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Biosurfactant-enhanced remediation of hydrocarbon contaminated mangrove swamp

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ABSTRACT

Crude biosurfactant extract produced by two microbial isolates, *Pseudomonas mallei* and *Pseudomonas pseudomallei* were used to enhance the biodegradation rates of petroleum hydrocarbon pollutants in a mangrove swamp in Nigeria. Nutrient application in combination with biosurfactants showed very significant biodegradation rates of over 99% of the total petroleum hydrocarbon (TPH) which was removed after 3 weeks of exposure. Nutrient application alone without the biosurfactants only recorded about 53% remediation of TPH while biosurfactant application alone without nutrient supplement had about 84% of the TPH removed after 3 weeks of exposure. The control plot did not show any remarkable biodegradation even after the 5 weeks exposure period, an indication that natural biodegradation without any form of enhancement can be a very slow process. The experimental data and the results obtained showed that the crude biosurfactant extract from *P. mallei* and *P. pseudomallei* were very effective in enhancing the biodegradation rates in the mangrove swamp especially when supplemented with nutrients.

Keywords: Biodegradation, Petroleum hydrocarbons, Hydrocarbon utilizing bacteria.

INTRODUCTION

Biosurfactants are a structurally diverse group of surface active molecules synthesized by microorganisms. These molecules reduce surface and interfacial tensions in both aqueous and hydrocarbon mixtures which make them potential candidates for enhancing oil recovery and de-emulsification processes (Desai and Banat, 1997).

Biosurfactants are synthesized by different organisms and are grouped into 6 major classes based on the producing microorganism and these classes are; glycolipids, phospholipids, polysaccharide lipid complexes, lipoprotein-lipopeptide, hydroxyllated and crosslinked fatty acids and the complete cell surface (Kosaric et al., 1987).

Almost all surfactants currently in use in some developed countries like the United States of America are chemically derived from petroleum hydrocarbon (Desai and Banat, 1997). However interest in biological surfactants has been steadily increasing in recent times due to their diversity, environmental friendly nature, lower toxicity, biodegradability and the possibility of their production in large quantity through fermentation. Their potential applications in environmental protection, crude oil recovery, health care and food processing industries has also been reported (Desai and Banat, 1997). Although biosurfactants have several advantages over chemical surfactants, their applications and industrial usage is limited (Sakamoto et al., 2006).

The relevance of biosurfactants in biodegradation is based on the fact that hydrophobic pollutants present in petroleum hydrocarbons require solubilisation before degradation by the microbial cells. Biosurfactants can increase the surface area of hydrophobic pollutants in petroleum hydrocarbons and in soil and water environment thereby increasing their water solubility and this makes microbial degradation easy (Desai and Banat, 1997). Biosurfactants can also increase bioavailability by reducing surface tension, which is the key parameter that facilitates degradation (Sakamoto et al., 2006).

Microbial remediation of hydrocarbon and crude oil contaminated soils using biosurfactants to enhance the process is an interesting and very effective technology. The effectiveness of enhancing hydrocarbon degradation through addition of microbial inocula prepared from non-indigenous populations (bioaugmentation) has been ambiguous (McCray et al., 2007), however the addition of biosurfactants stimulated the indigenous bacterial populations to degrade the hydrocarbons at rates higher than those which could be achieved through addition of nutrients alone (Desai and Banat, 1997). In another study, it was discovered that Rhamnolipid from *P. aeruginosa* removed substantial quantities of oil from contaminated Alaskan gravel from the Exxon Valdez oil spill (Harvey et al., 1990). Barathi and Vasudevan (2001) and McCray et al. (2001) studied the removal of single and double components of petroleum hydrocarbons through soil washing using aqueous surfactant solutions and concluded that it is important to evaluate the characteristics of the contaminated soil such as soil particle size distributions, organic and inorganic material content. Kosaric (1992) demonstrated the effectiveness of biosurfactants in bioremediation of contaminated soil, control of oil spills and biodegradation and detoxification of industrial effluents. When compared with chemical surfactants, Urum and Pekdemir (2004) demonstrated that a biosurfactant, Rhamnolipids is as effective as a chemical surfactant Sodium Dodecyl Sulphate (SDS) in removing about 80% of petroleum hydrocarbon from contaminated soil. Other researchers like Amiriyan et al. (2004) have also isolated microorganisms from oil reservoirs with very high potential for biosurfactant production.

In this study, crude biosurfactant extract from two bacterial isolates from produced water effluents were used to enhance the bioremediation process of petroleum hydrocarbons in the mangrove swamp. Mangrove swamp was chosen because of its environmental sensitive nature and the rampant cases of oil spillages it receives from the oil exploration and production activities in the oil rich Niger Delta region of Southern Nigeria.

The main objective of this study is to test the biosurfactant producing ability of some indigenous microorganisms associated with produced water discharges in the mangrove swamp with the aim of using such microorganisms and their products for proper remediation of the mangrove swamp whenever there is significant hydrocarbon pollution.

MATERIALS AND METHODS

Study area

The study area is located within the Escravos mangrove swamp (Longitude E, 4° 28’ 14’ and Latitude 6° 33’ 16’) within Ugborodo town, Warri, Delta State, Nigeria. It is a nearshore environment with moderate salinity (860 mg/L) and the environment was tested for indigenous concentration of petroleum hydrocarbon by gas chromatographic analysis to ensure that it is pristine and have not received any form of crude oil pollution in the past.

