

PAHs Utilizing Bacterial Population and Physico-chemical Variability in Oil Contaminated Soils

Kumar Arun ^{1,*}, Sawhney Rajesh ², Munjal Ashok ¹, Singh RN ³

1. Banasthali Vidyapith, Banasthali 304022, Rajasthan, India
2. Bhojia Institute of Life Sciences, Budh, Baddi, Distt. Solan (H.P) 173205, India
3. S.B.S (PG) Institute of Biomedical Sciences and Research, Balawala, Dehradun 248161, India
arunkaran84@yahoo.com, sawhneyrajesh@yahoo.com

Abstract: Poly-aromatic hydrocarbons (PAHs), the known xenobiotic agents, persist as potentially hazardous soil contaminants. Various bioremediation strategies (indigenous microflora, introduced microorganisms, priming with bioremediated soil), applied for their removal from polluted soils, have rarely been successful. Biostimulation (nutrient amendment) and bioaugmentation (suitable microbial population) have shown accelerating effect on naturally occurring biodegradation of PAHs. However, a plausible bioremediation tool is still awaited. A study was conducted to examine PAH utilizing population and physico-chemical characteristics of the oil contaminated soil from different geographical locations. This could evolve an effective soil inoculum, with defined soil properties, for use as a bioremediation and bioaugmentation tool. Sixteen oil contaminated soil samples (AS-1 to AS-16) were collected from different sites under varying geographical locations in India. All the samples were analyzed for moisture content, pH, carbon (%), total organic content (TOC), inorganic phosphate, nitrate, poly-aromatic hydrocarbons (PAHs) and PAHs utilizing bacterial populations. All the samples showed different soil characteristics. AS-15 exhibited maximum pH (9.82) where as minimum pH (3.47) was observed in AS-8. The soil sample (AS-4), with higher content of PAH, Chloride, inorganic phosphate, TOC, % carbon, moisture and pH (7.45) was found to harbor comparatively higher PAH utilizing bacterial population. The results of this study highlight the possible higher degradation potential of PAHs utilizing bacteria under natural conditions. This study provides a baseline to understand the population dynamics, possible biodegradation potential of oil contaminated soils, at different locations, and may be used to prepare an appropriate inoculum with defined soil characteristics to evolve a plausible bioremediation technology.

[Kumar Arun, Sawhney Rajesh, Munjal Ashok, Singh RN. PAH Utilizing Bacterial Population and Physico-chemical Variability in Oil Contaminated Soils Nature and Science 2011;9(6):32-37]. (ISSN: 1545-0740). <http://www.sciencepub.net>.

Key words: poly-aromatic hydrocarbons (PAHs), carbon, total organic content, oil contamination

1. Introduction

Poly-aromatic hydrocarbons (PAHs) have been identified as xenobiotic agents causing toxic, carcinogenic and tetragenic effects on life (Ruma et al., 2007). United States Environmental Protection Agency (USEPA) has designated sixteen PAHs (naphthalene, acenaphthylene, acenaphthene, fluorine, phenanthrene, anthracene, fluoranthene, pyrene, benz-a-anthracene, chrysene, benzo-b-fluoranthene, benzo-k-fluoranthene, benzo-a-pyrene, dobenz-a-h-anthracene, benz-g-h-i-perylene and indeno-1,2,3-cd-pyrene), as priority pollutants. They originate from incomplete combustion of fossil fuels, oil spoilage near to oil reservoirs, forest fire and pyrolysis of organics (Kanaly et al., 1997). These are lipophilic in nature and are relatively insoluble in water, and persist as potentially hazardous soil contaminants (Johnsen et al., 2005, Hafez et al., 2008). Several studies such as the use of indigenous microflora, introduced microorganisms, priming with bioremediated soil and treatment with

nitrogen, phosphorus and potassium have been carried out for the degradation or removal of PAHs from polluted soils (Juhasz et al., 2005, Johnsen et al., 2005, Johnsen et al., 2007, Hafez et al., 2008). These studies have rarely been promising. The physico-chemical factors control microbial metabolic and nutritional requirements, and the availability of pollutants to the microbial population (Lion, 1990, Amellal et al., 2001, Johnsen et al. 2005). Thus, de-aromatization of the PAHs could be influenced by the physico-chemical and biological characteristics of the soil. In present study, an effort has been made to determine the PAH utilizing population and the physical/chemical properties of different oil contaminated soils. This work provides a baseline to understand population dynamics and possible biodegradation potential of oil contaminated soils at different geographical locations. The findings would provide an appropriate soil inoculum with defined soil characteristics, and explore further possibilities to evolve a plausible bioremediation

technology.

