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A Feasibility Study for Assessment of *In-situ* Bioremediation Potential of a Crude Oil Degrading *Pseudomonas* Consortium

A. Mittal[1] **and P. Singh**

Department of Microbiology, Gurukul Kangri University, Haridwar, Uttarakhand, India

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Abstract

A microcosm study evaluating inoculums addition of mixed bacterial consortium to stimulate in-situ bioremediation of crude oil contaminated soil was conducted. In feasibility study, out of five treatments the application of bacterial consortium, nutrients and environmental factors resulted in 79.16% removal of TPH in 60 days, compared to 30.24% removal of TPH carried out by indigenous microflora. Gas chromatograms of original spilled oil and bioremediated oil by the addition of developed consortium shows that Pr/Ph ratio decreased progressively from 2.358 to 1.626. The results showed that the ratio of di/tri aromatics decreased from initial 0.63 to 0.25 with progressive treatment of nutrient addition, as nutrient + tilling, nutrient + tilling + microbial seeding. Similar effect was observed in di/di + tri aromatics ratios which also decreased 0.31 to 0.20 by bioaugmentation only.

Keywords: Hydrocarbons; *Pseudomonas*; Biodegradation; Gas Chromatogram.

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1. Introduction

Of late, there is increasing concern in the maintenance of soil quality and remediation strategies for management of soil contaminated with hydrocarbons. Oil spills may result from operational mishaps, equipment failure and sabotage. When oil spills onshore or near shore, the soil and the components of the terrestrial ecosystem are inevitably affected [1] Bioremediation is a technique that enhances the natural rate of biodegradation of pollutants through reactions carried out by selected microorganisms [2]. There are many definitions of 'microcosm' a typical one is that of an intact, minimally disturbed piece of an ecosystem brought into the laboratory for study in its natural state [3].

Bioremediation was made simpler and more practical in the late 1980s by a technique that induces colonies of oil-eating bacteria to enter a state of suspended animation–an inactive mode that the microbes normally adopt during extended periods of drought or freezing [4]. Many carrier materials, mostly agricultural byproducts, are used to transfer the bacterial consortium to the field effectively [5, 6].

[1] *Corresponding author* : anupma.garg@yahoo.com

A laboratory microcosm study was conducted to assess the short term bioremediation potential of a sandy loam soil of Haridwar region, freshly contaminated with un-weathered Sobhasan crude oil well (well # Y). An indigenous microbial consortium was developed by assembling selected microbial strains *Pseudomonas* strains PS-I, PS-II and PS-III, this developed consortium was used for the cleanup of oil contaminated soil. This work reports degradation of crude oil by Gas chromatographic (GC) analysis for saturate fraction and High pressure liquid Chromatographic (HPLC) analysis for aromatic fractions to test the decontamination and conservation of a garden soil polluted with crude oil after bioremediation using "adapted" strains of *Pseudomonas* PS-I, PS-II and PS-III.

2. Materials and Methods

2.1. *Source of bacterial consortium*

A bacterial consortium was developed in minimal salt medium [7, 8], with crude oil as the sole source of carbon and energy, from a sample of soil contaminated with crude oil. The soil sample was collected from an oil production site of ONGC, Sobhasan oil field project, Gujarat in western India, as described elsewhere [9]. The indigenous bacterial consortium consisting of three bacterial isolates was selected. The crude oil-degrading efficiency (qualitative and quantitative) of individual bacterial isolates *Pseudomonas* strains (PSI, PSII, PSIII) was screened on minimal salt medium as described elsewhere [9], using crude oil as the sole carbon and energy source [10]. These selected isolates were characterized and identified using biochemical tests and 16S rRNA sequencing from The Energy and Resources Institute (TERI) New Delhi, India. All the constituent strains of the selected consortium were stored in 25% glycerol at -70°C.

2.2. *Carbon substrates*

Crude oil samples from Sobhasan oil well # Y was collected with the help of O.N.G.C. authorities.

2.3. *Physico-chemical analysis of soil*

Physical and chemical properties of soil samples taken at the onset and at the end of the treatment were analyzed. The samples were drawn with a hollow pipe, 3.5 cm in diameter. Air-dried and pulverized soil samples were analyzed for pH, temperature, moisture level, organic carbon, nitrogen, and available phosphorus with standard methods.

