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Bioaugumentation of Crude Oil Contaminated Soil Using Bacterial Consortium

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Crude oil utilizing bacteria were isolated from crude oil polluted soil collected in Ekpan-Warri, Delta State, Nigeria. They were identified as species of *Pseudomonas*, *Bacillus*, *Streptococcus* and *Micrococcus*. These organisms utilized Agurra (Nigeria) Light crude oil as a source of carbon and energy at varying rates and formed unstable and less stable emulsion in the oil medium. Based on their high ability of utilization of the crude oil, three bacterial strains *Bacillus megaterium*, *Pseudomonas putida* and *Pseudomonas aeruginosa* were put together into a mixed bacterial culture (MBC) to decontaminate soil intentionally polluted with 30% (v/w) crude oil. The results revealed that the bioaugmentation caused changes in the pH of the soil and in the CO_2 evolution. The pH of the amended oil polluted soil ranged from 6.15 to 7.66 while that of the unamended oil polluted soil ranged from 6.57 to 7.12. 68.2 mg CO_2 was liberated in amended soil as compared to 26.4 mg CO_2 liberated in unamended soil after 16 days. GC-MS analysis of the residual oil revealed that the oil components were more extensively degraded in the soil amended with the mixed bacterial culture than the unamended soil. The resistant phytane and pristane were equally attacked in the soil. The results suggest that a consortium of *Bacillus megaterium*, *Pseudomonas putida* and *Pseudomonas aeruginosa* can be useful in reclaiming crude oil polluted soil in the tropics.

KEYWORDS: Bioremediation, Bacteria, Consortium, Bioaugmentation, Crude Oil.

1. INTRODUCTION

Crude oil is an extremely complex mixture of aliphatic and aromatic hydrocarbons, including volatile components of gasoline, petrol, kerosene, lubricating oil and solid asphaltene residues.[1] In developed and developing countries, contamination of soil and marine environment by crude oil and petroleum products has become a serious problem. The main sources of this are natural oil seepage and human activities including extraction, transportation, utilization of petroleum (crude oil and natural gas), oil field installations, petroleum plants (refining), liquid fuel distribution and storage devices, transportation equipment for petroleum products and illegal drillings in pipelines. The scale of the hazards imposed on the natural environment depends on the surface of the area contaminated by the petroleum products, their chemical composition, and the depth at which pollutants occur.[1–4]

Crude oil causes a variety of risks when released into the environment. It is physically, chemically and biologically harmful to soil because of the presence of many toxic compounds, such as polycyclic aromatic hydrocarbons (PAH), benzene and its substituted and cycloalkane rings, in relatively high concentrations. Under natural environment, crude oil pollution results in an increased percentage of organic carbon and a decreased percentage of phosphorus.[5,6] These effects lead to an alteration of the ecological equilibrium such as a change in biodiversity and soil biomass, and an alteration of the soil physicochemical status. Abandonment of such lands is the consequence, which leads to a reduction of the productive land area available to rural farmers in such zones. Oil spills disrupt the functions of the ecosystem, such as respiration and the nitrogen (N) cycle.[1,7] In addition, oils contain ingredients that are toxic to flora and fauna as well as to human health.[1,4,6,8,9] Crude oils have toxic, carcinogenic and mutagenic properties.[10,11] Workers exposed to hydrocarbons are known to be prone to scrotal cancer and a high level of exposure to PAHs can also lead to lung cancer, prostate cancer and kidney cancer Moreover, spilled oil damages and destroys the infrastructure and contaminates the landscape.[12]

The undesirable ecological and socioeconomic effects associated with oil spills have led to the development of remediation techniques aimed at reducing the adverse effects of oil in the environment.[2]

Biodegradation of hydrocarbon compounds is one of the most important processes involved in the weathering and