Physicochemical analysis of the study area

pH, temperature measurement, salinity and estimation of Phosphorus

The pH of the mangrove swamp was measured with a portable water proof pH meter (Jenway, 3150, USA); temperature was measured using portable thermometer (Hanana, H1-93510, USA). Salinity was measured as chloride using the Argentometric method as earlier described in (Eaton et al, 1995). Persulphate digestion method was used to estimate phosphorus (Eaton et al., 1995).

Determination of biological oxygen demand

BOD bottles were filled with appropriate dilutions of the samples (50 ml) and the initial dissolved oxygen was

measured. The BOD bottles with samples were sealed to exclude air followed by incubation at 20 °C for 5 days after which the BOD was computed from the difference between the initial and the final dissolved oxygen (Eaton et al., 1995).

Detection of heavy metals

Heavy metals were detected using the Atomic absorption Spectrophotometer (Perlkin Elmer 5100PC, England) after sample preparation and digestion as previously described (Eaton et al., 1995).

Moisture content

The moisture content of the mangrove soil was measured by simple gravimetric analysis. 10 grams of the soil sample containing water was dried in the oven at a temperature of 200 °C after which, the sample was weighed again and the difference in weight is the moisture content as previously described (Eaton et al., 1995).

Experimental design

A 50 square meter portion of mangrove swamp was clearly demarcated with an oil resistant polythene lined in a wooden platform of about 40 inches deep from the surface into 5 equal segments otherwise referred to as plots in this study and the design was in such a way that fluid movement from one plot to another was totally restricted. The entire plot was polluted with 50 liters of crude oil (Escravos light, obtained from Chevron Nigeria Limited) at the rate of 10 liters per plot which was evenly distributed in all the plots and the set up was allowed to stand for 48hrs undisturbed. This was followed by the application of 2.5 liters of oleophylic fertilizer enclosed in a semipermeable membrane to plots A, B, and C and 1.5 liters of crude biosurfactant A extract to plot A, and the same volume of biosurfactant B extract to plot B. Crude biosurfactant extracts A and B were mixed in the ratio of 1:1 and about 1.5 liters of the crude extract was applied to Plot E. Plot D served as a control with crude oil application only without any form of treatment. Biosurfactant A is the crude biosurfactant extract from *P. mallei* while biosurfactant B is the crude biosurfactant extract from *P. pseudomallei*. Both bacterial isolated were from produced water which at the time of this study was constantly being discharged into the recipient water near the mangrove swamp. The entire set up was thoroughly mixed and homogenized to allow for even distribution of the nutrients and the surfactants and the experiment was allowed to stay for 5 weeks. At the end of each weekly interval, samples were collected and analyzed for total petroleum hydrocarbon (TPH) and hydrocarbon utilizing bacteria.

Growth and biosurfactant production

The two bacterial isolates used for biosurfactant production P. *mallei* and *P. pseudomallei* were grown on the minimal salt medium of Rosenberg et al. (1988) with diesel oil as the sole carbon source. The composition of the medium in g/L, is as follows, NaCl (5), $NaHPO_4$ (13.7), KH_2PO_4 (7.26), $(NH4)_2S0_4$ (3), $MgS0_4$ (0.4) and diesel (2 ml). The culture medium was contained in a 1000 ml Wheaton glass bottle which was covered with a non absorbent cotton wool and placed in a slanted position to allow air passage through the pores of the cotton wool. The bottles were shaken at regular intervals to allow adequate homogeneity of contents and the incubation was at room temperature. At the end of the 6 days of incubation, the supernatant which consists of the microbial cells and the crude biosurfactant were aseptically collected and the microbial cells were separated from the biosurfactant by centrifugation.

Enumeration of hydrocarbon utilizing bacteria

Hydrocarbon utilizing bacterial counts were obtained by plating out at low dilutions $10^{-1} – 10^{-3}$ of samples on mineral salt medium of Mills et al. (1978). The composition of the medium in (g/L) is as follows NaCl (10), $MgSO_4.7H_20$ (0.42), KCl (0.29), KH_2PO_4 (0.83), Na_2HPO_4 (1.25), $NaNO_3$ (0.42), Agar bacteriological (15), distilled water (1000 ml), and pH (7.2). The medium was autoclaved at 1.1 kg/cm^2 for 15 mins. The inoculated mineral agar plates were then inverted over sterile membrane filters moistened with crude oil (Escravos light) and held in the lid of the petri dishes. The dishes were wrapped round with a masking tape so as to increase the vapor pressure within the Petri dishes while the plates were incubated at 29 °C for 6 days after which the growth of hydrocarbon degrading bacteria were observed and counted.

Solvent extraction of residual oil

One gram of the sample was introduced into a separating funnel containing 50 ml of Methylene chloride, this was followed by vigorous shaking for 10 min and filtered using Watman no.1 filter paper as previously described (Eaton et al., 1995) and the filtrate was collected in a clean conical flask.

Gas chromatography of oils

Degraded oil were analyzed by Gas chromatography using Hewlett Packard 5890 series 11 Gas chromatograph equipped with single flame ionization detector (FID) fitted with Perkin Elmer Nelson analog digital converter (900 series) and a Compaq deskpro computer. A J and W scientific DB-1 capillary column of 15 m length and an internal diameter of 0.32 mm wide bore of 1micron film thickness were used. A temperature program of 50-305 °C increasing at 3.5 °C per minute for 27.15 min was employed. Hydrogen with a flow rate of 2ml per min was used as a carrier gas while the flow rate of air was 400ml per min. The detector temperature was 325 °C while the injection port temperature was 305 °C. 1 ml of the residual oil extract was dissolved in methylene chloride at the ratio of 1:1 and a sample volume of 0.2 µl was injected into the GC.

