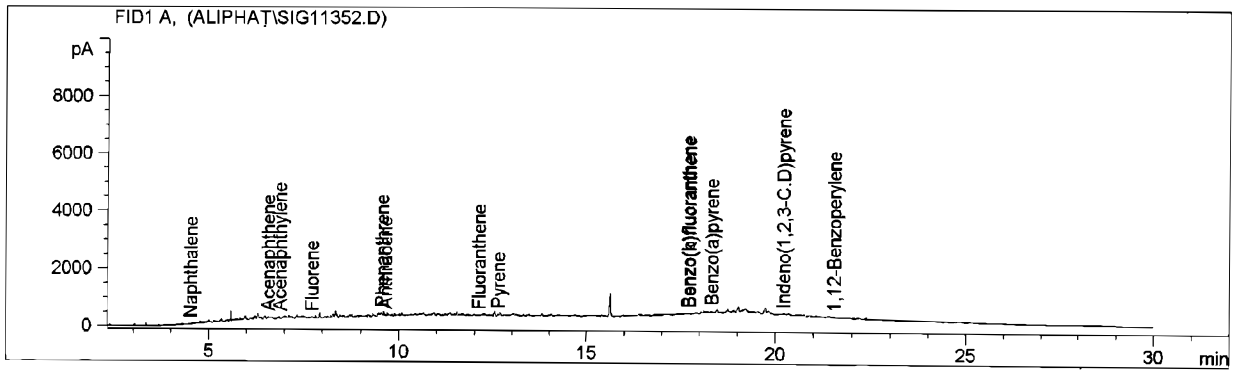

Figure 3 Polycyclic aromatic hydrocarbons after two weeks of incubation

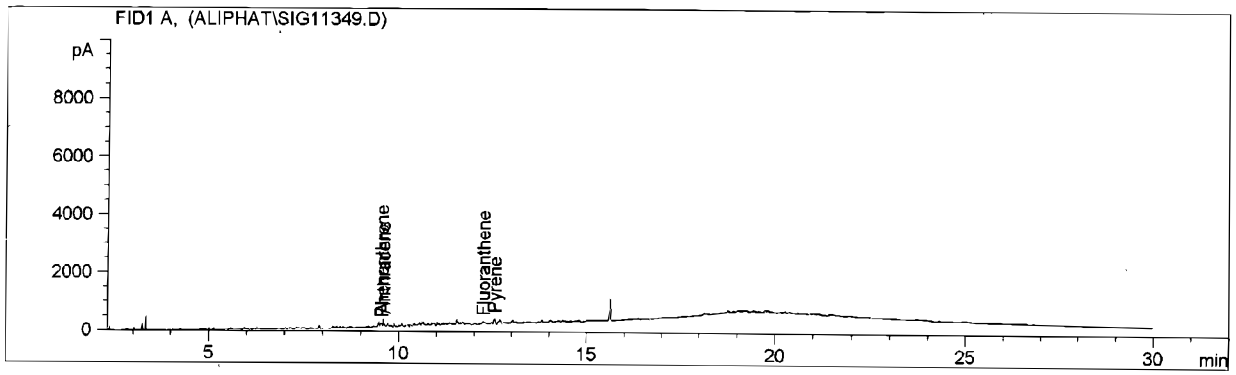

Figure 4 Polycyclic aromatic hydrocarbons after three weeks of incubation

Discussion

The effects of the consortium of the three isolates *Pseudomonas, Bacillus* and *Micrococcus spp* on polycyclic aromatic hydrocarbons were studied. Previous reports (Udeme and Antai, 1988; Chikere and Okpokwasili, 2003; and Ekpo and Ekpo, 2006) have shown the capability of these species to degrade crude oil. The low count in the total viable counts and low value of optical density on the day zero may be due to the fact that the organisms had not started utilizing the hydrocarbon components in the crude oil and thus were adjusting to the new environmental conditions and at the same time synthesizing new enzymes for the degradation of the crude oil (Bouchez *et al.,* 1995). The total viable counts and the values of the optical densities increased continuously from day zero to week three. The use of consortium in this research was in line with the report of Okpokwasili and Nnorom, (1990).