2. Materials and Methods

2.1 Chemicals and Reagents

All the chemicals used in this study, were of analytical grade. Anthracene was obtained from Sigma-Aldrich (Copenhagen, Denmark), and other chemicals and media were obtained from Himedia (India).

2.2 Collection of Soil Samples

Subsurface (1-10 cm below the surface) oil contaminated soil samples were collected in sterile plastic bottles from different sites. The samples were coded as AS-1 to AS-16 and were stored at low temperature (4°C) for further investigations.

2.3 Moisture Content

10 g soil of each sample was weighed (w_1) and dried at 100°C in a hot air oven (Narang Scientific Works, New Delhi.) for 24 hours. The final weight (w_2) of each sample was recorded using an electronic weighing balance (Sansui, Japan), and the moisture content (w_1-w_2) was determined as mg of moisture per gram soil.

2.4 pH

10% (w/v) suspension of air dried soil of each sample was prepared in de-ionized milli-Q water. This suspension was allowed to settle for one hour and filtered through Whatman filter paper no.42. pH for all the soil filtrates was checked using a calibrated pH meter.

2.5 Total Organic Content (TOC)

250 mg of air dried soil of each sample was taken in 250 ml conical flasks, and 5 ml of 1N potassium dichromate solution was added. After that, 10 ml concentrated sulfuric acid was added gradually and the contents were incubated for 30 minutes at room temperature. Then, 100 ml de-ionized water, 5 ml of concentrated Phosphoric acid, 0.1 g of dry sodium fluoride and 0.5 ml of diphenylamine indicator were added sequentially. The contents of the flask were titrated against 0.5 N ferrous ammonium sulfate. The end point was noticed as dull green through turbid blue to brilliant green. Distilled water blank was run simultaneously, and the TOC was calculated as described by Hooda and Kaur, 1999.

$$\text{TOC(mg/g soil)} = 6.791/W (1-T_1/T_2) \times 10$$

Where, T_1 = Volume of titrant used against samples (ml)

T_2 = Volume of titrant used against distilled water blank (ml)

2.6 Poly-aromatic Hydrocarbons (PAHs)

100 ml solvent (n-hexane & dichloromethane, v: v = 1:1) was added to 5 g of soil sample in a soxhlet. After 14 h, contents of the flask were evaporated and concentrated exactly to 3 ml. The PAH content in each soil sample was measured by observing absorbance at 220 nm. (Tsai et al. 2002).

2.7 Inorganic Phosphate Content (PO_4^{3-})

Stock solution was prepared by dissolving 136 mg of KH_2PO_4 in 100 ml distilled water, and diluted ten times. Different volumes viz. 0.2, 0.4, 0.6, 0.8 and 1.0 ml of this solution were taken and the final volume was made to 1.0 ml with distilled water. Simultaneously, the test sample was also prepared by adding 0.1 ml. soil suspension (10% w/v) to 1.0 ml. distilled water. Then, added 1 ml 5 N H_2SO_4 , 1 ml of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ and 0.1 ml ANSA to each test tube containing stock and test sample, and waited for 10 minutes. Final volume in all the test tubes was made to 10 ml and the absorbance (O.D) was measured at 690 nm. (Hooda and Kaur, 1999).

2.8 Nitrate Content (NO_3^-)

10% (w/v) soil suspension was prepared in de-ionized water and was filtered through Whatman filter paper no 42. The filtrate was treated with 0.4 ml concentrated aluminium hydroxide suspension to remove color and avoid the organic interference from the filtrate. The mixture was swirled; the suspension was allowed to settle for 5 minutes, and filtered through Whatman filter paper no 42. Then, 0.1 ml of 1 N HCL was added to 5 ml clear filtrate. A nitrate calibration curve was plotted using a standard curve in range of 0-350 μg of nitrate. The absorbance was recorded at 220 nm and 275 nm (Hooda and Kaur, 1999).

2.9 Chloride Content (Cl^-)

100 ml (10%) soil suspension was taken and the pH was adjusted to 7.0. Then, added 9.0 ml of 5% K_2CrO_4 , stirred well and titrated with 0.0282 N AgNO_3 to a permanent reddish tinge. The chloride content was calculated as outlined by Hooda and Kaur, 1999.

Chloride (mg/g) = [Volume of AgNO_3 used against samples (ml) - Volume of AgNO_3 used against blank (ml)] X [Normality of AgNO_3] X 35.46 X 1000/ mg of soil sample taken.

