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Hydrocarbon Degradation by Free-Living Nitrogen-Fixing Bacteria

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ABSTRACT

Free-living nitrogen-fixing bacteria that could utilize crude oil as sole carbon and energy source were isolated from agricultural soil samples. Four isolates identified as Paenibacillus lautus, Brevibacillus agri, Bacillus sp. and Paenibacillus polymyxa with the highest capability to utilize the substrate were selected for further studies. The isolates degraded crude oil significantly, achieving a percentage degradtion of 97.97% for Paenibacillus polymyxa, 97.00% for Bacillus sp., 95.54% for Brevibacillus agri and 94.13% for Paenibacillus lautus with corresponding increases in bacterial populations after 21 days of incubation. A loss of 10.74% crude oil was obtained in the control medium. The isolates were able to utilize n-hexadecane, diesel, n-dodecane, kerosene and crude oil, but none of the organisms could utilize phenol. In bioremediation experiments with soil microcosms polluted with crude oil over a 60-day period, hydrocarbon degradation of 92.72%, 90.6%, 89.25%, 89.92%, 92.26% and 23.19% was recorded in soils seeded with Brevibacillus agri, Paenibacillus lautus, Bacillus sp., Paenibacillus polymyxa, a consortium of the isolates and the non-bioaugmented control soil respectively. At 0.05 (95%) level of significance, there was a significant difference in the level of crude oil degradation between the bioaugmented soils and non-bioaugmented control soil. Cowpea seedlings grown on the bioaugmented soils had better growth in terms of percentage germination and stem height when compared with those grown on the polluted non-bioaugmented control soil.

Key Words: *Hydrocarbon, Biodegradation, Bioremediation, Nitrogen – fixing bacteria, Soil*

INTRODUCTION

The continuous demand for crude oil and its allied products come with their related problems such as spills resulting from oil production, refining and transportation; with an ultimate impact in pollution of the environment. Crude oil is known to exert adverse effects on plants, soil organisms and soil properties, which makes the soil condition unsatisfactory for plant growth, due to the reduction in the level of available plant nutrient or a rise in toxic levels of certain elements such as iron and zinc (Udo and Fayemi, 1995; Daniel-Kalio and Pepple, 2006; Omosun *et al.,* 2008). Nitrogen and phosphorus are the nutrients that most frequently limit bioremediation (Riser-Roberts, 1998). The presence of petroleum products in the soil can widen the C:N ratio, therefore limiting available nitrogen for degradation processes (Frick *et al.,* 1999). In addition, microbes able to metabolize hydrocarbons will quickly immobilize the mineral nitrogen that is available, leaving unfavourable conditions for other micro organisms and growing plants (Newman *et al.,* 2004). The absence of sufficient nitrogen in the soil will, in turn, slow the degradation process resulting from microbial metabolism (Riser-Roberts, 1998). Therefore, adding nutrients in the form of either organic or inorganic fertilizers can stimulate contaminant degradation (Huesemann and Moore, 1993). Free-living nitrogen-fixing bacteria can fix atmospheric nitrogen into a more usable form such as ammonia. Thus, the bioaugmentation of hydrocarbon polluted soil with free-living nitrogen-fixing bacteria may indirectly contribute to soil nitrogen by releasing nitrogenous biomass (Perez-Vargas *et al.,* 2000). This study was undertaken to isolate hydrocarbon-degrading free-living, nitrogen-fixing bacteria from the soil and to determine their potential in bioremediation of hydrocarbon polluted soil.

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MATERIALS AND METHODS

Sample Source

The Bonny light crude oil used was obtained from Shell Petroleum Development Company (SPDC) flowstation near Port-Harcourt, Nigeria. Soil samples were collected from different farmlands located in Lagos, Nigeria. The samples were collected randomly from 0–12 cm depth. Each sample was a composite collection from five different points of the site, homogenized and collected in sterile screw-capped bottles. Analysis commenced immediately upon arrival in the laboratory. The garden soil used for the microcosm experiment was obtained from the Biological Garden, University of Lagos, Nigeria.

