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Bioremediation of oil-spilled sites through seeding of naturally adapted *Pseudomonas putida*

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Abstract

Considering the government-imposed regulations in developing countries as well as the negative impacts on genetically engineered microorganism (GEM), a field study was conducted with the naturally adapted *Pseudomonas putida* isolated from oil-contaminated sites of BHEL, Tiruchirappalli, India, to evaluate the biodegradation of crude oil in the open environment. Replicate field trials comprising of oil, oil plus bacteria, oil plus fertilizer and oil plus bacteria and fertilizer were monitored for total viable count and rate of degradation of crude oil. There was an increase in microbial count and rate of degradation in oil plus bacteria and fertilizer plot. It also revealed that fertilizer stimulated the rapid multiplication of microbes which simultaneously increased the rate of degradation. The present work illustrates the successful bioremediation of oil in the open environment by the naturally adapted microbe which might serve as an important adjunct in oil spillage operations. In view of tropical climate prevailing during most part of the year, the rate of oil degradation was faster.

1. Introduction

Biodegradation of accidentally spilled crude oil in aquatic and terrestrial environments has been the subject of recent reviews (Prince, 1993; Tennyson, 1993; Baker and Herson, 1994; Lehmann, 1998). Unrefined crude oil accounts for majority of oil spills because this is the principle cargo of 'supertankers' which hold up to 84 million gallons of crude oil; Common sources of these are storage tank bottoms, oil-water clarifiers, the cleaning of processing equipment and biological waste water treatments (Raghavan and Vivekanandan, 1999). Microorganisms are considered to be the only biological source for degradation of hydrocarbons (Atlas, 1981). Bacterial species have been shown to degrade hydrocarbon. The degradation was reported to be stimulated with fertilizer in laboratory (Scherrer and Mille, 1989; Rosenberg et al., 1992).

As there is government-imposed restriction in utilizing Genetically Engineered Microorganisms (GEM) in India as well as due to inability of the microbial genome to degrade all the components of pollutants in different environments and climatic conditions, seeding of naturally adapted microbes in situ field trial is considered as an important approach in bioremediation (Shome et al., 1996). However, the information available in this area of research of naturally existing efficient microbes is rather scanty (Prince, 1993).

Crude oil causes imbalances in the C–N ratio at the spilled site. For the efficient growth of bacteria, the C–N ratio should be around 60–100:1 (Dibble and Bartha, 1979; Jobson et al., 1972a). If it is slower or greater, the growth of bacteria will be retarded. Urea is added at the oil-spilled site to correct deficiencies in the nitrogen content of the soil (Jobson et al., 1972b).

The site chosen for the experiment was BHEL (Bharat Heavy Electricals Ltd), Tiruchirappalli, Tamilnadu, India, which liberates a huge volume of aqueous oil-effluent into the Uyyakondan canal, a tributary of the river Cauvery. *Pseudomonas putida* was isolated from the oil-contaminated sites of BHEL (Raghavan and Vivekanandan, 1999) and its pure cul-

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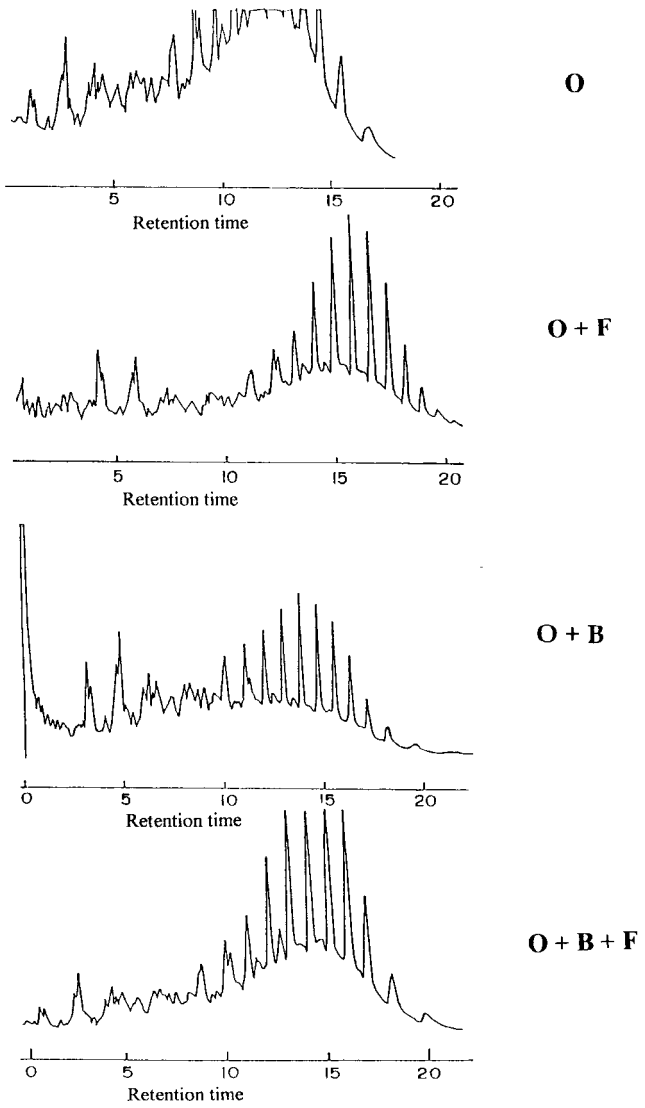