2.10 Total Colony Forming Unit (CFU)

10% (w/v) soil suspension in sterile normal saline was serially diluted under aseptic conditions. The dilution was inoculated on nutrient agar media using spread plate method, and incubated. The results were recorded as total colony forming units (CFU) per gram

of soil sample (Aneja, 2002).

2.11 PAHs Utilizing Bacterial Population

10% (w/v) soil suspension in sterile normal saline was serially diluted under aseptic conditions. The dilution was inoculated on a synthetic basal salt mineral medium (BSM medium: K_2HPO_4 - 0.38g/l, $MgSO_4 \cdot 7H_2O$ - 0.2 g/l, NH_4Cl - 1.0 g/l, $FeCl_3$ - 0.05 g/l; pH- 7.0) using spread plate method. 0.05% (w/v) anthracene in diethyl ether (DEE) was sprayed on the plates as a carbon source. The plates were incubated at 37°C. The results were recorded as PAH utilizing bacterial population (CFU) per gram of soil sample

(Aneja, 2002).

3. Results and Discussion

Table.1 describes all the sixteen soil sampling sites. Among all the samples analyzed for pH, four samples (AS-1, AS-12, AS-15, AS-16) were found to show pH above 8.0. The samples, AS-7 to AS-9 were acidic in nature. However, nine samples were found to have pH between 7 to 8 (Table 2). AS-8 showed minimum pH (3.47). The maximum pH (9.82) was measured for AS-15 (Table 2a , 2b).

Table 1. Description of Soil Samples

Sample code	Site & expected time of oil contamination	Locations(latitude/longitude/elevation)	Address
AS-1	Workshop & 21 years	30°17'41.52"N/78°03'25.99"E/2078ft	Dehradun, Uttarakhand, India
AS-2	Workshop & 20 years	30°17'39.20"N/ 78°03'49.08"E/2109ft	Dharampur, Dehradun, Uttarakhand, India
AS-3	Workshop & 21 years	30°17'27.55"N/78°03'42.90"E/2095ft	Dharampur, Dehradun, Uttarakhand, India
AS-4	Railway Engine repairing site & 100 years		Haridwar Uttarakhand, India
AS-5	Workshop & 15 years	29°22'51.30"N/78°88'22.82"E/771ft	Bijnor, Uttar predesh, India
AS-6	Workshop & 14 years	29°22'50.05"N/78°08'21.64"E/775ft	Bijnor, Uttar predesh, India
AS-7	Workshop & 16 years	29°22'25.05"N/78°48'29.04"E/774ft	Bijnor, Uttar predesh, India
AS-8	Service centre & 16 years	29°22'34.21"N/78°08'45.45"E/777ft	Bijnor, Uttar predesh, India
AS-9	Service centre & 15 years	29°18'52.69"N/79°30'16.98"E/769ft	Dhampur, Uttar predesh, India
AS-10	Workshop & 15 years	29°19'23.08"N/78°29'53.78"E/773ft	Dhampur, Uttar predesh, India
AS-11	Service centre & 12 years	29°19'17.00"N/78°29'51.45"E/772ft	Dhampur, Uttar predesh, India
AS-12	Service centre & 9 years	23°02'00.11"N/72°33'58.32"E/176ft	Ahemdabad, Gujrat, India
AS-13	Petrol pump & 8 years	23°02'60.41"N/72°13'51.12"E/172ft	Ahemdabad, Gujrat, India
AS-14	Petrol pump & 10 years	25.78°N / 87.47°E	Purnia, Bihar, India
AS-15	Petrol pump & 20 years	25.78 °N / 87.47°E	Purnia, Bihar, India
AS-16	Sulphur water & 100 years	30.385952°N / 78.130903°E	Shashtradhara, Dehradun, Uttarakhand, India

