

Polycyclic aromatic hydrocarbon degradation by developed consortium in microcosms study.

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Abstract

A feasibility study was conducted prior to onsite full-scale bioremediation study. This feasibility study has investigated the possibility of bioaugmentation of polycyclic aromatic hydrocarbon (PAH)-polluted soil with PAH-degrading strains or consortia. Five treatment options- Oil alone, Oil + Indigenous microflora, Oil + nutrients, Oil + nutrient + tilling, Oil + nutrient + tilling + developed consortium were evaluated. Out of five treatments the application of bacterial consortium, nutrients and environmental factors resulted in 79.16% removal of TPH in 60days, compared to 30.24% removal of TPH carried out by indigenous microflora. The results of High-pressure liquid chromatographic analysis 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 0.31 to 0.20 by bioaugmentation only.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) constitute a group of priority environmental pollutants, which are ubiquitous contaminants in soils and sediments and are of environmental concern because of their toxic, mutagenic and/or carcinogenic effects (Mastrangelo et al., 1996 Marston et al., 2001; Xue & Warshawsky, 2005, Mallick et al., 2007).

Bioremediation is a technique that enhances the natural rate of biodegradation of pollutants through reactions carried out by selected microorganisms (Providenti et al., 1993). Bioremediation of agricultural land polluted with polycyclic aromatic hydrocarbons (PAHs), can be achieved in two ways: by enhancing the growth and activity of microorganisms already present at the site of pollution through nutrient addition (a process, called by some "biostimulation"), and by adding more selected microorganisms (Bioaugmentation) to the pollution site (Ted and Udall, 1991; Andrew and Jackson, 1996). The use of bacteria to achieve these functions has been widely studied (Atlas, 1981; Rocha et al., 1992; Kampfer et al., 1991; Mercade et al., 1996). Various studies have investigated the possibility of bioaugmentation of polycyclic aromatic hydrocarbon (PAH)-polluted soil with PAH-degrading strains or consortia.

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 (Prichard and Bourquin, 1984). Microcosms can vary in complexity from simple static soil jars of contaminated soil to highly sophisticated systems designed to enable variations in various environmental parameters encountered on site to be more accurately simulated in the laboratory.

A feasibility 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 # Y. Five treatment options- Oil alone, Oil + Indigenous Microflora, Oil + Nutrients, Oil + Nutrient (Fertilizer) + Tilling (Aeration), Oil + Nutrient (Fertilizer) + Tilling (Aeration) + Developed consortium were evaluated. 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 aromatic fractions of crude oil by High pressure liquid chromatographic (HPLC) analysis 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.

MATERIAL AND METHODS

Isolation and characterization of bacterial strain: The test organisms used in this study (*Pseudomonas* (PSI, PSII, and

PSIII) were isolated from Oil production site of ONGC, oil wells from Sobhasan oil field project, Gujarat. in western India, as described elsewhere (Mittal et al., 2005). by the enrichment culture technique with crude oil as the sole source of carbon and energy. 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 (Mittal et al., 2005), using crude oil as the sole carbon and energy source. These three bacterial isolates could degrade aliphatic and aromatic fractions of the crude oil. These selected isolates were characterized and identified using biochemical tests and 16S rRNA sequencing from Tata Energy Research Institute (TERI) Labs, New Delhi, India. Three of these isolates were identified: *Pseudomonas* (PSI, PSII, and PSIII). All the constituent strains of the selected consortium were stored in 25% glycerol at -70°C .

CARBON SUBSTRATES

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

ANALYSIS OF SOIL AT THE BIOREMEDIATION SITE

Physical and chemical properties of soil samples 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.