Fig. 1. Gas chromatographic profiles of the crude oil recovered from the application site at zero time after various amendments. O: oil, B: bacteria, F: fertilizer.

ture was seeded, with and without concurrent application of fertilizers as a soil amendment. The physico-chemical parameters of the soil selected for field experiments were also characterized.

2. Materials and methods

Pure cultures of *Pseudomonas putida* were obtained by enrichment technique from the effluent-polluted soil which had been contaminated over a period of time (Raghavan and Vivekanandan, 1999). The cells were grown on a rotary shaker in one liter flasks each containing 500 ml of basal salts medium plus 0.5 ml of crude oil. The cells were recovered by centrifugation after 2–3 days of growth, washed and re-suspended in distilled water. The amount of inoculum added per square meter of soil was 0.5 g (fresh weight).

Table 1
Physico-chemical parameters of the soil[a]

Sl. No	Parameters	Soil
1.	Texture	Clay
2.	Consistency (when dry)	Slightly hard
3.	Consistency (when wet)	Sticky
4.	Bulk density (g/cc)	1.25
5.	pH	8.2
6.	EC (mS/cm)	1.82
7.	Moisture (%)	4.71
8.	Chloride (mg/g)	3.53 ± 0.12
9.	Carbonate (mg/g)	3.86 ± 0.12
10.	Bicarbonates (mg/g)	6.23 ± 0.07
11.	Nitrogen (mg/g)	2.47 ± 0.07
12.	Phosphorous (mg/g)	10.3 ± 0.05

[a] The data are mean values of three different experiments (Mean $\pm$ SE).

The crude oil was applied in mid-August, 1996, by sprinkling from a perforated can at a rate of 9-l of crude oil per square meter of the soil. The oil completely covered the surface of the plots. The bacterial inoculum added per m^2 of soil was 0.5 g, as stated previously. The applied crude oil penetrated to a depth of 6 inches. Fertilizer Urea (Southern Petrochemical Ltd, Chennai, India, $N = 41.1\%$) was added simultaneously to plots at a rate of 1.0 g/m^2. Replicate field plots for each treatment like control, plus oil, plus oil and fertilizer, plus oil and bacteria and plus oil, bacteria and fertilizer were formed. Of the eight plots, four received fertilizer supplement.

Soil samples (10 g) were collected periodically and analyzed for bacterial counts. The oil was extracted from soil, and its breakdown products were quantified.

2.1. Recovery of oil

Approximately 5 g of soil sample was extracted with 5 ml of CCl_4, and the extracts were evaporated to dryness. The dry residue was re-dissolved in 5 ml of CCl_4,

Table 2
Fertilizer effect on bacterial population at oil-spilled plots (for details refer to Materials and methods)[a]

Treatments	Days after bacterial seeding		
	0	14 (CFU)	21
Control	220×10^5	286×10^5	219×10^5
Plus oil	158×10^5	177×10^5	166×10^5
Plus oil and bacteria	162×10^5	252×10^5	122×10^6
Plus oil and fertilizer	202×10^5	212×10^5	219×10^5
Plus oil, bacteria and fertilizer	162×10^5	88×10^7	196×10^7

[a] All counts are the mean values from triplicate plate count.