Isolation of Free-Living Nitrogen-Fixing Bacteria from Soil

Aliquots of the appropriate dilution of the soil samples were plated on Ashby's Mannitol-Phosphate Agar (KH_2PO_4, 0.2 g/l; $MgSO_4.7H_2O$, 0.2 g/l; NaCl, 0.2 g/l; $CaCO_3$, 5.0 g/l; $CaSO_4$, 0.1 g/litre; mannitol, 10 g/litre; Agar, 15 g/litre; distilled water, 1000 ml; pH 7.2) and incubated at ambient temperature for 5-7 days. The isolates obtained were streaked on Luria Bertani (LB) agar to obtain pure cultures. The axenic cultures were screened for hydrocarbon-degrading ability by culturing in a nitrogen-fixing hydrocarbon oxidizers medium ($NaHPO_4$, 0.3 g/l; KH_2PO_4, 0.2 g/l; $MgSO_4.7H_2O$, 0.1 g/l; $FeSO_4.7H_2O$, 0.005 g/l; $Na_2MoO_4.2H_2O$, 0.002 g/l; distilled water; 1000 ml) with crude oil (1% v/v) as sole carbon and energy source for 21 days at ambient temperature.

Identification of Bacterial Isolates

The morphological and biochemical characteristics of the bacterial isolates were determined using the method described by Collins *et al.* (1989). Analytical profile index (API) kits (API 50 CHB/E and API 20E by Biomerieux, SA) were used to carry out further identification tests on isolates.

Biodegradation study

The ability of the isolates to degrade crude oil was studied over time by culturing each isolate in separate cotton wool-plugged 250 ml Erlenmeyer flasks containing sterile nitrogen-fixing hydrocarbon oxidizers medium with crude oil (1% v/v) as sole carbon and energy source with constant shaking for 21 days at ambient temperature. Control flasks containing sterilized isolates in minimal salts medium with crude oil were also set up. Samples were withdrawn in replicates of three from each flask for Total Viable Count (TVC) analysis on a 3-day interval. The residual hydrocarbon concentration after biodegradation was extracted with hexane: dichloromethane solvent system (1:1) and analysed by Gas Chromatography (GC-FID).

Determination of Total Viable Count (TVC)

The total viable count of bacteria was enumerated by plating aliquots (0.1 ml) of appropriate diluted culture samples on nutrient agar. Plates were counted after incubation at room temperature for 24 h.

Substrates specificity test

The ability of the isolates to utilize pure hydrocarbon substrates was tested by adding 1% (v/v) of liquid hydrocarbon to minimal salts medium containing 1.0 ml inoculum in Erlenmeyer's flasks to a final volume of 100 ml. The flasks were incubated with constant shaking at ambient temperature for 14 days. Control flasks were also set up. Hydrocarbon utilization was monitored by measuring increase in optical density at a wavelength (λ) of 540 nm.

Soil microcosm study

Two kilograms of air-dried garden soil was dispensed into six planting bags. The bags were kept in a glass house maintained at ambient temperature. The soil in each bag was polluted with 5% (v/w) crude oil (pollution level range suggested by Vidali, 2001 and mixed thoroughly. Bags 1-4 (bioaugmented) were each inoculated with 10 ml broth culture containing 10^{10} cfu/ml of an axenic culture of each isolate while Bag 5 was inoculated with 10 ml broth culture of a consortium of the four isolates containing 10^{10} cfu/ml of cells according to the method of Okerentugba and Ezeronye (2003). Uninoculated Bag 6 served as natural attenuation control.

Test for Soil Recovery

This was done by assessing the growth of cowpea (*Phaseolus vulgaris*) on 5% (v/w) crude oil-polluted bioremediated soils. Control experiments contaning 5% (v/w) crude-oil polluted and unpolluted garden soils were also set-up. The soils were tested for their recovery after 60 days remediation (bioaugmentation) in each test bag. The number of seeds (15 seeds per bag) that germinated from each soil was summed up after ten days. Morphological characteristics such as seed germination, stem diameter (cm) and height (cm) of the seedlings obtained from the bags were compared with those on the control soils.