2.4. *In-vitro microcosm study*

Five tin cans designated as Box1, Box2, Box3, Box4, and Box 5 with 45.72 cm x 45.72 cm x 20.32 cm dimensions were prepared. Soil was filled in cans up to the height of 15.2 cm.

In each can, garden soil (27 kg) was contaminated experimentally with 660 ml of Sobhasan crude oil (approximately 2% v/w). The initial and final concentration of crude oil, soil pH, temperature, moisture level, organic carbon, nitrogen and available phosphorus were also determined over a period of 60 days. The experiment was conducted in the premises of Kanya Gurukula Mahavidyalaya (G.K.U), Haridwar. The following five sets of treatment were conducted. Box 1 (Weathering control) - Soil was autoclaved at 121°C for 30 minutes and treated with 2% $HgCl_2$, after one month interval to monitor abiotic hydrocarbon losses. Box 2 (Indigenous biodegradation) - In the initial state, microcosm is left unmanipulated. In this Box no treatment was given except ½ to 1-liter water was added regularly. Box 3 (Nutrient addition) - Microcosm was amended with inorganic nutrients in the form of Diammonium phosphate (DAP) and urea fertilizer, in order to have a C: N: P ratio of 120:10:1 as per Gibb's formula (assuming crude oil is 78% carbon). Nutrients were applied two times, after one-month's interval. Box 4 (Nutrient + Tilling) - It entails the two or three times per weekly tilling of the microcosm contents to promote soil aeration, in addition to nutrient amendment. Box 5 (Nutrient + Tilling + Consortium) - The consortium of hydrocarbon degraders (*Pseudomonas* strain PS-I, PSII, and PSIII) was added in addition to nutrients and weekly tilling.

Throughout the study all microcosms were maintained open to the air, in field and sufficient water was added to hydrate the soil to 50% of its water holding capacity. In biostimulation treatment, a dry mix fertilizer (N: P) was applied to select boxes (Box 3, Box 4, and Box 5).

2.5. *Total petroleum hydrocarbons (TPH) in soil*

To assess the rate at which the TPH was being degraded, samples were collected at time Zero (just before initiating the bioremediation), 30 days later, and at the end of the study (60 days after initiating the process). The sample was weighed and mixed with anhydrous sodium sulfate in a 1:1 ratio and then used for soxhlet extraction. The sample weight was adjusted to account for the moisture and this weight was used for analysis.

Total petroleum hydrocarbon from 10 gm soil was then consecutively extracted with hexane, methylene chloride and chloroform (100 ml each) for 16 hours in soxhlet apparatus. The extraction cycle around the thimble were approximately 6~8 cycles per hour. The solvent extract containing soluble oil from the soil was evaporated in Rota evaporator. The residual oil content was estimated.

2.6. *Hydrocarbons fractionation and analysis of fractions*

Saturates, aromatics and NSO fractions were fractionated quantitatively by column chromatography following [11] method. The oil sample (approx. 100 mg/known weight) was dissolved in chloroform, adsorbed on silica gel and evaporated the excess chloroform at 80°C. The adsorbed sample was charged at the top and eluted saturates with 60 ml petroleum ether (40-60°C), aromatics with 90 ml benzene and NSO with 60 ml methanol

respectively. The solvents were evaporated in rotary vacuum evaporator at 60°C. Each fraction was transferred in tared sample vials, dried and weighed. Chemito - 1000 gas chromatograph was used for gas chromatographic analysis of saturate fraction on a FID detector. Conditions for saturate (alkanes) fraction: Column: BP-5 (30m × 0.25 mm), Initial temperature: 80°C, Final temperature: 300°C, Ramp rate: 4°C per min, Carrier gas: Nitrogen (40 ml/min), Injection temperature: 310°C. Aromatic fraction was further fractionated as follows, Shimadzu VP-3 HPLC system using normal isocratic mode was used for the fractionation of aromatic hydrocarbons into mono, di and tri-aromatics on a silica amino (NH_2) column (30cm×4.5mm). Elution was carried out using n-hexane (HPLC grade) and the separation was monitored with UV-visible absorbance detector operated at 245 µm wavelength. The flow rate was maintained at 6.5ml/min.

2.7. *Survival of introduced consortium*

Survival of introduced bacterial consortium during the course of study was indicated indirectly by observing the increase in population of consortium in soil. Soil samples (1 gm) were collected before and after the application of bacterial consortium i.e. after 15 Days, 30 Days, 45 Days and 60 Days, suspended in saline water (0.85% NaCl). The suspension after appropriate dilution was plated on Luria Bertani agar (LBA) media plates.