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eventual removal of oil from the environment, particularly for its non-volatile components. Thus, biodegradation can be used for the recovery of sensitive areas such as contaminated shorelines, marshes, and wetlands.[1] Bioremediation is a process in which microorganisms are used to degrade or transform contaminants to less toxic or nontoxic forms.[4] Microorganisms break down hazardous wastes and toxic materials and convert them into environmentally friendly end products usually carbon dioxide, water and biomass.[4] Bioremediation of soil contaminated with crude oil has been considered as a cost-effective technology.[13] The success of bioremediation highly depends on the presence of microorganisms with biodegrading capability.[14] If communities native to polluted sites lack significant populations of hydrocarbon degraders, microbes with the desired qualities can be added exogenously in a process known as bioaugmentation. This approach has been successfully used to remediate a wide range of waste products, from hydrocarbons to heavy metals.[15] Pure microbial strains or microbial consortiums for bioaugmentation process can be obtained from contaminated and pristine sites.[12, 16, 17] Due to the microbial complexity and diversity, a microbial consortium could work better and more stable than the pure culture for the bioremediation of crude oil contaminated soil. This could be effective mainly because crude oil is a complex mixture consisting of aliphatics, aromatics, resins and asphaltenes.[3, 18]

The activation of natural degradation potentials in environmental media is currently the challenge in the environmental research addressed to remediation methods. Ways to activate these potentials must consider that most degradation potentials are widely distributed among microorganisms,[19] but indigenous microbes are usually present in very small numbers. Bioaugmentation offers a way to provide specific microbes in sufficient number to complete the biodegradation.

The aim of this study was to enhance biodegradation of crude oil in the soil with a consortium of three bacteria isolated from oil polluted soil. Our concern has been to find a suitable microbial consortium for reclaiming oil polluted soil in oil producing areas of Nigeria.

2. MATERIALS AND METHODS

2.1. Collection of Samples

The soil sample from which the crude oil utilizing bacteria were isolated was collected at Ekpan–Warri, Delta State, Nigeria. The crude oil used was Agurra (Nigeria) light crude oil collected at the Warri Refining and Petrochemical Company (WRPC), Warri, Delta State, Nigeria. The soil sample used in the laboratory for biodegradation and bioaugmentation studies was collected from a farmland traversed by petroleum pipelines in Minna, Niger State, Nigeria.

2.2. Isolation of Crude Oil Degrading Bacteria

One hundred and fifty millilitres (150 ml) of mineral salts medium[20] contained in 250 ml capacity conical flask was sterilized by autoclaving at 121 °C for 15 minutes. After cooling to about 45 °C, 5 g of the soil sample was added. It was swirled for about 15 minutes to enhance homogeneity and then incubated at room temperature (28 ± 2 °C) with intermittent shaking at 250 rpm for 5 days. One millilitre was withdrawn from the enriched sample into a test-tube containing 9 ml of sterile distilled water. 0.5 ml of the enriched soil suspension of various diluents was inoculated into oil agar.[16] The plates were incubated at room temperature (28 ± 2 °C) for 5 days. Colonies, which developed on the plates were picked and subcultured repeatedly to obtain pure cultures.

2.3. Characterization and Identification of Isolates

The bacterial isolates were characterized based on their gram stain reaction and biochemical tests, including carbohydrate utilization profiles. The isolates were identified using the scheme of Bergey's Manual.[21]

2.4. Utilization of Crude Oil by Bacterial Isolates

The utilization of crude oil as a source of carbon and energy by the bacterial isolates was determined by the method of Okpokwasili and Okorie[22] using the mineral salts medium (MSM) of Zajic and Supplisson.[20] Nutrient broth grown culture of each isolate was inoculated into each test tube containing 5 ml of sterile MSM and 0.05 ml of crude oil. Control test tubes were set up without being inoculated with organism. The test tubes were incubated at room temperature without shaking for 14 days. The growth of the organism in the oil medium at the end of incubation was determined by visual examination of the turbidity of the oil medium.