Analytical procedure

The residual total petroleum hydrocarbons (TPH) in the soils were monitored at the beginning (day 0) and at the end of the experiment (day 60). TPH was extracted from soil samples and quantified using gas chromatograph-flame ionization detector (GC-FID) according to the methods of ASTDM 3921 and

USEPA 8270B (TPI, 2007). The residual TPH in the soil was extracted by mixing 10 g of the soil with 10 ml of n–hexane:dichloromethane solvent system (1:1) and stirred for five minutes. It was allowed to settle and filtered to separate the liquid mixture from the soil. The process was repeated thrice, the extract pooled and dried to remove the extracting solvent. The filtrate was concentrated to 1 ml and stored in a vial before GC-FID analysis.

Statistical analysis

The graphs and statistical analysis such as mean and analysis of variance were done using the GraphPad Prism version 5.0 computer software programme (GraphPad Software, San Diego, CA. USA).

RESULTS

Screening, characterization and identification of hydrocarbon degrading free-living nitrogen-fixing bacterial isolates

Four nitrogen-fixing bacterial isolates harvested on Ashby's Mannitol-Phosphate Agar exhibited the highest levels of turbidity in the nitrogen-fixing hydrocarbon oxidizers minimal salts medium containing crude oil as sole carbon and energy source. The isolates were identified as *Paenibacillus lautus*, *Brevibacillus agri*, *Bacillus* spp and *Paenibacillus polymyxa*. These isolates were used for further biodegradation tests.

Growth profile of isolates on crude oil

The liquid culture growth dynamics showed a steady increase in population density of *Paenibacillus lautus*, *Brevibacillus agri*, *Bacillus* spp and *Paenibacillus polymyxa* from days 0 to 15, 0 to 18, 0 to 12 and day 0 to day 15 respectively (Figure 1). *Paenibacillus polymyxa* had the best growth rate constant of 0.23 day^{-1} and a doubling time of 2.97 day (Table 1). Statistical analysis of variance showed no significant difference in the population density of the isolates. The gas chromatographic (GC) analysis of solvent extracts of culture fluids after 21 days (Table 1), showed a 97.97%, 97.00%, 95.54% and 94.13% reduction in the hydrocarbon components of the culture media containing *Paenibacillus polymyxa*, *Bacillus* spp, *Brevibacillus agri* and *Paenibacillus lautus* respectively. A 10.74% degradation was recorded in the control sample. Statistical analysis of variance at 0.05 (95%) level of significance, showed no significant difference in the comparative degradation dynamics of the isolates; however, a difference was observed between the degradation dynamics of the isolates and the control.

Substrate Specificity Test

The ability of the bacterial isolates to utilize different hydrocarbon substrates is shown in Table 2. All the isolates were able to utilize *n*- hexadecane, *n*-dodecane, kerosene, diesel and crude oil for growth. *Paenibacillus lautus* and *Paenibacillus polymyxa* both showed poor growth in hexane and *n*-decane; *Brevibacillus agri* showed moderate growth in them while *Bacillus* spp grew luxuriantly on both substrates. Only *Bacillus* spp demonstrated luxuriant growth in paraffin. Poor growth of all the isolates was observed in engine oil, xylene, benzene and cyclohexane. None of the isolates could utilize phenol.

Residual Hydrocarbon Concentrations of Nitrogen-Fixing Bacterial Augmented Crude Oil Polluted Soil

The gas chromatographic (GC) analysis showed that the bacterial isolates demonstrated a high rate of degradation of crude oil in soil (Table 3, Figure 2). The efficiency of extraction of oil from soil was 55%. The biodegradation potential of the consortium (92.26%) was not different from what was obtained for the pure isolates. There was however a relatively low level of degradation of crude oil (23.19%) in the attenuated control soil.

Test of Soil Recovery by Planting of Cowpea

The cowpea seedlings grown on bioaugmented soils showed better growth in terms of percentage seed germination and stem heights when compared with those grown on the polluted attenuated control soil (Table 4). The results also showed that the growth of seedlings in the treated soils compared favourably well with that grown in the unpolluted soil sample.