Table 2a. Physico-Chemical analysis of soil samples

Sample Codes	pH of the Samples	Moisture Content (mg/gm soil)	Carbon %	Total organic matter (mg/gm soil)
AS-1	8.09	116±0.48	6.93	119.5±0.015
AS-2	7.58	88±0.35	9.60	165.6±0.024
AS-3	7.40	111±0.38	12.12	209.13±0.042
AS-4	7.45	190±0.33	12.44	214.56±0.350
AS-5	7.59	114±0.39	5.04	86.91±0.078
AS-6	7.99	13±0.14	8.03	138.51±0.022
AS-7	3.55	58±0.22	5.67	97.77±0.042
AS-8	3.47	93±0.42	3.94	67.90±0.056
AS-9	6.27	101±0.28	3.30	57.03±0.072
AS-10	7.55	85±0.28	5.35	92.34±0.003
AS-11	7.51	86±0.54	3.78	65.18±0.054
AS-12	8.36	113±0.27	4.25	73.33±0.011
AS-13	7.48	23±0.32	9.13	157.52±0.028
AS-14	7.52	65±0.38	4.88	84.19±0.088
AS-15	9.82	44±0.26	6.30	119.50±0.008
AS-16	8.37	96±0.26	4.23	82.22±0.102

Table 2b. Physico-Chemical analysis of soil samples

Sample Codes	Inorganic PO ₄ ⁻³ (mg/gm soil)	NO ₃ ⁻ (mg/gm soil)	Cl ⁻ content (mg/gm soil)	PAHs Content (ppm)	PAHs utilizing Bacterial population
AS-1	12.40±0.004	2.56±0.002	123.60±0.35	27.817±0.004	102X10 ³
AS-2	17.60±0.014	0.15±0.006	476.98±0.31	56.759±0.008	150X10 ³
AS-3	10.20±0.026	0.001±0.000	527.09±0.45	85.701±0.009	43X10 ³
AS-4	18.60±0.048	0.002±0.000	544.44±0.50	131.567±0.002	163X10 ³
AS-5	01.60±0.002	2.5±0.004	12.06±0.64	31.471±0.001	19X10 ³
AS-6	15.20±0.066	2.4±0.005	56.24±0.66	56.759±0.009	17X10 ³
AS-7	10.40±0.089	3.5±0.004	102.04±0.24	120.22±0.007	63X10 ³
AS-8	07.40±0.033	3.54±0.004	66.82±0.54	62.432±0.008	13X10 ³
AS-9	14.60±0.018	1.0±0.007	65.67±0.33	120.22±0.004	6X10 ³
AS-10	04.20±0.018	2.52±0.003	114.0±0.31	120.22±0.002	4X10 ³
AS-11	01.40±0.049	0.32±0.006	86.87±0.19	20.028±0.007	57X10 ³
AS-12	02.80±0.076	1.42±0.008	96.0±0.19	29.836±0.008	42X10 ³
AS-13	02.00±0.008	3.42±0.006	50.36±0.34	46.663±0.004	24X10 ³
AS-14	00.08±0.002	2.51±0.004	147±0.64	30.798±0.002	54X10 ³
AS-15	00.06±0.002	4.53±0.003	42.66±0.44	104.548±0.006	8X10 ³
AS-16	02.55±0.004	2.33±0.003	42±0.26	39.451±0.004	11X10 ³

The moisture content measurement showed maximum moisture (190 mg/g) for AS-4. The moisture content in AS-6, recorded as the minimum, was about 1/15th of the highest value (Table 2). The carbon content ranged between 3.30 (AS-9) to 12.44% (AS-4) for different soil samples. Among all the soil samples, minimum total organic content was 57.03 mg/g soil (AS-9), and the maximum was found to be 214.56 mg/g soil for AS-4. The nitrate content of AS-3 and AS-4 was negligible; AS-15 showed highest (4.53 mg/g soil) value. The inorganic phosphate content for different soil

samples varied from 0.06 mg/g soil (AS-15) to 18.60 (AS-4). The chloride content was estimated to be maximum (544.44 mg/g) in AS-4.

The poly-aromatic hydrocarbon (PAH) content was 131.567 ppm in AS-4. The lowest PAH content was observed in AS-11. Similarly, the PAH population was highest (163X10³/g soil) in AS-4. Only 4X10³/g, PAH utilizing bacteria were found in AS-10. It was further noticed that there was significant variation of PAH utilizing population with respect to chloride/PAHs (Figure. 1), total organic matter/nitrate (Figure. 2) and

inorganic phosphate/moisture (Figure. 3). This variation can be attributed to different soil properties and geographical location.