2.5. Determination of Crude Oil Biodegradation by Bacterial Isolates

Mineral salts medium (5 ml) was dispensed into bottles with the addition of 0.05 ml of crude oil into each bottle and the mixture was sterilized by autoclaving at 121 °C for 15 minutes. When cooled, each bottle was inoculated with 1 ml (10^6 cells) of the nutrient broth grown culture of each of the three bacterial isolates as well as of the mixed culture of the organisms *(Bacillus megaterium*, *Pseudomonas putida* and *Pseudomonas aureginosa*) and swirled. The bottles were incubated at room temperature (28 ± 2 °C) with shaking at 250 rpm using an orbital shaker (SGM-300, Gallenkamp, England) for 16 days. Control bottles were set up (uninoculated). After every 4 days, the residual crude oil was extracted using diethyl ether. The oil solvent mixture was decanted into a container of known weight and allowed to evaporate overnight leaving the residual crude oil in the container. The weight of the container with

the residual oil was measured (Ijah and Ukpe).[23] The percentage of oil degraded was calculated using the formula:

$$\text{Biodegradation in } \% = \frac{\text{weight of oil (control)} - \text{weight of oil (degraded)}}{\text{weight of oil (control)}}$$

2.6. Determination of the Effect of Addition of the Bacterial Consortium to Oil Polluted Soil

Two hundred grams of soil was treated with 60 ml of crude oil (30% v/w) and the mixed bacterial culture *(Bacillus megaterium*, *Pseudomona putida* and *Pseudomonas aeruginosa*). The experiment was set up in duplicates in pots. The pots with their contents were incubated at room temperature (28 ± 2 °C). Control experiments were also set up without inoculation with the bacterial consortium. The pH of the oil polluted soil and the carbon dioxide (CO_2) evolution due to inoculation with the bacterial consortium were determined after every 4 days for a total duration of 16 days. The gas chromatographic analysis of the residual oil was also carried out.

2.7. pH Determination

The pH of the amended soil was determined by suspending 10 g of the soil sample in 25 ml of distilled water in a beaker, swirled and allowed to stand for 10 minutes. The pH meter was standardized with buffer solution of pH 4 and 7. The pH of the soil samples was determined by inserting the pH probe in the solution and noting the reading. The pH of the control soil samples was also determined.

2.8. Carbon Dioxide Determination

Carbon dioxide (CO_2) production in the treated samples was determined and calculated by the methods described by Cornfield[24] and Stotzky.[25] In the treated soil samples, 0.5 g of Barium peroxide with 5 ml of distilled water were introduced into plastic vials and placed on the soil surface in screw-capped bottles to absorb the CO_2 liberated during oil degradation. This experiment was set up for oil polluted soil (control) and oil polluted soil plus mixed bacterial culture *(Bacillus megaterium*, *Pseudomonas putida* and *Pseudomonas aeruginosa*). At the end of every 4 days of incubation period, the vials containing $BaCO_3$ and BaOH were washed with 40 ml of distilled water in a 250 ml capacity conical flask and the residual BaOH titrated with 1 N hydrochloric acid (HCl) using phenolphthalein as indicator. The amount of CO_2 produced was calculated by the formula of Stotzky,[25]

$$\text{amount of } CO_2 = (B - V)NE$$

where V = volume (ml) of acid used to titrate the alkali in the CO_2 collectors from treatment to end point, B = Volume (ml) of acid used to titrate the alkali in CO_2 collectors from control to end point, N = Normality of the acid and E = Equivalent weight, if data are expressed as CO_2, $E = 22$.

2.9. Determination of Crude Oil Biodegradation by Gas Chromatography

The residual crude oil from the soil sample was extracted using diethyl either. This was done by suspending 10 g of soil in 20 ml of diethyl either in a 100 ml capacity beaker. The beaker was shaken vigorously to extract the oil. The solvent oil mixture was exposed to allow the solvent to evaporate completely. The residual oil was collected in a McCartney bottle for GC analysis. One micro litre (1 μl) of the extractable crude oil was diluted with 1 μl of pentane and analysed on a 25-m cpsi15CB capillary column (Chrompack, The Netherlands), installed in a capillary gas chromatograph (Packard instruments, Delft, The Netherlands) equipped with a flame ionization detector (FID). A split injector was used with helium as carrier gas. The oven temperature was initially set at 45 °C for 2 minutes and increased at a rate of 10 °C 1minute to 280 °C.