Statistical analysis

One way analysis of variance (ANOVA) and Duncan tests were used to test for significant differences (5% level) in the mean concentrations of Total Petroleum Hydrocarbons (TPH) and Population densities of indigenous hydrocarbon degraders in the various plots (A, B, C, D and E) and the analysis was performed with a computer statistical package SPSS 10 for windows.

RESULTS

Physicochemical Properties of the Mangrove swamp

The mangrove swamp where the experiment was performed had the following physicochemical properties before the commencement of the experiment.

Atmospheric temperature (32 °C), Swamp temperature (28 °C), pH (7.4) BOD_5 (60 mg/g), Salinity (860 mg/g), Phosphorus (3.30 mg/g), TPH (0.056 ppm), Pb (0.006 mg/g), Zn (0.00 mg/g), Cr(0.12 mg/g) and Cd (0.0076 mg/g) and moisture content(56%).

Effects of biosurfactant A and nutrient application on plot A

Plot A had an initial TPH concentration of 4600 ppm which was reduced to 3.60 ppm after 5 weeks of application of biosurfactant A and Nutrients, the population density of the indigenous hydrocarbon utilizing bacteria also rose from 6 $x10^4$ cfu/g at week 0 to a peak level of $426x10^4$ cfu/g at week 2, after which it declined to $14x10^4$ cfu/g at week 5. The mean concentrations of residual total petroleum hydrocarbons in the experimental plots are shown in table 1 while the mean population densities of indigenous hydrocarbon degrading bacteria in the experimental plots are shown in table 2. The GC chromatograms of residual TPH concentrations in plot A are shown in Figure 1.

Effects of biosurfactants B and nutrient application on plot B

The initial TPH concentration in Plot B was 4208 ppm, which was reduced to 4.30 ppm after 5 weeks of application of nutrients and biosurfactant B. The population density of indigenous hydrocarbon degrading bacteria rose from $22x10^4$ cfu/g at week 0 to a peak level of $310x10^4$ at week 2, after which it declined to $23x10^4$ cfu/g at week 5. The mean concentrations of residual total petroleum hydrocarbons in the experimental plots are shown in table 1 while the mean population densities of indigenous hydrocarbon degrading bacteria in the experimental plots are shown in table 2. The GC chromatograms of residual TPH concentrations in plot B are shown in Figure 2.

Effects of nutrient applications alone without biosurfactant on plot C

The initial TPH concentration before Nutrient application was 4870ppm which was reduced to 48ppm after 5 weeks of application of Nutrients. The population density of indigenous hydrocarbon degrading bacteria rose from $12x10^4$ cfu/g at week 0 to $248x10^4$ cfu/g at week 2, after which it declined to $32x10^4$ cfu/g at week 5. The mean concentrations of residual total petroleum

Table 1: Mean Concentrations of residual Total Petroleum Hydrocarbons (TPH) in the experimental plots.

PLOTS	Mean TPH CONC.(PPM) Week 0 ± SD	Mean TPH CONC.(PPM) Week-1 ± SD	Mean TPH CONC.(PPM) Week-2 ± SD	TPH CONC.(PPM) Week-3 ± SD	TPH CONC.(PPM) Week-4 ± SD	TPH CONC.(PPM) Week-5 ± SD
A	4600 ±56.69^{A}	1650 ±42.42 ^{B}	110 ± 14.14^{A}	36 ± 2.83^{A}	5.80 ± 0.14^{A}	3.60 ± 0.28 ^{A}
B	4208 ± 11.31^{A}	1200 ± 70.7^{A}	146 ± 4.24^{A}	38 ± 2.83^{A}	6.50 ± 0.42^{A}	4.30 ± 0.42^{A}
C	4870± 42.42^{A}	3200 ±73.13^{C}	2520 ± 28.28^{C}	2300 ± 14.14 ^{C}	120 ± 28.28^{C}	48 ± 2.82^{B}
D	4685 ± 7.07^{A}	3476 ±8.48^{D}	3470 ± 42.43 ^{D}	2480 ± 28.28^{D}	2460 ± 14.14^{D}	2340 ± 14.14^{C}
E	4650 ± 70.7 ^{A}	3680 ±84.8^{E}	2180 ± 14.14^{B}	760 ± 28.28^{B}	46 ± 2.82^{B}	28 ± 9.90^{B}

*Means with the same superscript letter(s) in a column are not significantly different in the Duncan tests (at P=0.05), ± SD = Standard deviation at n=2. * ANOVA TEST: P<0.05 (Significant), P>0.05 (Not significant), n=2.
PLOT A: TPH Concentration after treatment with Nutrients and Biosurfactant A
PLOT B: TPH Concentration after treatment with Nutrients and Biosurfactant B
PLOT C: TPH Concentration after treatment with Nutrients ONLY
PLOT D: TPH Concentration without treatment (Control Plot)
PLOT E: TPH Concentration after treatment with Biosurfactants A and B

Table 2: Mean Population Densities of Indigenous Hydrocarbon Degrading Bacteria in the Experimental Plots.