SCALING UP OF BIOINOCULANT CONSORTIUM

To prepare the inoculums, bacterial isolates were grown separately in 2-liter Erlenmeyer flasks containing 500 ml of mineral medium and molasses (2%) as the sole carbon and energy source. All the isolates were grown to mid-log phase (108CFU/ml) and they mixed in equal proportions. The mixed culture was used as the inoculum (5%) for large-scale culture in a bioreactor (Bioflow 3000, New Brunswick) with a working volume of 10 liters. The same minimal salt medium with molasses (2% wt/vol) as sole carbon source was used in the bioreactor for large-scale culture. The growth conditions in the bioreactor were as follows: temperature 32°C ; aeration, 0.75 volume of air/volume of medium / min; agitation, 250 rpm; pH 7.0 (adjusted with 1N HCl-NaOH); and duration of growth, 15 h. Silicone oil was added to control excessive foaming in the bioreactor. After growth, the culture was immobilized onto the selected carrier material, corncob powder (a biodegradable

agriculture residue) by simple mixing of carrier material and culture in ratio of 1:3. The carrier-based culture was dispensed into sterile reusable polythene bags (4 kg of culture immobilized onto carrier material in each 10 kg polythene bag) and stored at 4°C after the bag was aseptically sealed. In previous study, various carrier materials were screened to determine the survival of carrier-based culture by monitoring the CFU counts (on LBA plates) at 15-days intervals, upto 90 days (Mittal et al., 2005).

EXPERIMENTAL DESIGN AND TREATMENTS

The total area (225 m²) of the feasibility study was divided into 20 Blocks (four replicate Block for each treatment), 1 by 1 m each and separated by a gap of 2 m. The experimental design chosen was a completely randomized block design. The treatments were as follows: (i) Oil alone; control where no treatment was done. (ii) Oil + Indigenous Microflora (iii) Oil + Nutrients (iv) Oil + Nutrient (Fertilizer) + Tilling (Aeration) (v) Oil + Nutrient (Fertilizer) + Tilling (Aeration) + Developed consortium. In each Block garden soil was contaminated experimentally with Sobhasan crude oil (approximately 2% v/w), up to the height of 6 inches. 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 soil used for microcosm study has a pH of 7.88, which is well within the range of optimal degradation. Therefore, no treatment for pH control is needed. The experiment was conducted in the premises of Kanya Gurukula Mahavidyalaya (G.K.U), Haridwar. The experiment was conducted during September to November months, in the year 2006. During the course of study, the following five sets of treatment were conducted.

Block 1-Oil alone: Soil was autoclaved at 121°C for 30 minutes and soil was treated with 2% HgCl_2 after one month time interval to destroy all microflora (Poisoned control). It is used to monitor abiotic hydrocarbon losses.

Block 2-Oil + Indigenous Microflora: It is essentially a no action control, wherein the initial state of the microcosm is left unmanipulated throughout the course of the investigation. In this Block no treatment was given except $\frac{1}{2}$ to 1-liter water was added regularly.

Block 3-Oil + Nutrients: This 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 (fertilizer) were applied two times, after

one-month time interval.

Block 4-Oil + Nutrient (Fertilizer) + Tilling (Aeration): It entails the two or three times per weekly tilling of the microcosm contents to promote soil aeration, in addition to nutrient amendment.

Block 5-Oil + Nutrient (Fertilizer) + Tilling (Aeration) + Developed consortium: The developed mixed culture consortium of hydrocarbon degraders (*Pseudomonas* strain PS-I, PSII, and PSIII) was also added in addition to nutrients and weekly tilling.

Throughout the course of study in field treatments were open to air 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 blocks (Block3, Block4 and Block5).

EXTRACTION OF TOTAL PETROLEUM HYDROCARBONS (TPH)

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 was then used for soxhlet extraction. Because the results were desired using dry-weight basis, 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.

FRACTIONATION OF TPH AND ANALYSIS OF FRACTIONS

The group type (saturate, aromatic and NSOs) analysis of whole oils has been carried out in chemito-1000 gas chromatography. For isolation of various compound class fractions (saturate, aromatic and NSOs), column chromatographic technique was used. The separation was carried out on dual packed activated silica-gel/alumina columns by successive elution with petroleum ether for saturates, benzene for aromatics and methanol for NSO compounds.