In this study, the polycyclic aromatic hydrocarbons (PAH) decreased continuously from day zero to week three. The decrease might be as a result of increase in microbial population of hydrocarbon-degrading bacteria that used hydrocarbon components of the crude oil as sources of carbon and energy, thus releasing more enzymes that can degrade polycyclic aromatic hydrocarbon (PAH) components of crude oil. This observation agrees with the report of Walker and Colwell (1977) that carried out a study of the rates of biodegradation of crude oil and reported that there was a consistent decrease in PAH component of the crude oil within the first four weeks. The high values of PAH on the day zero may be due to the fact that the organisms had not started utilizing the components of the crude oil and were adjusting to the new environmental conditions and at the same time synthesizing new enzymes for the degradation of the crude oil (Bouchez *et al.,* 1995). According to Miller *et al.* (1987), oil biodegradation typically; raises oil viscosity, reduces oil gravity, increases the asphaltene content (relative to the saturated

and aromatic hydrocarbon content), reduces PAH, reduces TPH, decreases the concentration of certain heavy metals, increases the sulfur content, increases oil acidity and adds compounds such as carboxylic acids and phenols. This research observed reduction of PAH and also changes in the composition of the PAH as a result of biodegradation. These observations agreed with the report of Miller *et al.* (1987).

Conclusion

The effects of the consortium of the three bacteria isolates on polycyclic aromatic hydrocarbons were studied. This research observed reduction of the PAH and concluded that the consortium of these bacteria can effectively be used for the decontamination of PAH-polluted sites.

References

Atlas, R.M. (1981). Microbial Degradation of Petroleum Hydrocarbons: An Environmental Perspective, *Microbiology Rev.*, 45: 180-209.

Atlas, R.M. (1977). Measurement of Hydrocarbon Biodegradation Potentials And Enumeration of Hydrocarbon-utilizing Microorganisms using Carbon 14 Hydrocarbon –spiked crude Oil In: Costerton, J.W. and Colwel, R.R. (eds). Native Aquatic Bacteria: Enumeration, Activity And Ecology, ASTM, Philcidelphia, PA.pp. 196-204.

Brock, T.D. (1974). Biology of Microorganisms. 2nd edition, Prentice- Hall, Inc England Cliffs, News Jersey pp. 66-69.

Bouchez, M., Blancher, D. and Vandecasteele J.P. (1995). Degradation of Polycyclic Aromatic Hydrocarbons by Pure Strains. Appl. Microbial. Biotechnol., 43: 156-164.

Buchanan, R.E. and Gibbons, N.E. (1974). Bergey's Manual of Determinative Bacteriology (8th ed.) Williams and Wilkings Co. Baltimore.

Cheesbrough, M. (2005). District Laboratory Practice in Tropical Countries. Part 1 (2nd ed), Cambridge University Press, U. K. pp.143-157.

Chikere, B. O. and Okpokwasili, G. C. (2003). Enhancement of biodegradation of petrochemicals by nutrient supplementation. Nig. J. Microbiol. 17(2):130-135.

Connan, J. (1986). Biodegradation of Crude Oils in Reservoirs. Microbial Problems in Off-Shore Oil Industry. In Hill, E.C, Scheman, J.I and Watkinson, R.J (Eds). Environmental Microbiology, 4th edition. Cambridge University Press, UK, pp 49-55.

Ekpo, M.A. and Ekpo, E.I. (2006). Utilization of Bonny -light and Bonny medium crude oils by microorganisms isolated from Qua Iboa River estuary. *Nig. J. Microbiol*. 20 (1): 832-839.

Jobson, A., Cook, F. D. and Westlake, D. W.S. (1972). Microbial utilization of crude oil. *Appl. Microbiol*. 23: 1082 – 1089.

Miller, R.M., Mill,B. and Joe, T. (1987). Biodegradation of Oil Polluted Soil. Academic Press, New York, Pp 97-103.

Okpokwasili, G. C. and Amanchukwu, S. C. (1988). Petroleum Hydrocarbon Degradation by *Candida.* Species. *Environ. International*. 14: 243 – 247.

Okpakwasili, G.C. and Nnorom, E.E. (1990). Microbial Degradation of Petroleum Hydrocarbon by Brackish Water Isolate. In Akpata.T.V.T and Okali, D.U.U. (Eds). Environmental Microbiology, 5th edition. Nigeria Wetlands Press. Pp 138-146.

Prescott, L.M, Herley, J.P and Klein,D.A. (2005). Microbiology, 6th ed. McGraw-Hill Publishers, New York, pp. 554-555.

Udeme, J.J. and Antai, S.P. (1988). Biodegradation and Mineralization of Crude Oil by Bacteria. Nig.J. Biotechnol. 5: 79-86.

Walker, J.D. and Colwell, R.R. (1977). Ecological Effects of Oil Pollution On Littoral Environment. Applied Science Publishers, London. Pp 152-158.

Wang, X., X. Yu, and Bartha, R. (1990). Effect of bioremediation on polycyclic aromatic

hydrocarbon residues in soil. Environ.Sci. Technol. 24:1086-1089.

Watkinson, R.J. (1978). Developments in Biodegradation of Hydrocarbons. Appl. Science 3: 146-148.

Zengler, T, Joel, G.T. and Larter, W. (1991). The Rate Hydrocarbon Absorption by Genera of Bacteria. African J. 2:174-179.