3. Results and Discussion

3.1. *Indigenous microflora*

The first and foremost criterion for designing a bioremediation program is to study the native microflora of the system and to analyses the physico-chemical composition of soil. For microcosm study, soil samples (garden soil of Kanya Gurukula Mahavidyalaya, Haridwar) were analyzed for detection of different groups of microbes. The soil contains 8×10^8 cfu/g of total heterotrophs, 4.5×10^5 cfu/g of actinomycetes and 1.4×10^4 cfu/g of fungi. The hydrocarbon degrader's count is 2.2×10^4 cfu/g of soil.

3.2. *Physico-chemical analysis of soil*

Physical and chemical properties of soil samples taken were analyzed for pH, temperature, moisture level, organic carbon, nitrogen and available phosphorus in each box at zero time intervals i.e. initially (Table 1). *In-situ* bioremediation approach was adopted, after optimizing the factors under laboratory conditions. These optimized conditions were attempted to be maintained during microcosm study. The soil used is sandy loam in texture and its pH was 7.88. Moisture content of soil was 7.30 %, while water holding capacity was 40.55. The temperature recorded during the study varied from 25°C- 33°C. Organic carbon content (TOC) of soil was 1.23 % (before amendment) and 2.79% (after amendment by

crude oil), nitrogen content - 0.126%, while phosphorous content was measured as 34.70 ppm initially.

Table 1. Variation in soil parameters in different treatment.

Parameters	Initial	After 60 days of treatment				
		Weathering control	Indigenous biodegradat-ion	Nutrient	Nutrient +Tilling	Nutrient +Tilling+ Consortium
Soil pH	7.88	7.3	7.71	7.62	7.6	8.14
Moisture content (%)	7.3	7.65	7.47	7.45	7.30-7.14	7.14
Organic carbon	2.79	2.7	2.79-1.942	2.79-1.73	2.79-1.61	2.79-0.566
Nitrogen	0.126	0.121	0.126-0.896	0.235-0.201	0.235-0.194	0.235-0.180
Phosphoro-us (ppm)	34.7	34.63	34.70-28.15	0.026-0.013	0.026-0.011	0.026-0.010

3.3. *Crude oil used*

The crude oil deployed for microcosm study (Sobhasan crude oil #Y) was analyzed for its gross composition. Saturates, aromatics and NSO percentage was found to be 71.7%, 20.0% and 7.4%, respectively.

3.4. *Microcosm Studies*

Box 1: Weathering Control: The hydrocarbon utilizing bacteria (HUB) count was also found to be zero, though some heterotrophic bacterial activity in an open environment is not possible. It is because the soil was sterilized and 2% $HgCl_2$ treatment also arrested the soil microbial activity. The minimum loss of TPH (5.02%) can be attributed to the abiotic losses like evaporation of low volatile fraction of crude oil and photo-oxidation etc. (Table 2). Several workers have reported hydrocarbon losses due to volatilization and photochemical oxidation [4, 12].

Box 2: Indigenous Biodegradation: The box 2, which shows the biodegradation by indigenous microflora, is containing 8×10^8 total heterotrophs and 2.2×10^4 hydrocarbon degraders. It has been reported previously that bioremediation is less if the population of hydrocarbon degrading microorganisms is less than 10^5 cfu/g in soil [13]. This warranted that a bacterial consortium was needed for effective bioremediation. The TPH decreased from 0.1885 gm to 0.1315 gm in 60 days, indicating 30.24% degradation of crude oil (Table 2). During the course of biodegradation, the pH dropped from 7.88 - 7.71. Many other workers have also been reported the pH alteration during crude oil degradation [14, 15].

Box 3: Effect of Nutrient addition: In this treatment, loss in TPH was 49.89% in 60 days, which indicate that, fertilizer addition increases the rate of biodegradation. After 60 days, pH dropped from 7.88 to 7.62. This lowering of pH can be attributed to the formation of various carboxylic acids during breakdown of hydrocarbons. The values of nitrogen and phosphorous determined during the study indicate that the soil was richly amended by Diammonium phosphate (DAP) and Urea fertilizer during study. Nitrogen content was depleted from 0.235gm to 0.201gm because the fertilizer was amended as per concentration of hydrocarbons remained in the soil after biodegradation. It can be concluded that microbial activity has consumed nitrogen amended in the form of fertilizer and nitrogen was available in plenty to the microbes at all the times. The concentration of available phosphorous was not dropped significantly because of addition of DAP fertilizer (Table 1). Nutrient amendment over a wide range of concentrations significantly improved oil degradation, confirming the low concentration of nitrogen and phosphorous limited degradation [16].