PLOTS	MBPD {cfu/g x 10^4} ± SD	MBPD {cfu/g x 10^4} ± SD	MBPD {cfu/g x 10^4} ± SD	MBPD {cfu/g x 10^4} ± SD	MBPD {cfu/g x 10^4} ± SD	MBPD {cfu/g x 10^4} ± SD
	Week-0	Week-1	Week-2	Week-3	Week-4	Week-5
A	6 ± 2.83 ^{A}	138 ± 11.31^{C}	426 ± 8.48 ^{D}	110 ± 7.07^{B}	32 ± 2.82^{A}	14 ± 1.41^{A}
B	22 ± 8.48^{A}	164 ± 8.48^{D}	310 ±14.14^{C}	86 ± 5.65^{B}	46 ± 1.41^{A}	23 ± 7.07^{AB}
C	12 ± 2.82^{A}	128 ± 5.65^{C}	248 ± 9.90^{B}	214 ±19.80^{C}	88 ± 9.90^{B}	32 ±2.82^{B}
D	14 ± 5.65^{A}	08 ± 2.82^{A}	26 ± 5.65^{A}	38 ± 7.07^{A}	48 ± 14.14^{C}	86 ± 2.82^{C}
E	26 ± 5.65 ^{A}	86 ± 1.41 ^{B}	266 ± 8.48 ^{B}	386 ± 2.82^{D}	158 ± 9.90^{D}	108 ±11.31^{D}

*Means with the same superscript letter(s) in a column are not significantly different in the Duncan tests (at P=0.05) , ± SD = Standard deviation at n=2. * ANOVA TEST: P<0.05 (Significant), P>0.05 (Not significant),
PLOT A: Population Density of Indigenous Hydrocarbon Utilizing Bacteria after Treatment with Nutrients and Biosurfactant A
PLOT B: Population Density of Indigenous Hydrocarbon Utilizing Bacteria after Treatment with Nutrients and Biosurfactant B
PLOT C: Population Density of Indigenous Hydrocarbon Utilizing Bacteria after Treatment with Nutrients Only
PLOT D: Population Density of Indigenous Hydrocarbon Utilizing Bacteria without Treatment (Control Plot)
PLOT E: Population Density of Indigenous Hydrocarbon Utilizing Bacteria after Treatment with Biosurfactants A and B
MBPD = Mean Bacterial Population Density.

hydrocarbons in the experimental plots are shown in table 1 while the mean population densities of indigenous hydrocarbon degrading bacteria in the experimental plots are shown in table 2. The GC chromatograms of residual TPH concentrations in plot C are shown in Figure 3.

Effects of natural biodegradation on plot D

Plot D served as control without any form of treatment. Both the hydrocarbon degradation and the bacterial growth rates were very slow as can be seen in tables 1 and 2, the GC Chromatograms also showed little or no degradation as can be seen in Figure 4.

Effects of biosurfactant applications alone without nutrient supplement on plot E

Plot E had an initial TPH concentration of 4650 ppm which was reduced to 28 ppm after 5 weeks of application of biosurfactant A and B. The population density of indigenous hydrocarbon degrading bacteria also rose from $26x10^4$ cfu/g to a peak level of 386 $x10^4$ cfu/g at week 3 and declined to 108 x 10^4 cfu/g at week 5 as shown in tables 1 and 2.

DISCUSSION

It is noteworthy that mangrove swamp plays a very vital role in the ecosystem by providing nurseries, shelter and feeding grounds for many commercially important