3. RESULTS AND DISCUSSION

The bacterial isolates in the soil were identified as species of *Bacillus, Pseudomonas, Streptococcus* and *Micrococcus*, as shown in Table I. The bacterial isolates had the following frequencies of occurrence: *Pseudomonas* (53.3%), *Bacillus* (33.3%), *Streptococcus* (6.7%) and *Micrococcus* (6.7%). Microorganisms are the main degraders of petroleum hydrocarbons in contaminated ecosystems because hydrocarbons are a excellent growth substrate for many microorganisms. These organisms have been implicated in crude oil biodegradation by several investigators.[26–29] The growth of these bacteria in the oil medium indicated their utilization of crude oil as a sole source of carbon and energy. Three of the bacterial isolates, *Bacillus megaterium*, *Pseudomonas putida* and *Pseudomonas aeruginosa* from the crude oil polluted soil were found to effectively degrade crude oil. This may be due to the efficient hydrocarbon-degrading enzyme system that these organisms possess. The identification of these species is in

Table I. Utilization of crude oil by bacterial isolates.

Isolates	Utilization of crude oil after 14 days
Bacillus megaterium	++
Bacillus subtilis	+
Bacillus cereus	+
Pseudomonas pseudomallei	+
Pseudomonas fluorescens	+
Pseudomonas putida	++
Pseudomonas aeruginosa	+++
Streptococcus faecalis	+
Micrococcus luteus	+

Notes: +++: Maximum growth; ++: Moderate growth; +: Minimal growth.

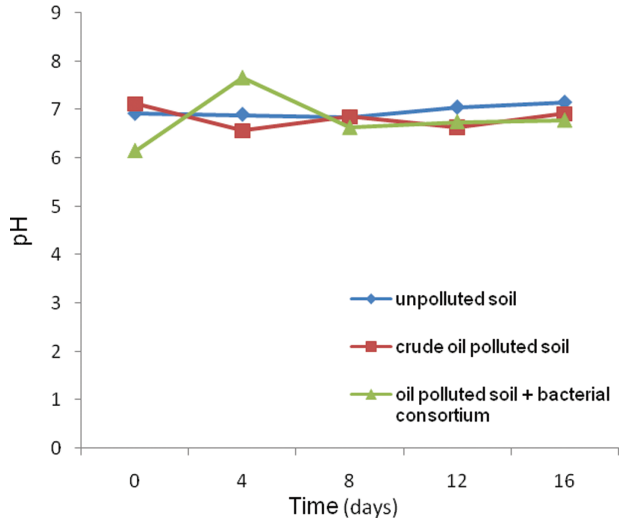

Fig. 1. pH of oil polluted soil amended with bacterial consortium.

line with previous investigations carried out by Abioye et al.[30] The isolates that utilized the crude oil at maximum and moderate rates were chosen for the bioaugmentation study.

The pH of the soil contaminated with oil and inoculated with a consortium of *Bacillus megaterium*, *Pseudomonas putida* and *Pseudomonas aureginosa* is presented in Figure 1. The pH values obtained ranged from 6.85 to 7.15 in the uncontaminated control soil while it ranged from 6.64 to 7.12 in oil polluted soil. The pH of the oil polluted soil amended with mixed bacterial culture ranged from 6.15 to 7.66 (Fig. 1). Higher pH values were observed in inoculated soil than in uninoculated soil. This could be due to accumulation of acidic metabolites caused by the degradation of the crude oil by soil microorganisms.[22] Since strong acidity is a limitation in biodegradation, the alkaline pH would have contributed to the enhanced crude oil degradation in the soil since crude oil degrading bacteria grow and utilize hydrocarbons better at slightly alkaline pH.[30, 31]

The CO_2 production in soil treated with crude oil and mixed bacterial culture is presented in Figure 2. The CO_2 production increased after 4 days, particularly in bacterial amended soil. 65.3 mg of CO_2 was produced in oil polluted soil amended with bacterial culture as compared to 46.4 mg of CO_2 liberated in unamended oil polluted soil. The values were significantly different ($P < 0.05$). This reflects greater oil biodegradation in that soil. It has been reported that breakdown of oil results in CO_2 and water.[27, 28]

The percentage of the oil degraded by the bacterial isolates and the mixed culture is presented in Figure 3. The rates of oil degradation by the organisms increased gradually from the 4th to the 16th day. The results revealed that the mixed bacterial culture caused the highest degradation (80.5%) of the oil followed by *Pseudomonas aureginosa* (65.6%) after 16 days. The least biodegradation rate (40.8%) was caused by *Bacillus megaterium*. *Pseudomonas putida* degraded 50.5% of the crude oil after 16 days. The high rate of degradation of crude oil by the bacterial consortium could have been due to the synergistic action of the microbes in the mixture. Since crude oil is a complex mixture consisting of aliphatic, aromatic, resins and asphaltenes, the complex and diverse nature of a microbial consortium could work better and more stable than a pure culture for the biodegradation of crude oil.