Box 4 - Effect of nutrient (Fertilizer) addition and tilling (aeration): The loss of TPH was 54.16% (Table 2); the increased loss in this box as compared to other boxes was due to (i) aeration done weekly in this box (from the bottom) through out the soil matrix, which has helped in evaporation of volatile part of crude oil present in the whole soil system. (ii) Aeration does help in growth of microbes by overcoming the oxygen limitations in soil matrix leading to more degradation of crude oil. The soil tilling stimulates the microbial activity due to breakdown of soil aggregates and better exposure and aeration of degradable material [17].

Box 5 - Effect of nutrient addition, tilling and developed consortium: In Box 5, maximum of 79.16% loss of total petroleum hydrocarbon (TPH) was obtained in 60 days (Table 2). This treatment has been bioaugmented with potent hydrocarbon degraders improvised from soil microflora. This treatment has significantly increased the rate of degradation as the number of potential hydrocarbon utilizing bacteria (native of the soil) was artificially raised. The maximum loss of TPH could be attributed to the addition of laboratory-grown inoculum of mixed consortium of native soil microflora (Hydrocarbon utilizers) [18, 19]. The organic carbon decreased from 2.79% to 0.566%, because of consumption of crude oil. Nitrogen and phosphorous content also decreased dramatically. It can be concluded that microbial activity has consumed nitrogen and phosphorous during crude oil degradation (Table 1).

The temperature recorded during the study varied from 25 - 33°C. Since maintenance of temperature in open soil system is not feasible, the bioremediation efforts should be concentrated during such a period of year when the temperature is suitable for treatment. Moisture content of soil is critical for microbial activities, since nutrients, organic constituents, oxygen and metabolic wastes are transported to and from microbial cells in water. The optimum level of soil water for microbial activity is 10-13% i.e. 65–80% of

water holding capacity [20]. To maintain this sufficient water was added regularly to hydrate the soil to 50% of its water holding capacity.

Table 2. Crude oil degradation in various treatments Boxes.

Treatment	Crude oil				
	Incubation period(days)	Initial conc.(gm)	Final conc.(gm)	Degrada-tion	% degrada-tion
Weathering control	15	0.1989±0.054*	0.1949±0.073*	0.004	2.01
	30	0.1989±0.054*	0.1919±0.069*	0.007	3.71
	45	0.1989±0.054*	0.1909±0.037*	0.008	4.02
	60	0.1989±0.054*	0.1889±0.011*	0.01	5.02
Indigenous biodegradation	15	0.1885±0.081*	0.1762±0.061*	0.0123	6.53
	30	0.1885±0.081*	0.1615±0.014*	0.027	14.32
	45	0.1885±0.081*	0.1405±0.050*	0.048	25.46
	60	0.1885±0.081*	0.1315±0.012*	0.057	30.24
Nutrient addition	15	0.1904±0.023*	0.1534±0.037*	0.037	19.43
	30	0.1904±0.023*	0.1224±0.091*	0.068	35.71
	45	0.1904±0.023*	0.1904±0.036*	0.081	42.54
	60	0.1904±0.023*	0.0954±0.058*	0.095	49.89
Nutrient + Tilling	15	0.1754±0.043*	0.1380±0.021*	0.037	21.09
	30	0.1754±0.043*	0.1084±0.071*	0.067	38.2
	45	0.1754±0.043*	0.0984±0.007*	0.077	43.9
	60	0.1754±0.043*	0.0804±0.015*	0.095	54.16
Nutrient+Tilling+ Consortium	15	0.1857±0.061*	0.1287±0.098*	0.057	30.69
	30	0.1857±0.061*	0.0837±0.010*	0.102	55.93
	45	0.1857±0.061*	0.063±0.021*	0.123	66.07
	60	0.1857±0.061*	0.039±0.017*	0.147	79.16

*****values are the mean ± SE of 3 replications.

3.5. *Survival of introduced consortium*

Survival of the microorganisms in the soil after their application is a deciding factor in rate of degradation of hydrocarbons [21]. Continuous increase in microbial count, cfu/g soil indicated survival of introduced consortium in the soil. Before bioaugmentation the population of hydrocarbon degraders which was 2.2×10^4 increased and reached up to 9.2×10^8 during 60 days of bioremediation. This increase in microbial count indirectly indicates survival of introduced consortium.