DISCUSSION

The screening for nitrogen-fixing hydrocarbon degraders resulted in the isolation of *Bacillus* spp., *Paenibacillus lautus*, *Paenibacillus polymyxa* and *Brevibacillus agri.* These Gram-positive sporeformers of the order Bacillales have displayed great ubiquity and diversity in physiology and phylogeny in nature (Scheldeman *et al.,* 2004). Their collective features include degradation of most substrates derived from plant and animal sources, including their ability to degrade a variety of macromolecules such as proteins (Ash *et al.,* 1993), polysaccharides (Claus and Berkeley, 1986; Kanzawa *et al.,* 1995) and polyaromatic hydrocarbons (Daane *et al.,* 2001, 2002). Sporogenesis enables members of *Bacillus*, *Paenibacillus* and related organisms to withstand environmentally harsh conditions, allowing long-term survival (Scheldeman *et al.,* 2004; Setlow,

1994;). Both genera of *Bacillus* and *Paenibacillus* have the ability to fix nitrogen (Ding *et al.*, 2005; Jin *et al.*, 2011). They have been isolated from a variety of sources including the soil, the rhizosphere, water, diseased insect larvae and foods (Daane *et al.* 2002, Jin *et al.*, 2011). Species of *Brevibacterium* and *Bacillus* were common in hydrocarbon-contaminated soil investigated by Dominguez-Rosado *et al.* (2004). There is however, a dearth of information available on hydrocarbon degradation by *Brevibacillus*. Although, information is available on its ability to tolerate high concentrations of a number of organic solvents and aromatic solvents, but they cannot utilize them as a sole carbon source (Kongpol *et al.*, 2009).

Crude oil is a mixture of hydrocarbons of various molecular weights and other liquid organic compounds. The degradation studies showed that the results obtained for the isolates were comparable with the observations of Lal and Khanna (1996). They remarked that microbial degradation of crude oil is often shown to occur by attack on alkanes or light aromatic fractions, while the higher molecular weight aromatics, resins and asphaltenes are considered recalcitrant. The increase in hydrocarbon degradation with corresponding increase in bacterial population density during the growth studies shows that the bacterial isolates were responsible for the oil degradation. Similar observations have also been reported by Adebusoye *et al.* (2007), Mandri and Lin (2007) and Obayori *et al.* (2009). The slight loss of hydrocarbon components recorded in the control sample may be attributed to evaporation, which occurred during the period of the experiment.

The susceptibility of hydrocarbons to microbial degradation which varies with the type and sizes of the hydrocarbon molecule was effectively demonstrated by our result. The isolates grew well on crude oil, diesel, kerosene and liquid aliphatic hydrocarbons such as *n*- hexadecane ($C_{16}H_{34}$) and *n*-dodecane ($C_{12}H_{26}$). Kerosene and diesel are a mixture of linear and branched alkanes with different chain lengths and a variety of aromatic compounds. Many of these compounds, especially linear alkanes, are known to be easily biodegradable (Ciric *et al.*, 2010; Sticher *et al.*, 1997). Saturated aliphatic hydrocarbons such as hexane (C_6H_{14}) and decane ($C_{10}H_{22}$) were able to sustain growth of the isolates but were not an excellent source of carbon for growth. The isolates showed poor growth in aromatic hydrocarbons such as benzene and xylene. Poor growth was also observed in cyclic hydrocarbon such as cyclohexane and engine oil which is a complex mixture of Long-chain saturated hydrocarbons (C_{16}-C_{36}) and cyclic alkanes (Bagherzadeh-Namazi *et al.*, 2008; Koma *et al.*, 2003) and other organic compounds, including some organometallic constituents (Butler and Mason, 1997). None of the isolates could utilize phenol; an aromatic hydrocarbon. Long-chain saturated hydrocarbons (C_{16}-C_{36}) and cyclic alkanes are known as recalcitrant to microbial degradation (Bagherzadeh -Namazi *et al.*, 2008; Koma *et al.*, 2003). It is generally accepted that the susceptibility of hydrocarbons to microbial attack decreases in the following order: n-alkanes > branched alkanes > low-molecular weight aromatics > cyclic alkanes (Obbard *et al.*, 2004; Labud *et al.*, 2007). Similar patterns of degradation has been observed with hydrocarbon substrates by Adebusoye *et al.* (2007) and Obayori *et al.* (2009).