The chromatographic analysis of the crude oil revealed that the undegraded oil had many hydrocarbons of varying peaks (Fig. 4). About twenty different peaks representing different compounds (mostly alkanes) were

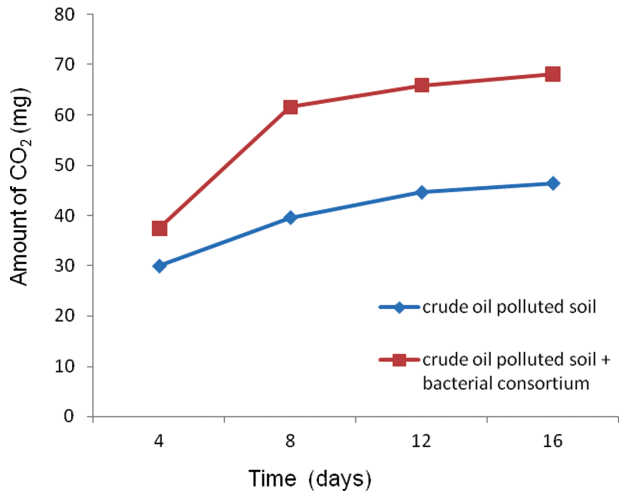

Fig. 2. Carbon dioxide production in oil polluted soil amended with bacterial consortium.

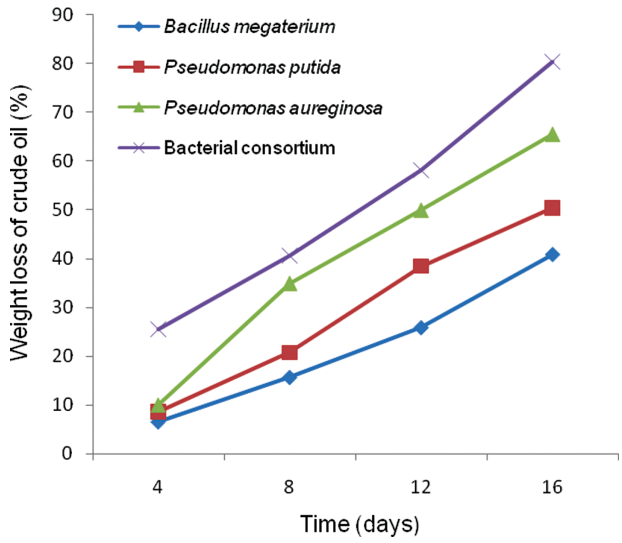

Fig. 3. Biodegradation of crude oil by bacteria and a consortium of the isolates.