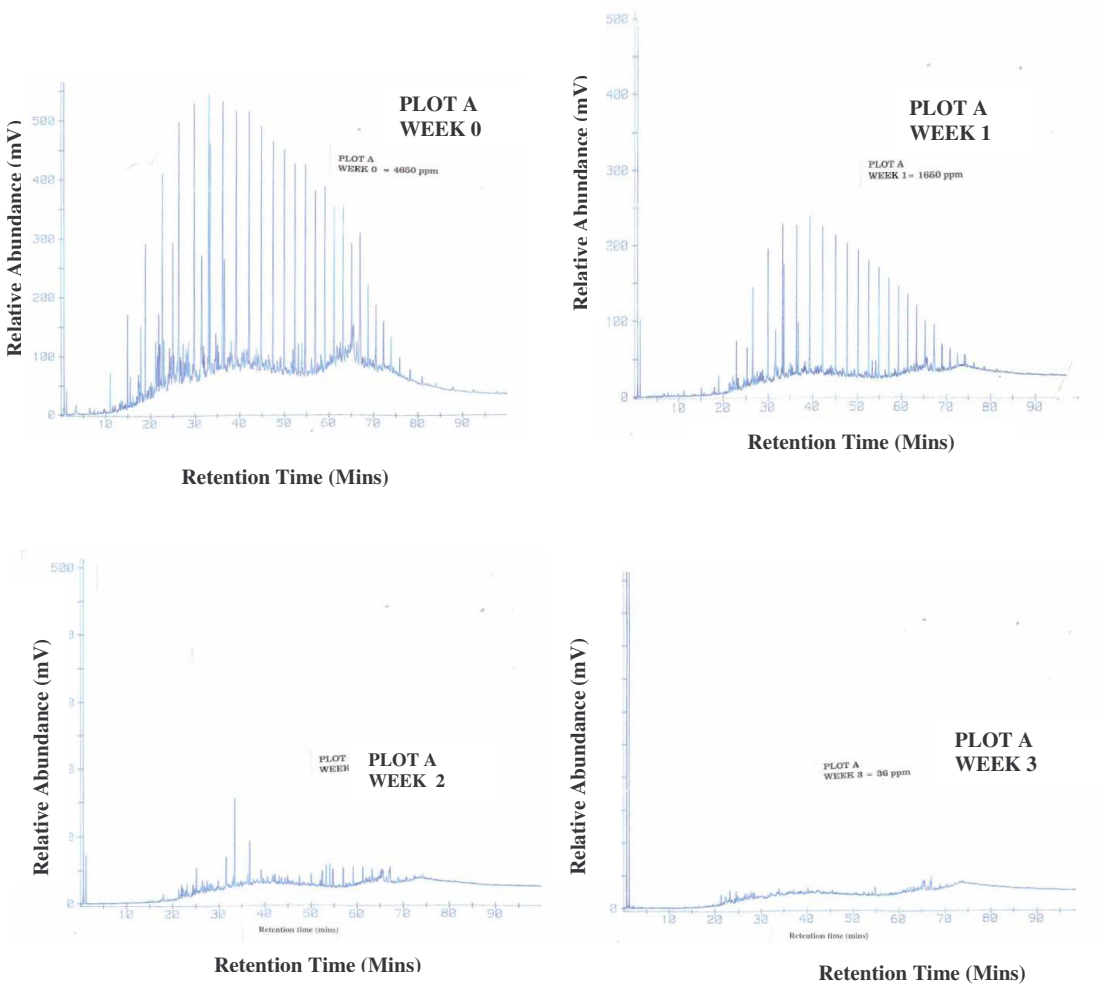

Figure 1: GC chromatograms of residual TPH concentrations in plot A.

species of fish and crustaceans. The silt roots, lower trunks and mud surface usually support a varied fauna of oysters, snails, crabs and other invertebrates. The upper parts of the mangrove trees are an essential terrestrial environment with varied fauna of birds, mammals and insects. All these factors indicate that the mangrove swamp is a very unique and essential environment that must be protected from the devastating effects of oil pollution.

Biosurfactants were chosen for the remediation program because the use of chemicals for the treatment of hydrocarbon polluted site may contaminate the environment with their bye products whereas biological treatment will efficiently destroy pollutants while they are biodegradable and, moreover, interest in microbial surfactants has been steadily increasing in recent years due to their diversity, environmentally friendly nature, possibility of large scale production, selectivity, performance under extreme conditions and potential application in environmental protection (Banat et al., 2000; Rahman et al., 2002).

Biosurfactants enhance the emulsification of hydrocarbons because they have the potential to solubulize hydrocarbon contaminants and increase their availability for microbial degradation, this process is very essential for any significant biodegradation to occur in the mangrove swamp. In the mangrove swamp of the Nigerian Niger Delta, crude oil pollution is very rampant and the

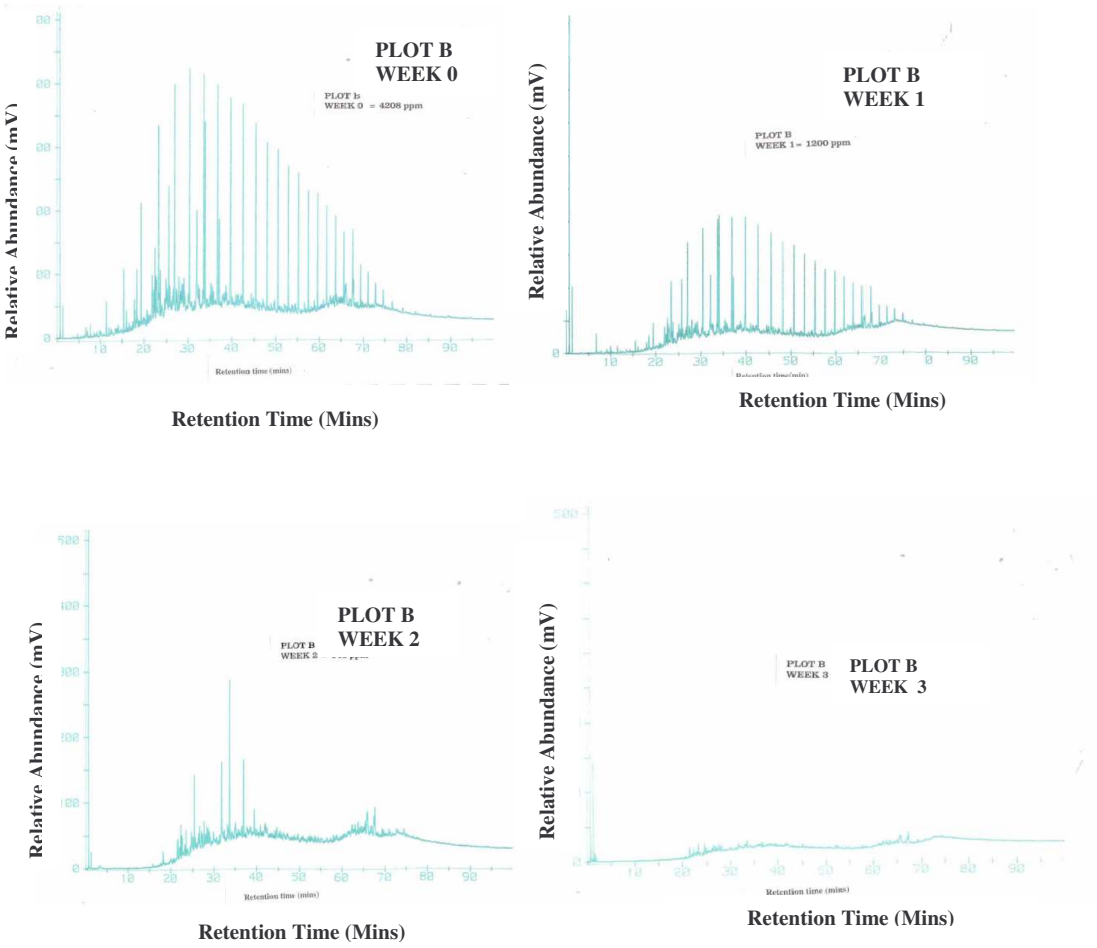

Figure 2: GC chromatograms of residual TPH concentrations in plot B+.