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 (30cmX4.5mm). Elution was carried out using n-hexane (HPLC grade) and the separation was monitored with UV-visible absorbance detector operated at 245 nm wavelength. The flow rate was maintained at 6.5ml/min.

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 over the period of study. 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.

RESULTS AND DISCUSSION

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. The soil sample taken for feasibility study (garden soil of Kanya Gurukula Mahavidyalaya, Haridwar) was analyzed for detection of different groups of microbes. The soil contains 8×10^8 /g of total heterotrophs, 4.5×10^5 /g of actinomycetes and 1.4×10^4 /g of fungi. The hydrocarbon degrader's count is 2.2×10^4 /g of soil.

CHARACTERIZATION OF SOIL AT THE BIOREMEDIATION SITE

Physical and chemical properties of soil samples taken were analyzed for pH, temperature, moisture level, organic carbon, nitrogen and available phosphorus in each block at zero time intervals i.e. initially and after 60 days of bioremediation after application of microbial consortium.

In- situ bioremediation approach was adopted, after optimizing the factors under laboratory conditions. These optimized conditions were attempted to be maintained during feasibility 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%, nitrogen content - 0.126%,

initially. After contamination with crude oil Organic carbon content (TOC) of soil reached 2.79% and amendment by fertilizer nitrogen content reached to 0.235%.

Figure 1

Table 1. Physical and chemical properties of soil at bioremediation site

^a Mean of 4 mixed soil samples of Block 5.

Property	Mean ^a ± SD at:	
	Time zero	After 60
days		
Moisture content (%)	7.3±0.10	7.14 ± 0.12
pH	7.88 ± 0.20	8.14 ± 0.10
Organic carbon (%)	2.79 ± 0.15	0.56 ± 0.11
Total nitrogen (%)	0.235 ± 0.02	0.180 ± 0.03

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.

FEASIBILITY STUDY

Block 1-Oil alone: The hydrocarbon utilizing bacteria (HUB) count during the study was also found nil, though some heterotrophic bacterial activity in an open environment is not possible. It is because the soil was sterilized and 2% HgCl₂ 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 (Smith et al., 1998; Dutta et al., 2002). The total organic carbon (TOC %) of original soil (before contamination) and contaminated soil was found to be 1.23% and 2.79% respectively. The organic carbon, nitrogen and phosphorous dropped down from 2.79%, 0.126% and 34.70 ppm (available phosphorous) to 2.70%, 0.121% and 34.63 ppm respectively in 60 days, in sterile control box. The pH of the soil dropped from 7.88 to 7.30 (Table-1). This could be due to that HgCl₂ solution is acidic in nature, which was used to hinder microbial activity. Bossert et al., (1984) also included poisoned controls (2% HgCl₂) to monitor abiotic hydrocarbon losses.

Block 2-Oil + Indigenous Microflora: The box -2, which shows the biodegradation by indigenous microflora, is containing 8 X 10⁸ total heterotrophs and 2.2 X 10⁴ hydrocarbon degraders. It has been reported previously that bioremediation is less if the population of hydrocarbon degrading microorganisms is less than 10⁵ cfu/g in soil

(Forsyth et al., 1995). 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 (Zaidi et al., 1988; Kastner et al., 1998; Sims et al., 1992) (& Fig.1). The TOC content reduced from 2.79 to 1.942, which was due to biodegradation of crude oil present in soil. The nitrogen and phosphorous concentrations also decreased (Table-1, Fig.4.2).

Block 3-Oil + Nutrients: In this Box, loss in TPH was 49.89% in 60 days, which indicate that, fertilizer addition increases the rate of biodegradation as compared to box-1 and box-2 in which no fertilizer treatment was given (Table-2). In Box-3, 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. Secondly, tap water (from over head tanks) that was being used for treatment of soil boxes had pH of 7.60. The source of water was deep tube well of Kanya Gurukula Vishwavidyalaya. If such a high pH water is being used, it will cause in drop of pH. 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 & Fig.4.3). Roling (2002) reported that nutrient amendment over a wide range of concentrations significantly improved oil degradation, confirming the low concentration of nitrogen & phosphorous limited degradation.