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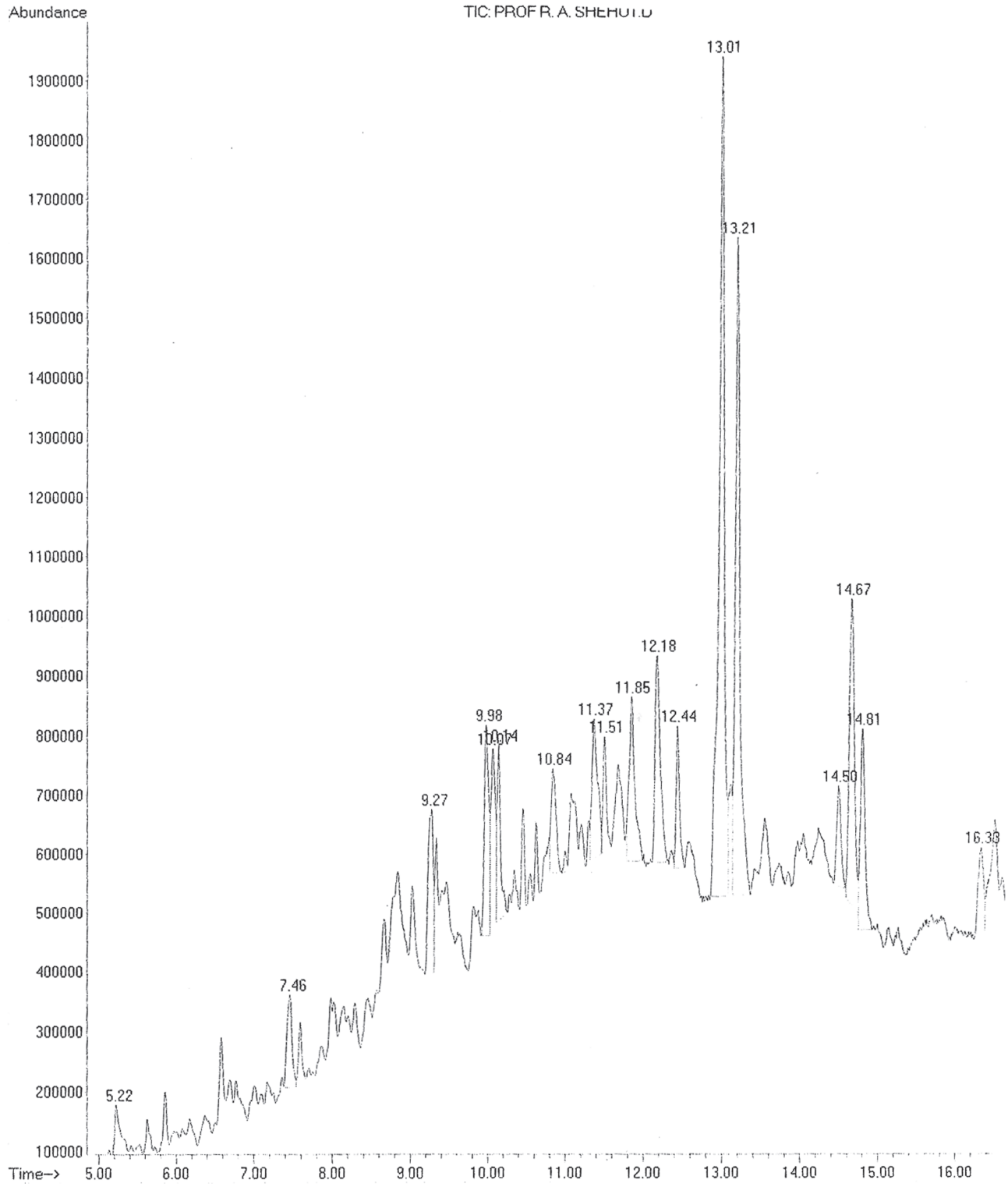

Fig. 4. Chromatographic profile of Agurra light crude oil (undegraded).

identified. For the oil extract from the soil it was observed that about 35 peaks were discernable. The additional peak might have been degradation products of the oil (Fig. 5). In oil polluted soil amended with mixed microbial culture, the peaks identified were about 22, representing various compounds. The hydrocarbons were highly degraded (Fig. 6). Chromatographic profiles of the crude oil showed that biodegradation was enhanced when a mixed culture of the indigenous microorganisms was added to the oil polluted soil. Biodegradation