The soil microcosm study indicates that the isolates are excellent candidates in bioremediation exercise because of their ability to fix nitrogen and also degrade hydrocarbons with high efficiency. These results show that seeding (bioaugmenting) the polluted soil with these isolates enhanced appreciably the rate of bioremediation of the soil. Nitrogen-fixing bacilli belonging to the genera *Bacillus* and *Paenibacillus* have been isolated and identified by Ding *et al.* (2005).

The percentage of seed germinated and the marked difference in the apperance of the germinated plants after bioremediation of the hydrocarbon polluted soil through planting with cowpea showed that bioaugmentation of hydrocarbon polluted soil with free-living nitrogen-fixing bacteria is an effective means of restoring soil fertility after pollution. Similar studies have beeen demonstrated by Mukherjee and Bordoloi (2011). Seed germination of several grass, legume, and cereal species have been shown to decline with an increase in used oil concentration at oil rates greater than 1.0% (w/w) and decreases in germination for most species were significantly ($p < 0.05$) below control rates (Dominguez-Rosado *et al.*, 2004). According to Gunderson (2006), the poor yield response in the polluted soil may be attributed to the exclusion of air from the soil due to the contaminants interfering with soil/water relationships, and also to the depletion of oxygen by microbial degradation of the contaminants.

On the basis of the results obtained, soils which have lost their fertility due to hydrocarbon pollution can be

restored back to become fertile soils by bioaugmentation with free-living nitrogen-fixing bacteria isolated from such environments. The use of these bacteria also have the added advantage of reducing the need for supplementation of the affected soils with nitrogenous compounds.

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Table 1: Growth kinetics of hydrocarbon-utilizing free-living nitrogen-fixing bacteria on crude oil

Sample*	Specific growth rate (day^{-1})	Mean generation time (day)	Residual hydrocarbon concentration (mg/l)	% Hydrocarbon degradation
Control	0.00	0.00	122.411	10.74
Paenibacillus lautus	0.19	3.72	8.0567	94.13
Brevibacillus agri	0.23	3.11	6.114	95.54
Bacillus spp	0.16	4.16	4.126	97.00
Paenibacillus polymyxa	0.23	2.97	2.786	97.97

*, After 21 days of incubation

Table 2: Substrate specificity test of hydrocarbon-utilizing free-living nitrogen-fixing bacteria on hydrocarbons

Substrates	Growth of Isolates*			
	Paenibacillus lautus	***Brevibacillus agri***	***Bacillus* spp.**	***Paenibacillus polymyxa***
Hexane	+	++	+++	+
Cyclohexane	+	+	+	-
n-decane	+	++	+++	+
n-dodecane	++	+++	+++	+++
n-hexadecane	+++	+++	+++	+++
Benzene	+	-	+	+
Phenol	-	-	-	-
Paraffin	+	+	+++	++
Xylene	+	+	+	+
Engine oil	+	+	+	+
Kerosene	++	++	+++	++
Diesel	+++	+++	+++	+++
Crude oil	+++	+++	+++	+++

- *, OD 520 nm; -, No growth; +, Poor growth (<0.2); ++, Moderate growth (0.2-0.3);
- +++, Luxuriant growth (>0.3).

Table 3: Residual Hydrocarbon concentrations of crude oil - polluted soil augmented with free-living nitrogen-fixing bacteria

Treatment	Residual hydrocarbon concentration (mg/l)	% Hydrocarbon degradation
Pure Crude	4037.33	0.00
Control (natural attenuation, Day 0)	2222.85	0.00
Control (natural attenuation, Day 60)	1707.37	23.19
Bag 1(*Paenibacillus lautus*), Day 60	208.86	90.60
Bag 2 (*Brevibacillus agri*), Day 60	161.92	92.72
Bag 3 (*Bacillus* spp), Day 60	239.03	89.25
Bag 4 (*Paenibacillus polymyxa*), Day 60	224.09	89.92
Bag 5 (consortium), Day 60	172.08	92.26