3.6. *Gas chromatography*

GC of extracted oil from each box of microcosm study has been depicted in Fig.1 (a), (b) and reveals degradation (bioremediation) of contaminated oil in increasing order. Abiotic

losses due to evaporation and photo-oxidation are observed to the extent of 5.02% in Box-1, though not apparent in gas chromatogram. The microcosm study indicated that spilled oil was degraded with the help of developed consortium and by adjusting the nutrients and environmental factors to the extent of 79.16% against the bioremediation, which could have been carried out by indigenous microflora (30.24%). It is note worthy to mention here that the developed consortium is potent hydrocarbon degrader and could degrade normal alkanes, as well as isoprenoidal alkanes to a greater extent. Gas chromatograms of original spilled oil, bioremediated oil by indigenous microflora as well as by the addition of developed consortium shows the degradation of alkanes and isoprenoidal alkanes in decreasing order as substantiated by nC_{17}/ Pr, nC_{18}/ Ph and Pr/Ph ratio (Table 3) of various boxes oils.

Table 3. Biodegradation indices.

Parameters	Initial(ct.)	Box 1	Box 2	Box 3	Box 4	Box 5
nC_{17}/Pr	2.0433	2.0149	1.7349	0.3893	0.3060	0.1827
nC_{18}/Ph	4.7733	4.5620	4.4543	1.2012	0.7819	0.7143
Pr/Ph	2.358	2.321	1.959	1.798	1.687	1.626

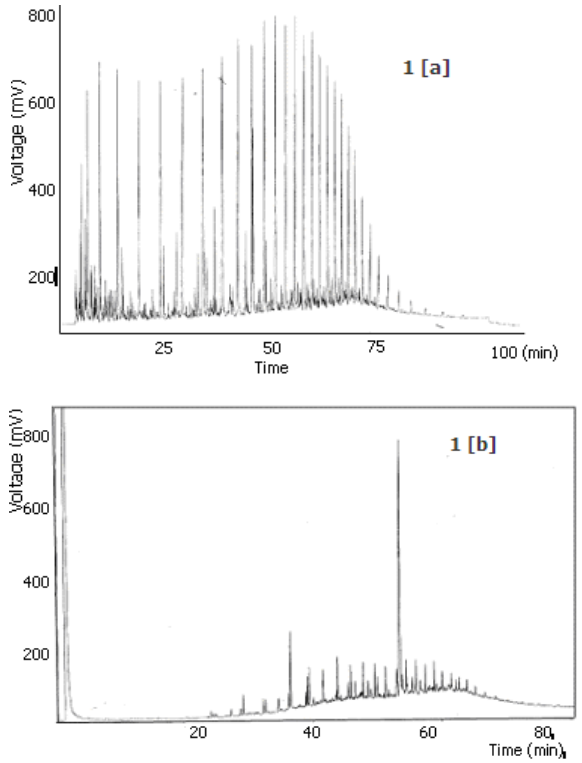


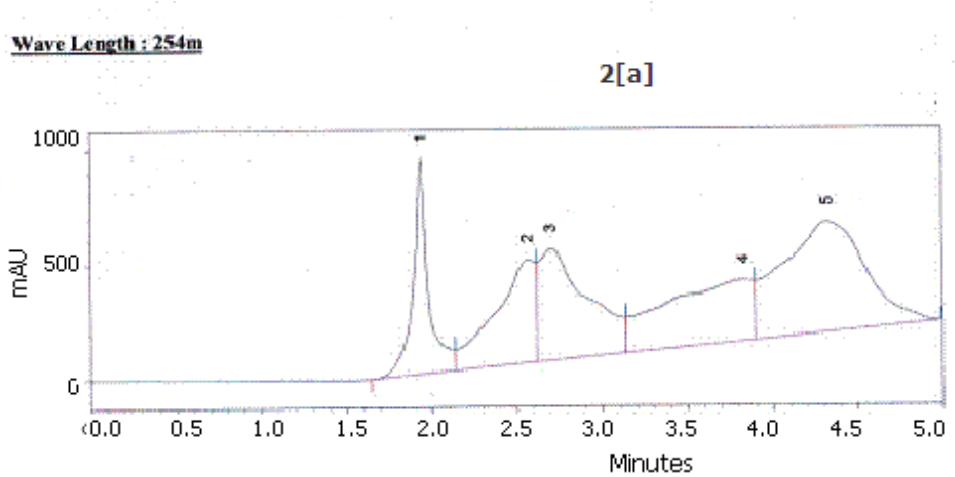
Fig 1. Gas chromatography fingerprinting of (a) Initial (Zero Day) Sobhasan Crude Oil and (b) after 60 days of biodegradation of crude oil obtained from Box 5 (Nutrient +Tilling +Consortium).