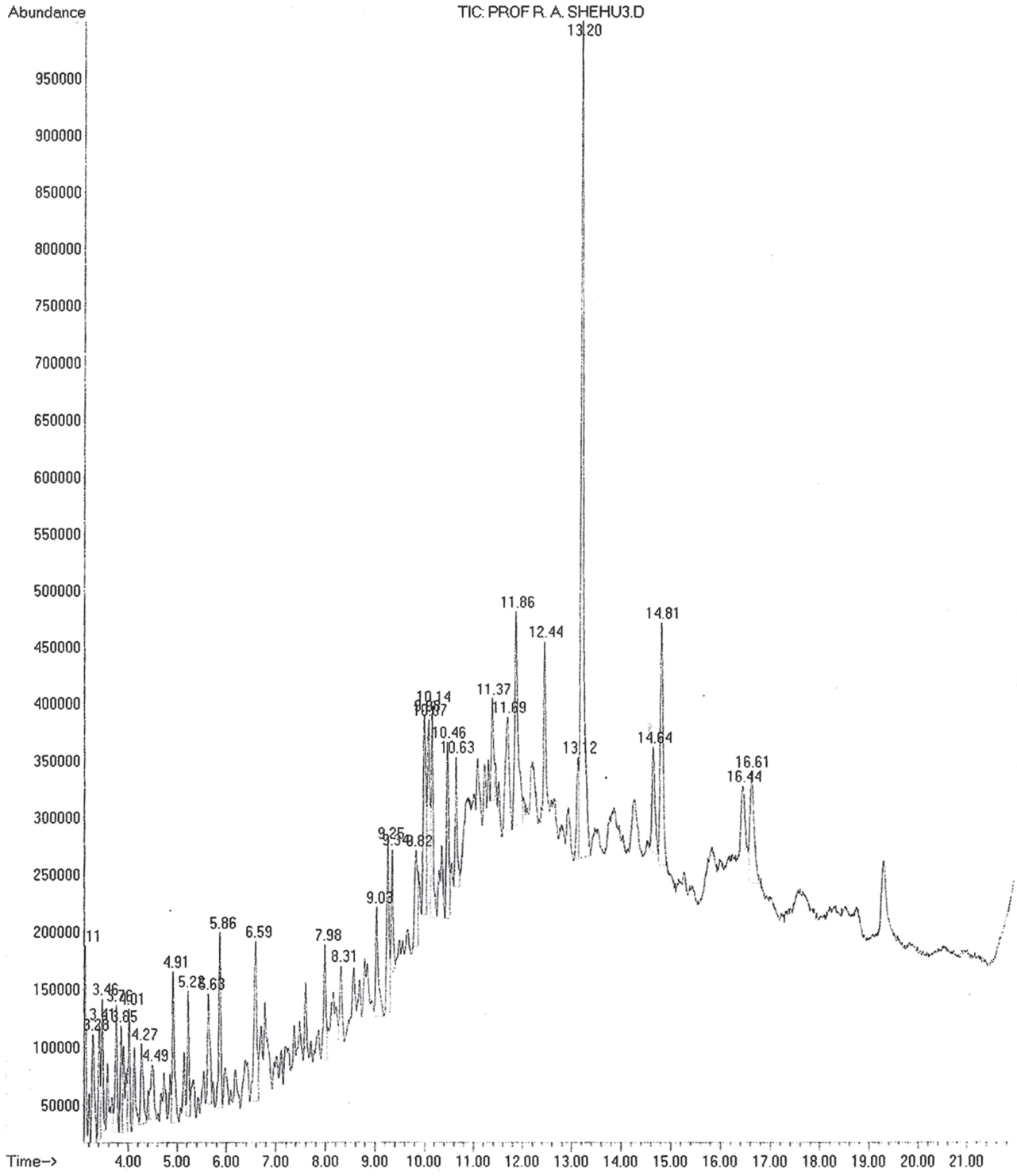

Fig. 5. Chromatographic profile of Agurra light crude oil extracted from soil after 16 days.

of oil components usually occurs in the following order alkanes, branched alkanes, the aromatic compounds and finally cycloalkanes.[32] The hydrocarbon components were extensively degraded after 16 days of exposure. The varying rates of biodegradation of the hydrocarbons in the soil indicated that bacteria in the soil varied in their biodegradative enzymes system. The hydrocarbon components were more extensively degraded in soil, which received the bacterial consortium than the unamended polluted soil, meaning that the bacterial consortium has enhanced the biodegradation process. The isoprenoids were attacked greatly further reflecting the competent oil degrading ability of the microorganism in the soil.[16]