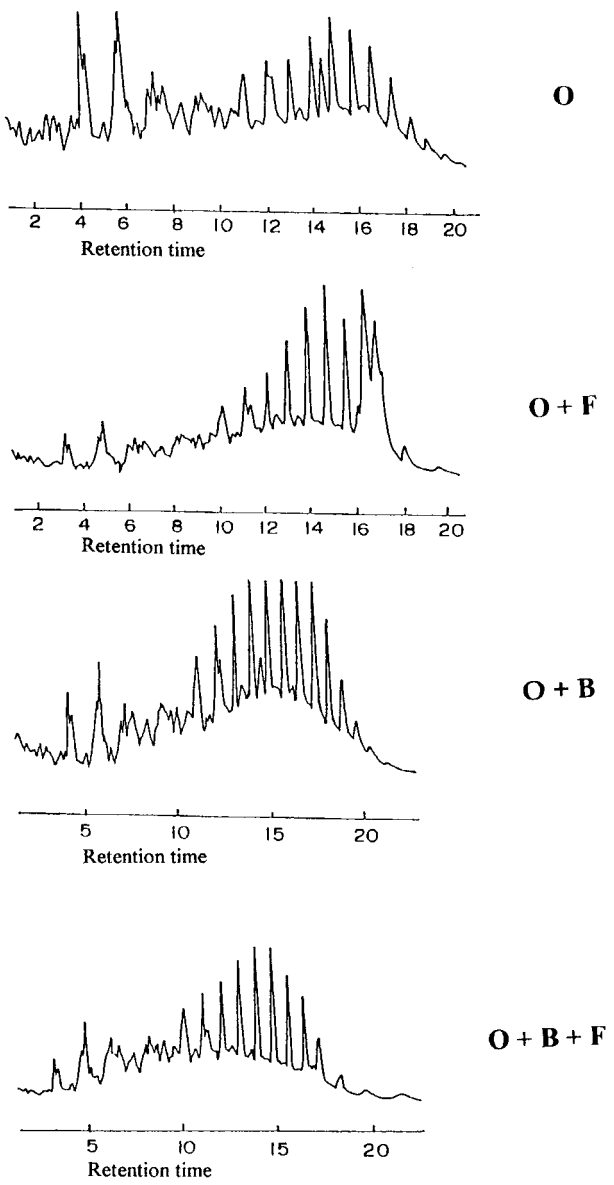

Fig. 2. Gas chromatographic profiles of the residual oil after 14 days contact with the soil in various amendments. O: oil, B: bacteria, F: fertilizer.