3.7. *Aromatic hydrocarbon degradation study*

HPLC analyses of aromatic fractions of Sobhasan crude oil (deployed in microcosm study) were done after 60 days period only (Figs. 2a,b). The effect of indigenous microflora as well as seeded consortium was observed. The widely accepted ratios of biodegradability di/tri aromatic and di/di + tri aromatic were calculated and are given in Table 4. The results showed that the ratio of di/tri aromatics decreased from initial 0.63 to 0.25 with progressive treatment of nutrient addition, nutrient+tilling, nutrient+tilling+microbial seeding. Similar effect was observed in di/di+tri aromatics ratios which also decreased from 0.38 to 0.20. The results indicate that the developed consortium could help in degradation of higher aromatics, as ratio was decreased from 0.31 to 0.20 by bioaugmentation only. Thus developed consortium could help in detoxification of the harmful and carcinogenic aromatics specially polyaromatic hydrocarbons.

Table 4. Microcosm study by degradation of aromatics.

Treatments	Monoaromatic (%)	Diaromatic (%)	Triaromatic (%)	Di/tri aromatic (%)	Di/di+triaromatic (%)
Initial (Sobhasan oil)	11.87	34.36	53.77	0.639018	0.3898785
Weathering control	10.14	32.68	57.18	0.5715285	0.3636768
Nutrient addition	11.97	28.2	59.83	0.4713354	0.3203453
Nutrient+tilling	17.73	25.75	56.51	0.4556715	0.3130318
Nutrient+tilling+ consortium	11.96	17.45	67.71	0.2577167	0.2049084



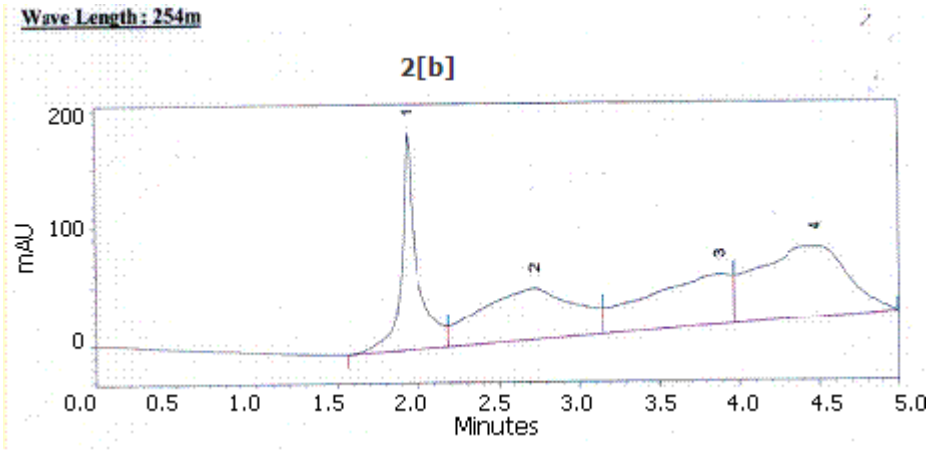


Fig. 2. HPLC analysis of aromatic fraction of (a) Unaltered preserved sobhasan crude oil and (b) after 60 days bioremediation of crude oil obtained from Box 5 (Nutrient+Tilling+Consortium).

4. Conclusion

The present study clearly demonstrates that application of a carrier-based bacterial consortium, improvised after isolation of potent hydrocarbon degraders from the oil contaminated site, can be used to remediate soil contaminated with crude oil. Maintenance of proper environmental factors is an essential aspect for treating an oil spill by biotechnological methods. In view of synergistic and antagonistic relationship among the various strains of bacteria, bioremediation is considered as site-specific approach.

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