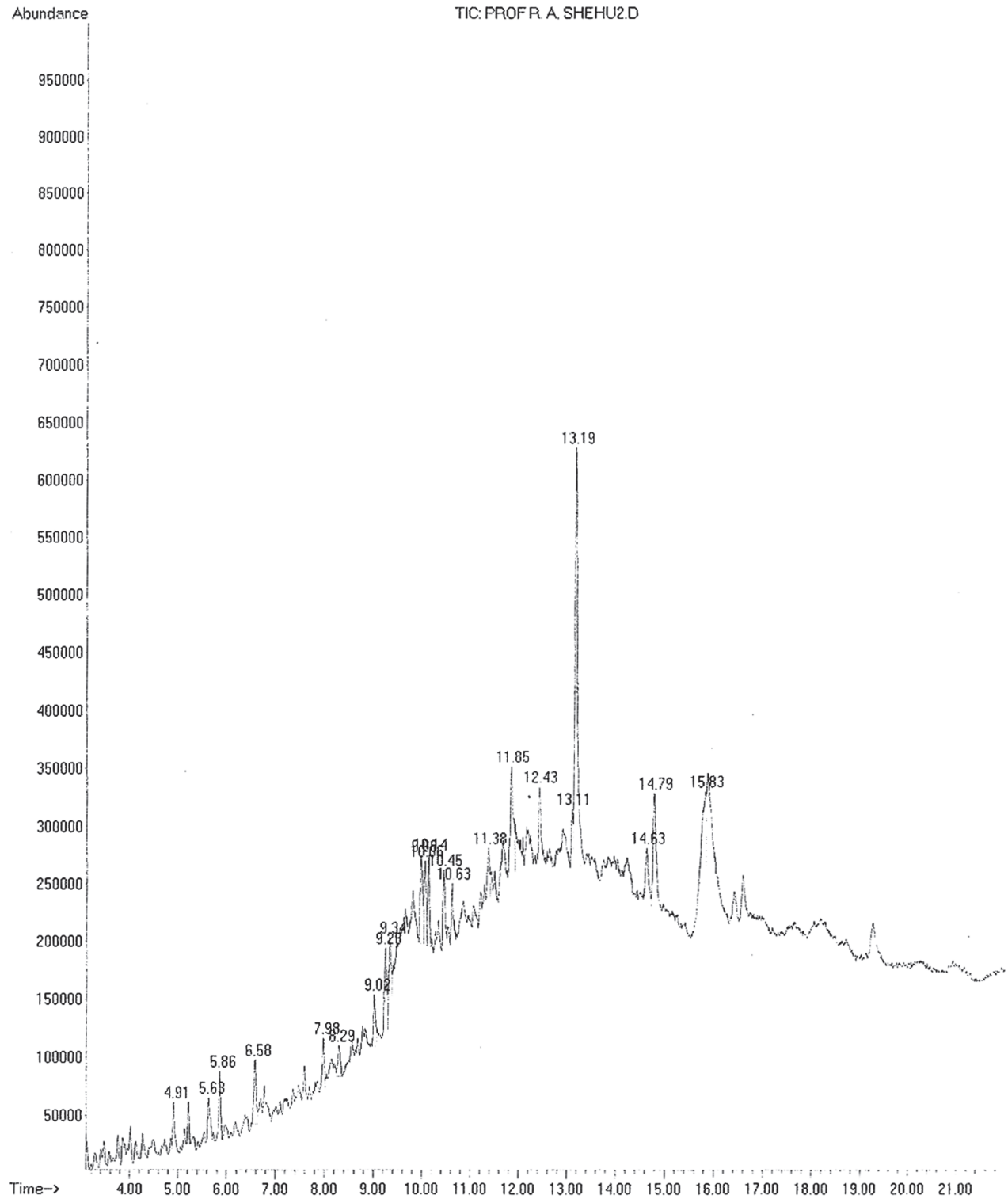

Fig. 6. Chromatographic profile of Agurra light crude oil extracted from soil inoculated with bacterial consortium (*Bacillus megaterium*, *Pseudomonas putida* and *Pseudomonas aeruginosa)* after 16 days.

4. CONCLUSION

In developing bacterial inoculants for oil spill remediation three bacterial isolates obtained in this study could be considered as possible candidates. The mixed culture of these isolates *(Bacillus megaterium*, *Pseudomonas putida* and *Pseudomonas aeruginosa*) was able to degrade the crude oil faster than each of the individual isolates. Thus, the bacterial consortium can enhance the degradation of crude oil spilled in soil better than the individual isolates.

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