Table 4: Growth of Cowpea on Bioaugmented Soil with free-living nitrogen-fixing bacteria

Treatment	Germination (%)	Stem height (cm)	Stem diameter (cm)
Bag 1(*Paenibacillus lautus*)	100	14.1	0.48
Bag 2 (*Brevibacillus agri*)	100	14.9	0.49
Bag 3 (*Bacillus* spp)	100	13.5	0.47
Bag 4 (*Paenibacillus polymyxa*)	100	13.8	0.48
Bag 5 (consortium)	100	14.2	0.49
Control (polluted)	53	5.4	0.50
Control (unpolluted)	100	15.0	0.48

Bag 1, *Paenibacillus lautus;* Bag 2, *Brevibacillus agri;* Bag 3, *Bacillus* spp; Bag 4, *Paenibacillus polymyxa;* Bag 5, consortium of the isolates; control (polluted); Control (unpolluted).

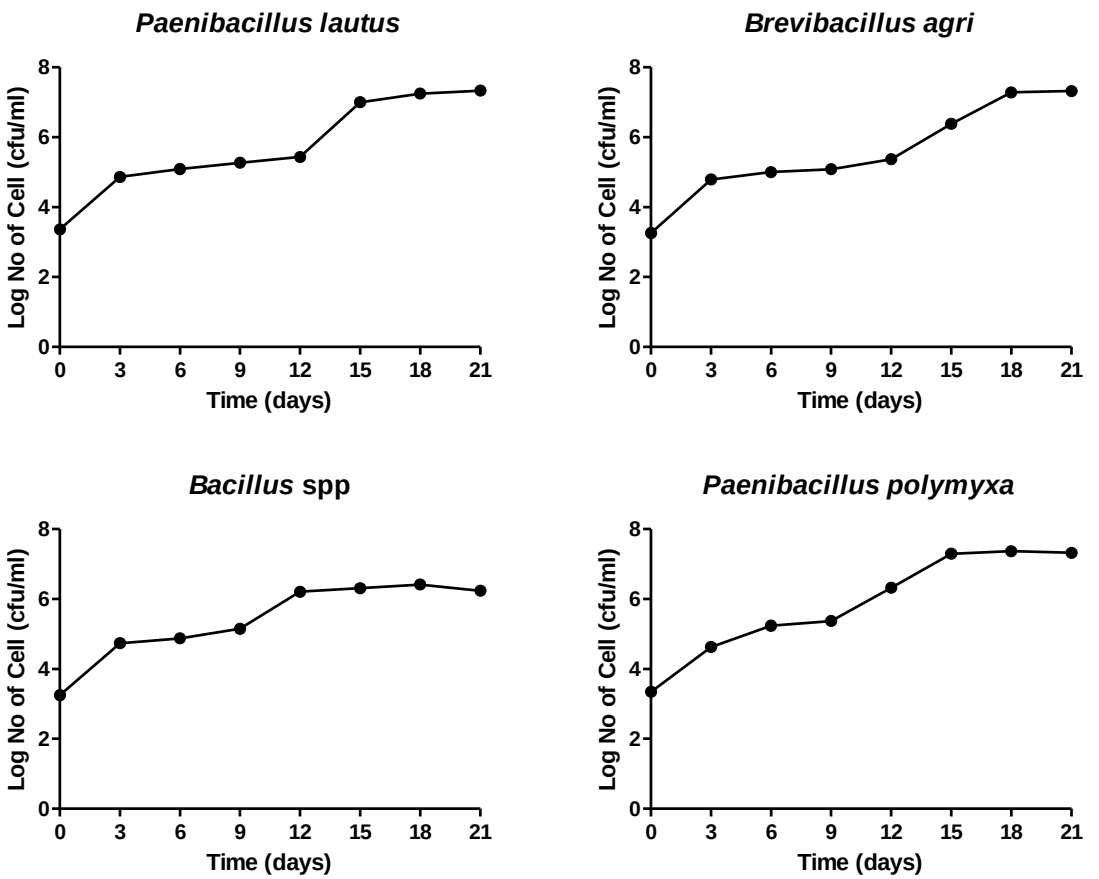

Fig. 1 Crude oil degradation by free-living nitrogen-fixing bacteria isolated from different farmlands. Crude oil was supplied at 1% (v/v) concentration. Data represent averages of three means.