The present studies highlight that all the samples, collected from different locations, vary in their soil parameters. The distribution of organic matter, humus substances, xenobiotics and microorganisms have been found to be affected by soil structure (Jocteur-Monrozier et al, 1991; Christensen, 1992; Kukkonen and Landrum, 1996). It is evident from the findings that the samples collected from site AS-4 (pH 7.45) were rich in soil properties viz. PAH content, chloride, inorganic phosphate, total organic content, carbon (%) and moisture content, as compared to all concentration and the number of PAH degrading bacteria has earlier been reported (Amellal et al., 2001). The highest values of PAH and PAH utilizing bacteria in AS-4 are also suggestive of intermediate aggregate size soil, as evidenced by Amellal et al., 2001. The results indicate possible higher PAH degradation potential of AS-4. Johnsen and Karlson, 2005 found that the PAH degradation potential of PAH polluted soil was also linked to the level of PAH pollution. Our results corroborate with earlier findings that the indigenous PAH degraders respond favourably, when the growth conditions improve (Hestbjerg et al, 2003, Juhasz et al., 2005). The improved levels of soil parameters might be a favorable set of growth conditions for the PAH utilizing bacteria.

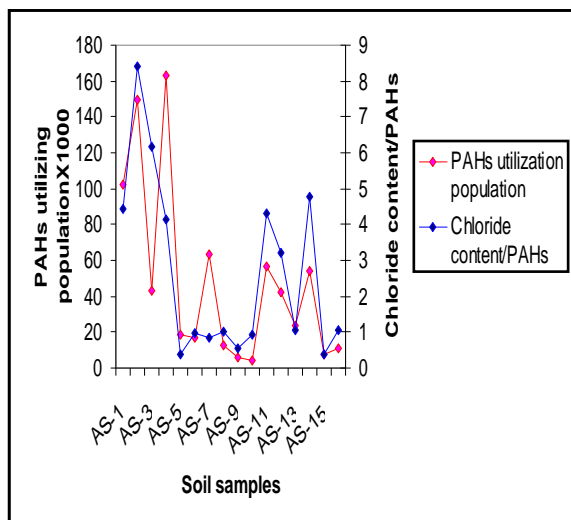


Figure 1: PAHs utilizing population kinetics with Chloride content/PAHs.

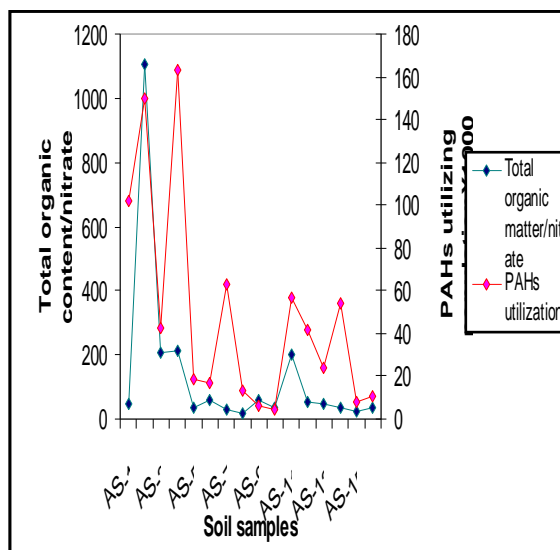


Figure 2: PAHs utilizing population kinetics with Total organic content/nitrate.

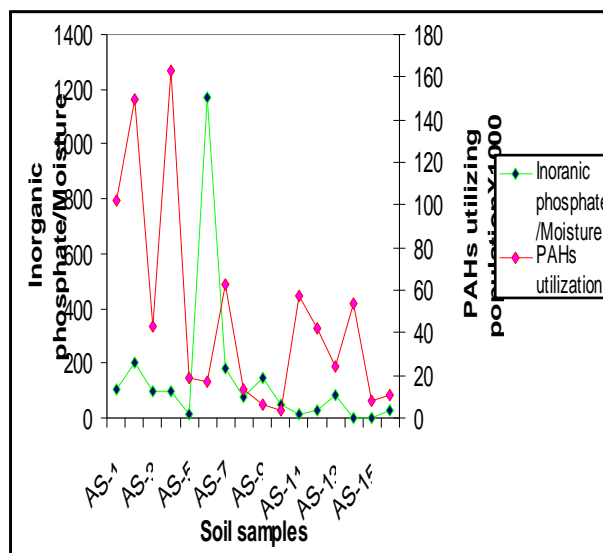


Figure 3: PAHs utilizing population kinetics with Inorganic phosphate/Moisture.

4. Conclusion

Results of this study emphasize on the importance of soil characteristics on PAH utilizing population. This can contribute to develop an effective soil inoculum for use in bioaugmentation and bioremediation of oil contaminated soils. However, in depth studies would be required to establish the bioavailability of the polluting PAHs and their effective biodegradation, to design new protocols and strategies, which could curb the adverse effects of hazardous soil pollutants.