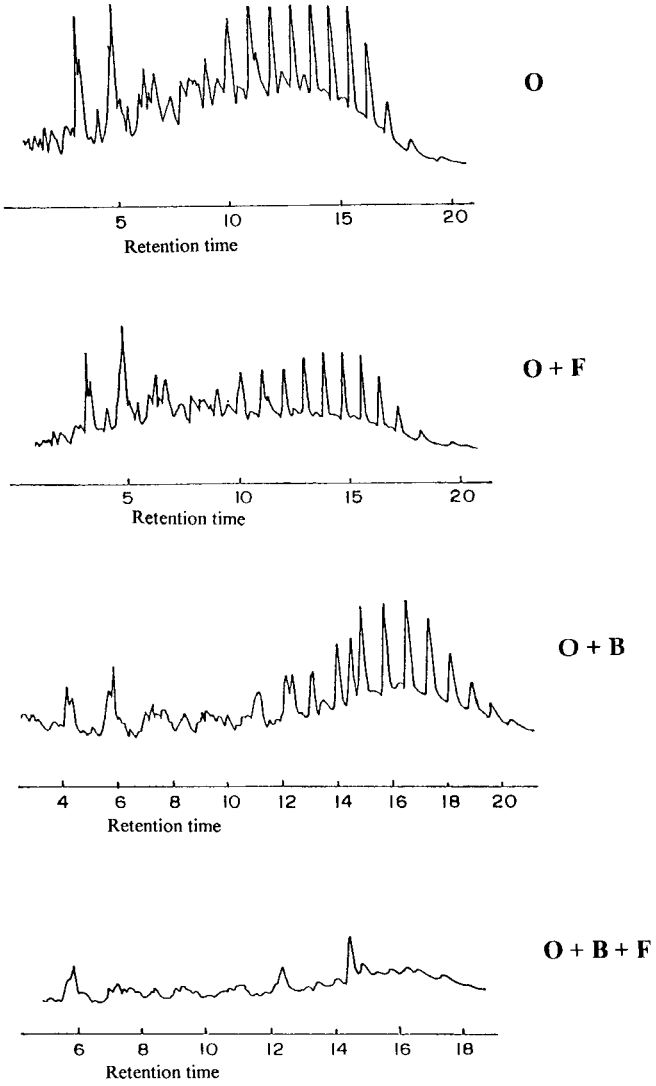

Fig. 3. Gas chromatographic profiles of the residual oil after 21 days contact with the soil in various amendments. O: oil, B: bacteria, F: fertilizer.

and the extracts were evaporated to dryness. The final residue was re-dissolved in 5 ml of CCl_4 and analyzed for GC profiles.

2.2. Microbial count

The microbial count was monitored by random sampling and standard serial dilution technique using agar spread plate method.

2.3. Gas chromatography

The hydrocarbons were detected by gas chromatography (GC). The Schimadzu gas chromatograph was equipped with a flame ionization detector and a 10 ft stainless steel column containing chromosorb P precoated with 3% OV-1. The operation conditions were as follows: Oven initial temperature (50°C); Oven final temperature (270°C); Injection port temperature (90°C); N2 carrier gas flow rate (30 ml/min); H2 gas pressure (1.5 kg/cm^2); Air pressure (2.0 kg/cm^2); Chart speed (1 cm/min); and Volume of sample injected (0.5 μl).

3. Results

The physicochemical parameters of the soil in the site are described in Table 1. Initially the pH of the soil was 7.8–8.2 in the seeded and unseeded plots but it gradually decreased to the range of 6.4–6.9 in the

plot seeded with bacteria and fertilizer. Changes in the bacterial count observed after 0, 14 and 21 days of treatment are presented in Table 2. The GC profiles of the oil extracted immediately after application in the soil is presented in Fig. 1. A comparison of the GC profiles 14 days after oil application (Fig. 2) revealed that application of fertilizer plus microbial inoculum resulted in a marginally higher rate of substrate utilization. However, most of the crude oil fractions were totally utilized after 21 days of treatment with the bacteria plus fertilizer (Fig. 3). The viability is an indication of the survival of the active phase of the microbe in the natural environment. The utilization of crude oil is evident by the increase in the number of viable cells (Table 2) as well as in the rate of degradation at 21 days (Fig. 3) in oil plus bacteria and fertilizer plot.

4. Discussion

The hypothesis that a nutrient deficient condition is responsible in part for the persistence of the oil applied to the soil has been substantiated from the data presented. There was no increase of bacterial count in oil-amended and oil plus fertilizer plots and this cannot be attributed to the toxicity of the oil. The marginal increase in the rate of oil utilization by the bacteria at the oil-spilled site could be a result of too low level of bacterial application or the inability of these bacteria to survive under natural field conditions (Jobson et al., 1972b). It also implied the pre-conditioning of microbes to the open environment. The application of urea resulted not only in a rapid multiplication of bacteria but also accelerated the disappearance of the hydrocarbons (Garg et al., 1994; Prince, 1993). The increase in bacterial mass can be correlated to the disappearance of the components of crude oil, which simultaneously increased the rate of recovery of oil-contaminated soil. In this work, fertilizer alone was ineffective in stimulating the indigenous microflora. In fact, the application of naturally adapted strains along with the fertilizer was highly effective for clean-up operations.

Bioremediation by application of oil-degrading bacteria, an appealing idea to the general public, did not meet with any clear-cut success without the extraneous addition of fertilizers and seeding with the right type of microbe. Bioremediation by applying nutrients along with the microbes well adapted to a particular environment, should be considered as an effective 'tool' for combating oil spills (Raghavan and Vivekanandan, 1999: Shome et al., 1996). The results presented further confirm the successful use of 'bacterial seed or cocktails' well adapted to natural environment to deal with oil spills (Jobson et al., 1972b). It is also evident that oil spills on land could be effectively degraded by bacterial applications (Tennyson, 1993; National Research Council, 1993) which may be attributed to the prevailing tropical environment conditions. All these effects can be directly related to the application of fertilizers along with the well adapted local microbial consortia to the 'oil soaked plots'.

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