Biodegradation of Polycyclic Aromatic Hydrocarbons (PAHs) in Petroleum Contaminated Soils

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ABSTRACT*: Polycyclic aromatic hydrocarbons are a class of potentially hazardous chemicals of environmental and health concern. PAHs are one of the most prevalent groups of contaminants found in soil. Biodegradation of complex hydrocarbon usually requires the cooperation of more than single specie. In this research biotreatment of PAH (phenanthrene) was studied in a solid-phase reactor using indigenous bacteria isolated from two petroleum contaminated sites in Iran, (i.e., Tehran refinery site with clayey-sand soil composition and Bushehr oil zone with silty-sand soil composotion). Phenanthrene ($C_{14}H_{10}$) was made in three rates (100, 500, and 1000 mg/kg of soil) synthetically and was conducted with two bacterial mixed cultures for a period of 20 weeks. Highest removal (more than 85 %) of phenanthrene with rates of 100, 500 and 1000 mg/kg in clayey-sand soil with BMTRS (Bacterial Mix of Tehran Refinery Site) consortium was achieved within 3, 5 and 14 weeks, respectively as for silty-sand soil composition with BMBOZ (Bacterial Mix of Bushehr Oil Zone) consortium was achieved within 10, 17, and 19 weeks, respectively. Results for phenanthrene biotreatment in solid phase reactor revealed a significance relationship between concentration and type of microbial consortium with the removal efficiency of phenanthrene over the time (P value<0.001). Furthermore, there was a significant relationship between soil type with removal efficiency of phenanthrene over the time (P value=0.022). That means the bioremediation of the lower concentrations of phenanthrene needs shorter time compared with the higher concentrations. Microbial analysis using confirmative series tests and analytical profile index (API) kit tests showed the Pseudomonas fluorescence, Serratia liquefaciens, Bacillus and Micrococcus strains as dominant bacteria in the mixed cultures.*

KEYWORDS: *Polycyclic aromatic hydrocarbons (PAHs), Bioremediation, PAH-degrading microorganisms, PAH contaminated soils.*

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INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) comprise a large and heterogeneous group of organic contaminants which are formed and emitted as a result of the incomplete combustion of organic material [1]. Anthropogenic contamination sources, such as road traffic and combustion of fossil fuels predominate, but there are also natural sources, e.g. volcanic eruptions and forest fires [2, 3].

The U.S. Environmental Protection Agency currently regulates 16 PAH compounds as priority pollutants in water and generally considers them as "total PAH" (tPAH) in contaminated soils. The 16 regulated PAH comprise both low and high molecular weight species, and seven of them are designated as known human carcinogens. All of the carcinogenic PAH (cPAH) are high molecular weight compounds [4, 5].

Bioremediation, which is based on ability of certain species of microorganisms to metabolize PAHs either completely or partially, is an economic and effective means to decontaminate PAH-contaminated soils. It has been shown to be a viable and extremely cost-effective remediation technique for soils contaminated with petroleum-based compounds [4-8].

Phenanthrene, a three-ring angular PAH, is known to be a human skin photosensitizer and mild allergen and is mutagenic to bacterial system [9]. Several different microorganisms with an innate ability to degrade phenanthrene based contaminants have been identified and extensively studied [5, 7-10].

As phenanthrene contains *bay*-and *k-region*, it is used as a model substrate for studies on the metabolism of bay-region and k-region containing carcinogenic PAHs such as benzo[a]pyrene, benzo[a]anthracene and chrysene [11-14]. In this study phenanthrene was selected as representative of PAH compounds for biodegradation purposes.

The purposes of this investigation were the isolation and identification of PAH-degrading microorganisms from petroleum-contaminated soils in some contaminated sites in Iran.

MATERIALS AND MOTHOD

Site investigation and soil sampling

In order to isolate PAH-degrading microorganisms, soil samples were collected from two different petroleum contaminated sites as follows:

1- Tehran Oil Refinery Site

The oil refinery of Tehran is located in the southern part of the city of Tehran, which has produced a variety of oil products e.g., gasoline, fuel oil and other oils from crude oil for the past 36 years.