Block 4-Oil + Nutrient (Fertilizer) + Tilling (Aeration): The loss of TPH is 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 (Paul and Clark, 1989).

Block 5-Oil + Nutrient (Fertilizer) + Tilling (Aeration) + Developed consortium: 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). (Eriksson et al., 1995, Lal et al., 1996). 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 & Fig.4.5).

The temperature recorded during the study varied from 25°C - 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. Brady (1990) suggested that biodegradation rates increase with increasing soil temperature upto a certain maximum temperature, which would be optimum for indigenous hydrocarbon degraders (Barker and Neumann, 1993). 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. (Bossert and Bartha, 1984; Sims et al., 1989). To maintain this sufficient water was added regularly to hydrate the soil to 50% of its water holding capacity.

Figure 2

Table-2 Crude oil degradation in various treatments Boxes

Treatment	Crude oil				
	Incubation period (days)	Initial conc.(gm)	Final conc.(gm)	Degrad -ation(gm)	%Degrad -ation
Oil alone	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
Oil+Indigenous microflora	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
Oil+Nutrients	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
Oil+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
Oil+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

SURVIVAL OF INTRODUCED CONSORTIUM

Survival of the microorganisms in the soil after their application is a deciding factor in rate of degradation of hydrocarbons (Ramos et al., 1991). 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 9.20×10^8 during 60 days of bioremediation study. This increase in microbial count indirectly indicates survival of introduced consortium.

AROMATIC HYDROCARBON DEGRADATION STUDY

HPLC analysis of aromatic fractions of Sobhasan crude oil (deployed in microcosm study) was done after 60 days period only (fig.1). 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 & are given in table 3. 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.

Figure 3

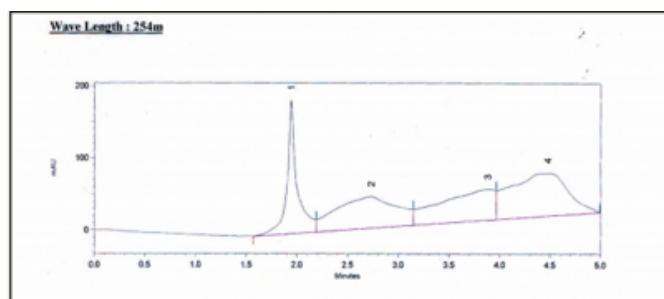
Table 3: Microcosm study: Degradation of Aromatics

Treatment	Monoaromatic (%)	Diaromatic (%)	Triaromatic (%)	Di/tri aromatic (%)	Di/di+tri aromatic (%)
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	28.20	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

Crude oil used: Sobhasan crude oil well # Y.

Figure 4

Fig1. HPLC analysis of aromatic fraction of Sobhasan crude oil obtained after 60 days of bioremediation from Block 5(Oil + Nutrient + Tilling + Developed consortium).



A carrier-based formulation made it easy to transfer the microorganisms from the laboratory to the field. Corncob powder (agricultural byproduct) was found to be the best carrier for this purpose as survival of 7.2×10^8 cfu/g was recorded during storage over 3 months. Corncob powder, being a good porous material facilitates growth and exposes a greater surface area to microbial action.

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.

References

- r-0. Andrew, R.W. J., and Jackson, J.M. (1996). Pollution and waste management. In: Environmental Science. The Natural Environment and Human Impact. Longman, Singapore Publishers Ltd.
r-1. Atlas, R. M. (1981). Microbial degradation of petroleum hydrocarbon: An environmental perspective. Microbiol. Rev.

45:180-209.