volume of pollution usually comes in very unimaginable proportions and in most cases the availability of petroleum hydrocarbons to microorganisms for degradation is usually difficult due to the nature of the mangrove swamp. Microbial surfactants are therefore required to facilitate microbial degradation in the mangrove swamp.

Several microorganisms are known to synthesize a wide range of biosurfactants which can emulsify hydrocarbon substrate and facilitate its transport into the cells. The two microbial isolates used in the present study; *P. mallei and P. pseodomallei* were isolated from produce water which was constantly being discharged into the Escravos river which flows directly into the mangrove swamp. Some earlier studies on these microorganisms showed that they have great potential for production of biosurfactant which was earlier classified as Glycoproteins (Okoro et al., 2002). Okoro (1999) also reported that the biosurfactant produced by these two microbial isolates are closely related as they contain both protein and carbohydrate moieties of very close molecular weight with no traces of lipid. Since these microorganisms are already familiar with the mangrove swamp environment and have great potential for biosurfactant production, an attempt was made to practically use the crude biosurfactant produced by these organisms to enhance the remediation of hydrocarbon contaminated mangrove swamp. In Plot A and B, where biosurfactants and nutrient application were used, over 99% of the residual TPH concentration was removed after 3 weeks of treatment. A similar trend was applicable to Plot E where only biosurfactants were applied without nutrients, about 83.7% of the residual

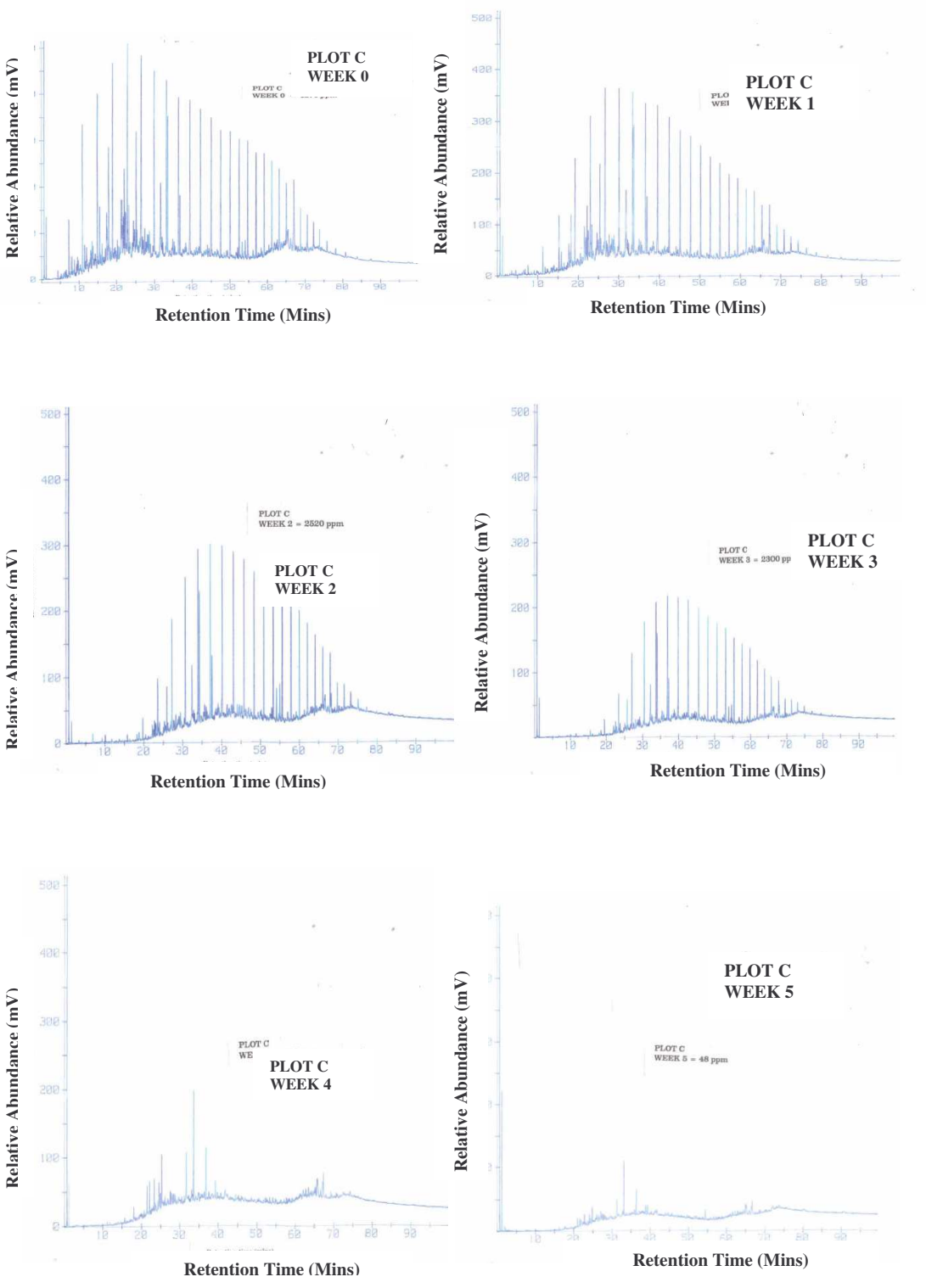

Figure 3: GC chromatograms of residual TPH concentrations in plot C.