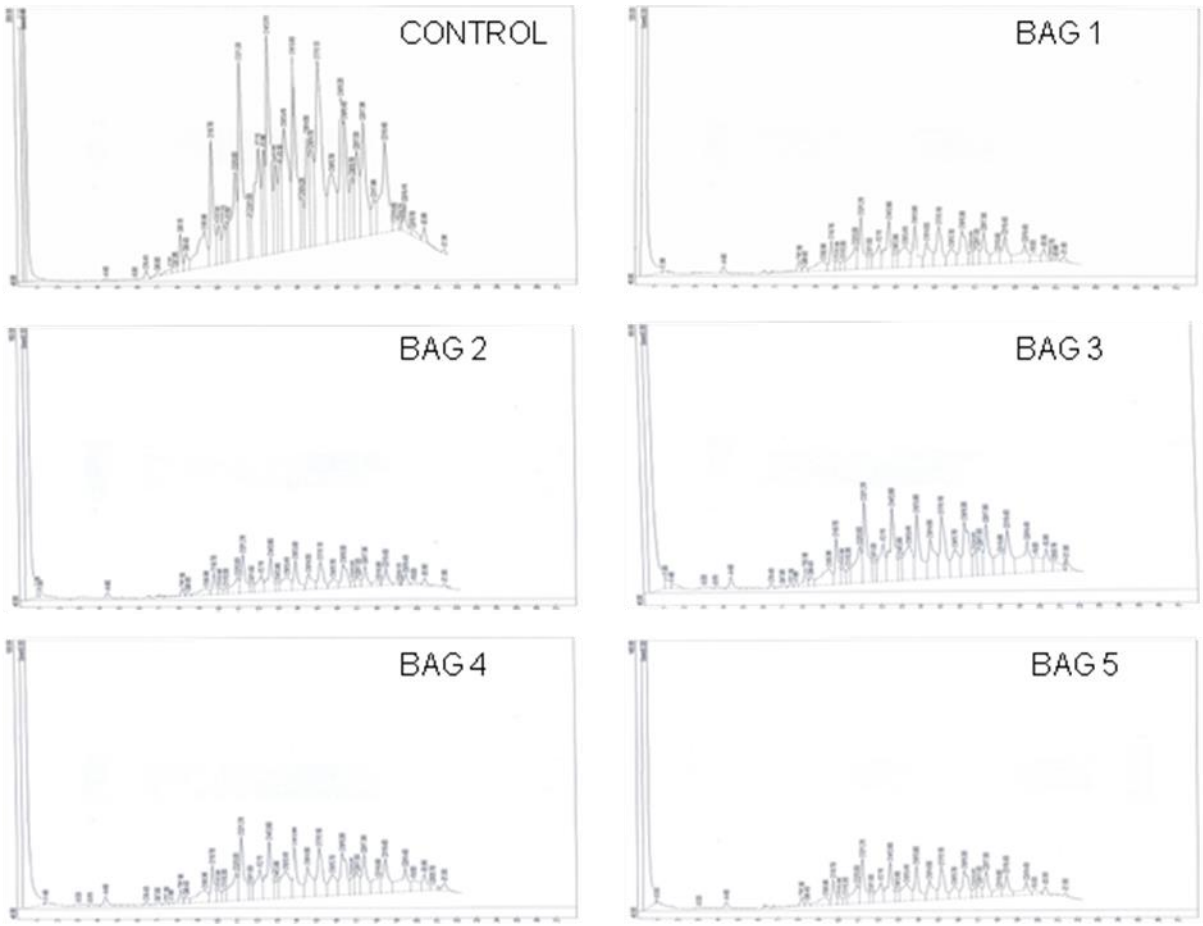

Fig. 2 Gas chromatogram of residual hydrocarbon concentrations of nitrogen-fixing bacterial augmented crude oil polluted soil at day 60.