- r-2. Barker, G.W., Neumann, S.N. (1993). Bioremediation of hydrocarbon contaminated surface soil at production site. Proceedings of the CoBioReM (Cooperative Bioremediation Research for Michigan) on bioremediation of petroleum hydrocarbons in soils and groundwater. Michigan oil and Gas association. Lansing, Michigan.
r-3. *Brady, N.C. (1990). The nature and properties of soils. Macmillan Publishing Co. New York.
r-4. Bossert, I. and Bartha, R. (1984). The fate of Petroleum in soil ecosystems. In : Petroleum microbiology, pp. 434-476. (Ed. R.M. Atlas), Macmillan Publishing Co. New York.
r-5. Dutta, V. (2002). Bioremediation for oil pollution. Science Reporter, 4: 22-25.
r-6. Erickson, M.G., Dalhammar, A.K., Borg-Karson, (1995). Aerobic degradation of hydrocarbon mixture in natural contaminated potting soil in indigenous microorganisms at 20°C and 6°C. Appl. Microbiol. Biotechnol. 51: 532-535.
r-7. Forsyth, J.V., Taso, Y.M., Bleam, R.D. (1995). Bioremediation: when bioaugmentation needed? In: Bioaugmentation for sites remediation (Eds. R.E Hinchey, J. Fredrickson, B.C. Allrman). Columbus, OH : Battelle Press, pp 1-14.
r-8. Kampfer, P., Steiof, M. and Dott, W. (1991). Microbial characterization of a fuel oil contaminated site, including numerical identification of heterotrophic water and soil bacteria. Microbiol. Ecol. 21: 227-251.
r-9. Kastner, M., Jammali, B.M., Mahro, B. (1998). Impact of inoculation protocols, salinity, and pH on the degradation of polycyclic aromatic hydrocarbons (PAHs) and survival of PAHs degrading bacteria introduced into the soil. Appl. Environ. Microbiol. 64 (1) 359-362.
r-10. Lal, B., Khanna, S. (1996). Degradation of crude oil by Acinetobacter calcoaceticus and Alcaligenes odorans. J. Appl. Bacteriol. 81:355-362.
r-11. Mallick, S., Chatterjee, S., and Dutta, K. T., (2007). A novel degradation pathway in the assimilation of phenanthrene by Staphylococcus sp. strain PN/Y via meta-cleavage of 2-hydroxy-1-naphthoic acid: formation of trans-2,3-dioxo-5-(2'-hydroxyphenyl)-pent-4-enoic acid.
r-12. Marston, C. P., Pereira, Z. C., Ferguson, J., Fischer, L., Hedstrom, O., Dashwood, W. M. & Baird, W. M. (2001). Effect of a complex environmental mixture from coal tar containing polycyclic aromatic hydrocarbons (PAH) on tumor initiation, PAH-DNA binding and metabolic activation of carcinogenic PAH in mouse epidermis. Carcinogenesis 22, 1077-1086.
r-13. Mastrangelo, G., Fadda, E. & Marzia, V. (1996). Polycyclic aromatic hydrocarbons and cancer in man. Environ Health Perspect 104, 1166-1170.
r-14. Mercade, M.E., Monleon, L., de Andres, C., Rodon, I., Martinez, E., Espuny M.J., and Manresa, A. (1996). Screening and selection of surfactant-producing bacteria from waste lubricating oil. J. Appl. Bacteriology, 81: 161-166.
r-15. Mishra, S., Lal, B., Jyot, J., Kuhad, R.C., Rajan, S., Khanna, S. (1999). Field Study: in-situ bioremediation of oily sludge contaminated land using "oilzapper." 31st Mid-Atlantic Industrial and Hazardous Waste Conference at University of Connecticut.
r-16. Mittal A, Studies on biodegradation of Onland oil spill, Ph. D. Thesis, Gurukul Kangri University, Haridwar, India, 2005.
r-17. Osuji, L. C., Opiah, U.C. (2007). Hydrocarbon contamination of a terrestrial ecosystem: the case of oshire-2 oil spill in Niger delta, Nigeria. Environmentalist. Springer Science. 27: 337-340.