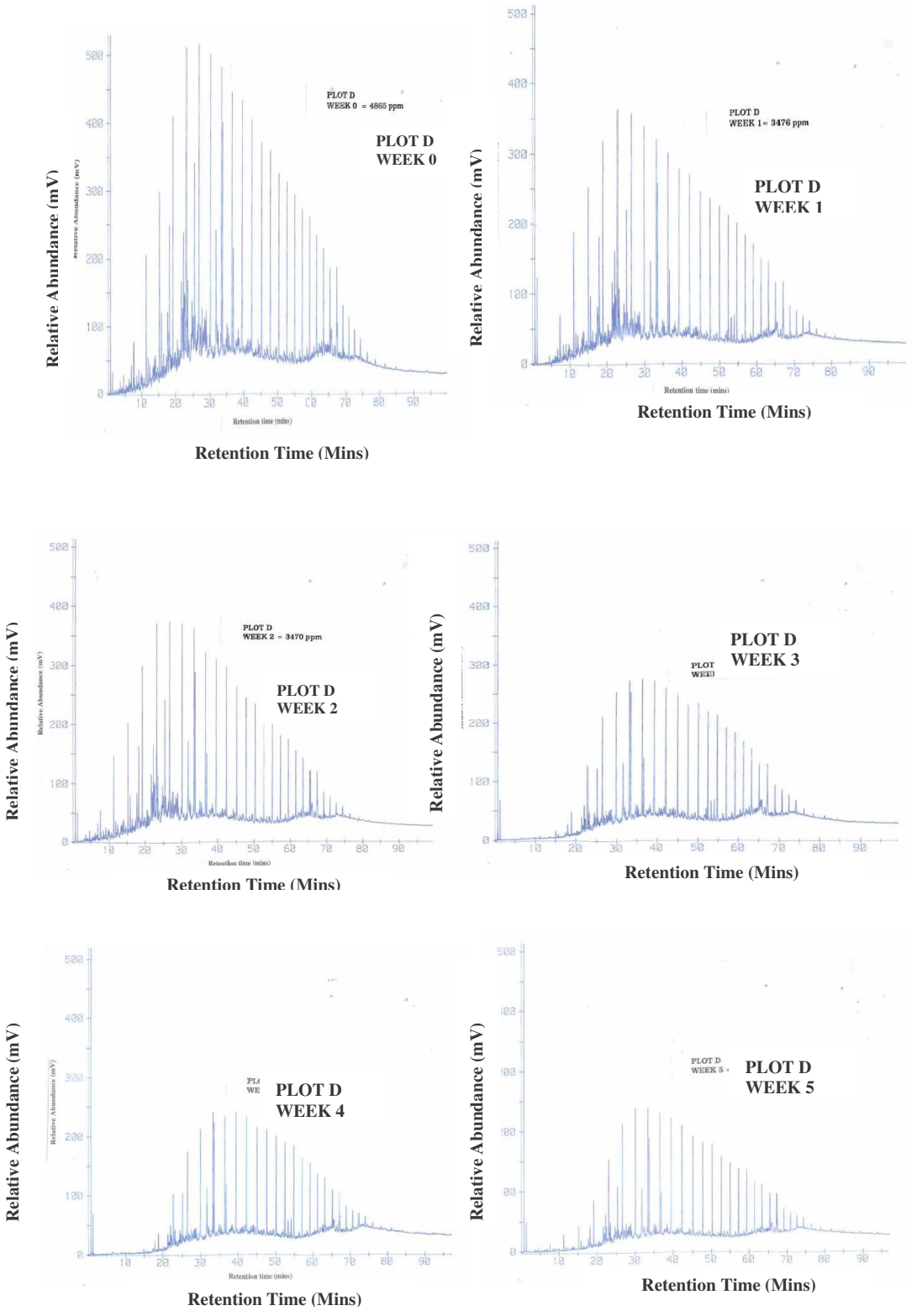

Figure 4: GC chromatograms of residual TPH concentrations in plot D.