Acknowledgement:

We gratefully acknowledge the support provided by Banasthali Vidhyapith, Banasthali-304022, Rajasthan, India. The help rendered by Bhojia Group of Institutes is also gratefully acknowledged.

Correspondence to:

Arun Kumar
Bhojia Institute of Life Sciences
Budh, Baddi. Distt. Solan (H.P) 173205, India
Telephone: Mobile No.
Phone No. +91- 1795-247621 (Extn. 342)
Fax. No. +91-1795-247621
Cellular phone: +91-9805556005
Emails: arunkaran84@yahoo.com

References

- [1] Aneja KR. Experiments in microbiology, Plant Pathology, Tissue culture and Mushroom production technology. Edition 3, New age international (P) Ltd. Publishers, New Delhi. 2002; 71.
- [2] Amellal N, Portal JM, Vogel T, Berthelin J. Distribution and location of polycyclic aromatic hydrocarbons (PAHs) and PAH-degrading bacteria within polluted soil aggregates. *Biodegradation* 2001;12:49-57.
- [3] Christensen BT. Physical fractionation of soil and organic matter in primary particle size and density separates. *Advances in Soil Science*. 1992;20:1-90.
- [4] Hafez EE, Rashad M, Abd-Elsalam HE, EL-Hanafy AA. The polyaromatic hydrocarbons as a serious environmental pollutants and the role of bioremediation to overcome this problem. Accepted for publication In: Basu SK, Datta Banik S, editor. *Environment, Healthy and Nutrition-Global Issues 2008*; APH Publishing Corporation, New Delhi, India.
- [5] Hestbjerg H, Willumsen PA, Christensen M, Andersen O, Jacobsen CS. Bio-augmentation of tar-contaminated soils under field conditions using *Pleurotus ostreatus* refuse from commercial mushroom production. *Environmental Toxicology and Chemistry*. 2003;22:692-8.
- [6] Hooda S, Kaur S. Laboratory manual for environmental chemistry. Edition 1, S. Chand & Company Ltd.: New Delhi, 1999; 80-85.
- [7] Jocteur Monrozier L, Ladd JN, Fitzpatrick AW, Foster RC, Raupach M. Components and microbial biomass content of size fractions in soils of contrasting aggregation. *Geoderma* 1991;49:37-62.
- [8] Johnsen AR, Karlson U. PAH degradation capacity of soil microbial communities-does it depend upon exposure? *Microbial Ecology*. 2005;50:488-94.
- [9] Johnsen AR, Wick LY, Harms L. Principle of microbial PAH-degradation in soil. *Environmental Pollution*. 2005;133:71-84.
- [10] Johnsen AR, Schmidt S, Hybholt TK, Henriksen S, Jacobsen CS, Andersen O. Strong impact on the polycyclic aromatic hydrocarbon (PAH)- degrading community of a PAH-polluted soil but marginal effect on PAH degradation when priming with bioremediated soil dominated by Mycobacteria. *Applied and Environmental Microbiology*. 2007;73(5):1474-80.
- [11] Juhasz, AL, Waller N, Lease C, Bentham R, Stewart R. Pilot scale bioremediation of creosote contaminated soil-efficacy of enhanced natural attenuation and bioaugmentation strategies. *Bioremediation Journal*. 2005;9:141-57.
- [12] Kanaly R, Bartha R, Fogel S, Findlay M. Biodegradation of (¹⁴C) benzo(a)pyrene added in crude oil to uncontaminated soil. *Applied and Environmental Microbiology*. 1997;63:4511-15.
- [13] Kukkonen J, Landrum PF. Distribution of organic carbon and organic xenobiotics among different particle-size fractions in sediments. *Chemosphere* 1996;32:1063-76.
- [14] Lion LW. Sorption of hydrophobic compounds in aquifer materials: Analysis of methods and effects of organic carbon. *Journal Contaminant Hydrology*. 1990;5:215-34.
- [15] Ruma R, Ray R, Chowdhury R, Bhattacharya P. Degradation of polyaromatic hydrocarbons by mixed culture isolated from oil contaminated soil-A bioprocess engineering study. *Indian Journal of Biotechnology*. 2007;(6):107-13.
- [16] Tsai PJ, Shih TS, Chen HL, Lee WJ, Lai CH, Liou SH. Assessing the contents of polycyclic aromatic hydrocarbons in the tollbooths of a highway toll station via direct and indirect approaches. *Environmental Science and Technology*. 2002;36(22): 4748-53.

3/6/2011