- r-18. *Paul, E.A., Clark, F.E. (1989). Soil Microbiology and Biochemistry, Academic press, New York, pp. 46-48.
- r-19. Pfenning, N., Widdel, F & Pruper, H. Q. (1981). Isolation and Identification of bacteria, in the dissimilatory sulphate reducing bacteria. Prokaryotes A- Handbook of habitats, edited by M P Starr, H Stolp, HG Pruper, A Balow & HG Schigel (Verlin, Springer, Verlag) 926-940.
- r-20. Pometto, A.I., Oulman, C.S., Dispirito, A.A., Johnson, K.E., Baranow, S. (1998). Potential of agricultural by-products in the bioremediation of fuel spills. J. Indust. Microbiol. Biotechnol. 20:369-372.
- r-21. Pritchard, P.H., Bourquin, A.W. (1984). The use of microcosm for evolution of interactions between pollutants and microorganisms. In: Advances in microbial ecology. vol.7, pp 133-215. (Ed. K.C. Marshall), Plenum press, New York.
- r-22. Providenti, M.A., Lee, H. and Trevors, J.T. (1993). Selected factors limiting the microbial degradation of recalcitrant compounds. J. Ind. Microbiol. 12:379-395.
- r-23. Ramos, J.L., Duque, E., Ramos-Gonzalez, M.I. (1991). Survival in soils of an herbicide-resistant *Pseudomonas putida* strain bearing a recombinant TOL plasmid. Appl. Environ. Microbiol. 57:260-266.
- r-24. Ramos, L.S., Brown, D.W., Jenkin, R.G. and Macleod, J.W.D. (1979). Modification of conventional gas chromatographic inlets for the use of glass capillary columns. National Bureau of standards special Publication No. 519.
- r-25. Rocha, C., San-Blas, F., San-Blas, G. and Vierma, L. (1992). Biosurfactant production by two isolates of *Pseudomonas aeruginosa*. World J. Microbiol. Biotechnol. 8: 125-128.
- r-26. Roling, W.F.M., Milner, M.G., Jones, D.M. Lee, K., Daniel, F., Swannell, R.J.P., and Head, I.M. (2002). Robust hydrocarbon degradation and dynamics of bacterial communities during nutrient enhanced oil spill bioremediation. Applied and Environmental Microbiology, Vol.68, No. 11, pp. 5537-5548.
- r-27. Sathishkumar, M., Binupriya, A. R., Baik., Sang-Ho., Yu, Sei-Eok. (2008). Biodegradation of Crude Oil by Individual Bacterial Strains and a mixed bacterial consortium isolated from hydrocarbon contaminated areas. Clean-Soil air water. Wiley-Inter science. 36: 92 – 96.
- r-28. Sim, J.L., Sim, R.C. and Matthews, J.E. (1989). Bioremediation of contaminated surface soils. EPA-600/9-89/073. Robert S. Kerr. Environmental Research Laboratory. Oklahoma.
- r-29. *Sims, J.L., Sumtilita, J.M. and Russell, H.H. (1992). In-situ Bioremediation of contaminated ground water, EPA/540/5-92/003.
- r-30. Smith, V.H., Graham, D.W. and Cleland, D. (1998). Application of resource-ratio theory to hydrocarbon biodegradation. Environ. Sci. Technol. 32:3386-3395.
- r-31. Ted, S. and Udall, M.K. (1991). The use of genetically engineered microorganisms, plants and animals In: Impacts of applied Genetics of microorganisms, plant and animals. Congress of the U.S.A., Office of Technology Assessment, Washington DC, 1: 117-132.
- r-32. Whittenbury, R., Phillip, K.C., and Wilkinson, J. E. (1970). Enrichment, isolation and some properties of methane utilizing bacteria. J. Gen. Microbiol. 61:205-218.
- r-33. Xue, W. & Warshawsky, D. (2005). Metabolic activation of polycyclic and heterocyclic aromatic hydrocarbons and DNA damage: a review. Toxicol Appl Pharmacol 206, 73–93.
- r-34. Zaidi, B.R., Sucki, G. and Alexander, M. (1988). Low chemical concentration and pH as factors limiting the success of inoculation to enhance biodegradation. Environ. Toxicol. Chem. 7:143-151.

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