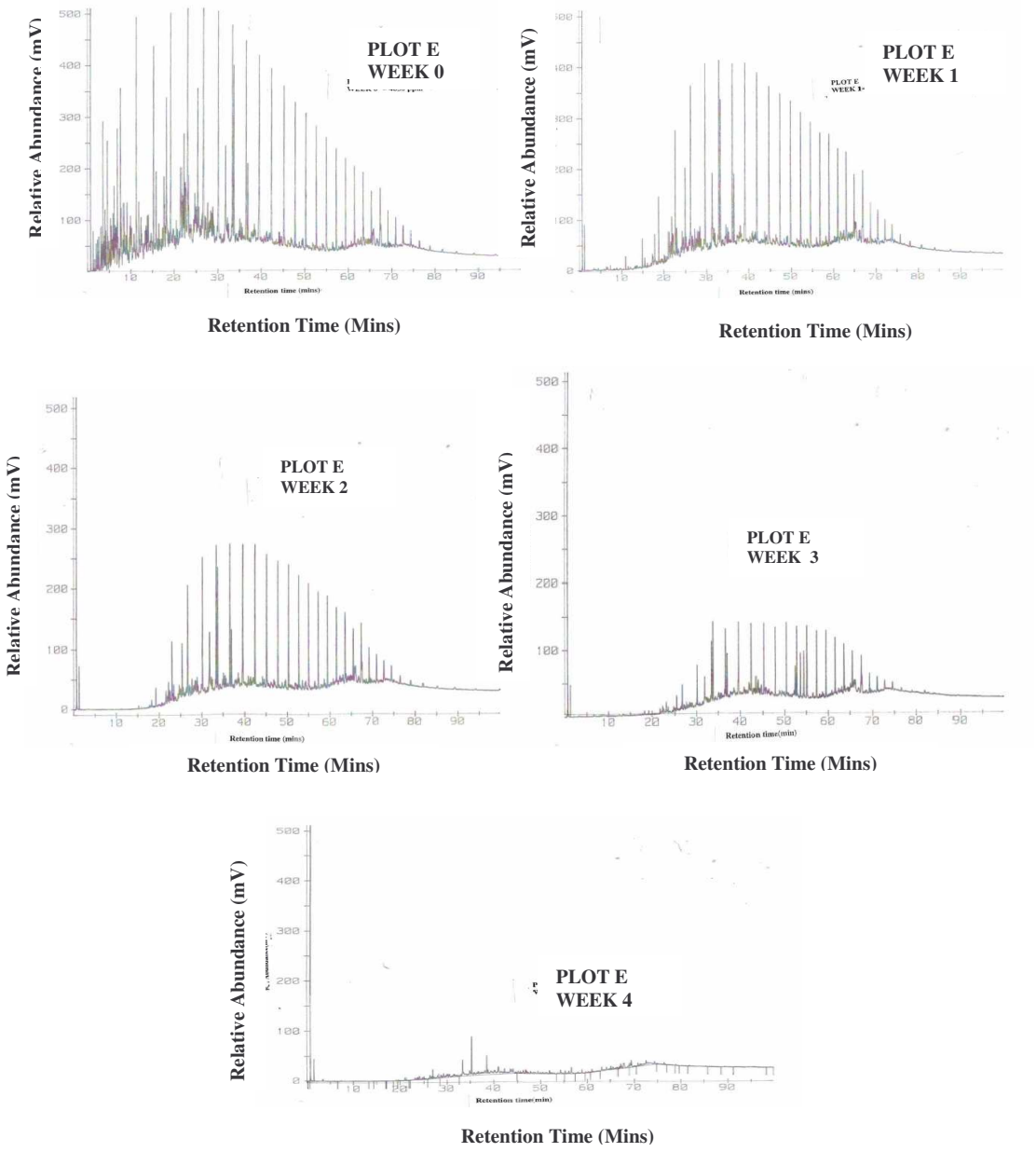

Figure 5: GC chromatograms of residual TPH concentrations in plot E.

TPH concentration was removed after 3 weeks of treatment. Comparatively, the results observed in Plot E was better than what was observed in Plot C where only nutrient application was used as only about 52% of the residual TPH concentration was removed after 3 weeks of treatment. In Plot D that served as control, a very slow biodegradation rate was observed throughout the 5 weeks period the experiment lasted. Only about 52% of the residual TPH concentration was removed at the end of the fifth week when the experiment was terminated.

It is evident from the results that both the surfactants and the nutrients played very active roles in petroleum hydrocarbon degradation but the role of the surfactant was more evident as can be seen in the results from Plots C and E. When a pure biosurfactant extract, Rhamnolipid was used

by Urum and Pekdemir (2004) in oil removal and the result was compared with a chemical surfactant SDS, both the biosurfactant and SDS were able to remove about 80% of the oil. However in the present study, the approach was different because biosurfactant was used to complement the activities of indigenous hydrocarbon degraders and over 99% of the original TPH concentrations were removed after a relatively short period of 3 weeks. In a similar study carried out by Harvey et al., 1990, a microbial surfactant produced by a *Pseudomonas sp.* was used to enhance the remediation of spilled oil from Alaskan gravel after the Exxon Valdez oil spill in 1989. Similarly, the biosurfactant used in their study complemented the degradation potential of the indigenous hydrocarbon degrading bacteria originally present which led to a faster degradation process of the petroleum pollutants.

In case of large scale production of biosurfactants, the substrate used in this study, diesel oil may not be economical. Industrial wastes such as mollases will be preferable as a cheaper substrate for large scale biosurfactant production and secondly using industrial wastes will help to alleviate many processing industrial waste management problems (Maneerat, 2005).

Conclusion

Conclusively, this study has demonstrated that microbial surfactants from *P. mallei* and *P. Pseudomallei* can be used to enhance the biodegradation rates in the polluted mangrove swamp and the enhancement was faster when a combination of nutrients and biosurfactants were used to facilitate the activities of indigenous hydrocarbon degrading microorganisms already present in the mangrove swamp. However an alternative substrate such as mollases is being suggested for large scale biosurfactant production and its effectiveness will depend on how well the biosurfactant producing microorganisms can grow and proliferate in it. This is another promising area for further research.

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