According to field studies, the site is polluted by oily water effluents and emissions from combustion stacks. This site was chosen as an example of oil refinery site.

2- Bushehr Oil Zones

Bushehr oil zone, located on the coasts of Persian Gulf, is one of the most important crude oil extraction zones in Iran. The soil samples were collected from petroleum-contaminated soil which is located between Genaveh and Daylam ports, near the Imam Hassan village. This site was selected as an example of oil extraction site.

Baseline soil samples for contaminant concentrations, soil chemistry and microbiology were collected from aged contaminated soils around the above mentioned two sites. The samples were collected in the range of 3-4 kg from surface and 10 cm deep layer of petroleum-contaminated soil. Prior to conducting any analysis on collected soils, the coarse pieces e.g., stones and debris were separated and the remaining were mixed well. The sub-samples were kept cold (3-5) ºC for isolation of microorganisms. Microbial analyses were conducted within 24-48 hrs after sampling.

With respect to two different compositions of soils in two selected contaminated sites, two microbial mixed cultures were separated and used for pilot plant studies. Consortium of BMTRS was derived from Tehran refinery site with clayey-sand mixture (CS) and consortium of BMBOZ was derived from Bushehr oil zone with silty-sand (SS) mixture.

Soil microbial analysis

Initial microbial isolation from contaminated soils was carried out by mixing of 1g soil with 10 mL of sterile $Na_2P_2O_7$ solution 0.025 M (2.8 g/L) in 50 mL capacity sterile Erlenmeyer which was mixed by a magnetic shaker for 2 hrs in 250 rpm [14]. The soil particles were allowed to settle for 30 minutes. The supernatant was diluted and plated on solid media. Initial microbial analysis was conducted both for fungi and bacteria. Fungi determination test was carried out on solid media of

HPC (heterotrophic plate count agar) containing chloramphenicol and cyclohexamide compounds for inhibition of bacterial growth [15].

Total CFU (Colony Forming Unit) was determined using plate count agar using HPC (Heterotrophic Plate Count) and BHI (Brain Heart Infusion Agar) media. Also MPN (Most Probable Number) analyses were conducted using lactose broth media with 15 tubes method [15]. After dilution of extracted polluted samples by 10^{-3} - 10^{-5} times with sterile distilled water, they were plated on solid media (BHI agar). The number of bacterial colonies on plate was counted based on standard methods [15]. For identification and isolation of individual bacteria confirmative series tests and also analytical profile index (API) kit test were applied [15, 16].

Microbial Adaptation and enrichment procedure

Adaptation and enrichment procedures involved an initial addition of 1 g of contaminated soil containing origin bacterial concentration around of 10^4 as CFU/g with synthetic concentration of 10, 50 and 100 mg/L phenanthrene, to 50 mL sterile MSM (Mineral Salt Medium), which maintained at 30 ^oC and 200 rpm in shaker incubator. After 7 days, 5mL of each sample was withdrawn and added to new samples with the same concentration of phenanthrene and MSM. Enrichment procedure was repeated for 8 times. After a two month period, bacterial population number and phenanthrene concentration were analyzed. Results showed a reasonable reduction of phenanthrene with bacterial population of 10^5-10^8 CFU in all samples.

A mixed consortium was used as bacterial source. The consortium was revived in culture medium which consisted of MSM based on phenanthrene concentration and ratio of C/N/P in the range of 100/10/2 in the soil samples [17]. MSM composition is presented in table 1.

Soil physico-chemical analysis

Fundamental soil physico-chemical properties were analyzed through following standard methods:

Soil particle size distribution was determined based on the Unified Soil Classification System (USCS), Organic carbon by ISO/DIS 11277 and ISO/DIS 10694 methods [18]; Soil moisture content [15]; Polycyclic aromatic hydrocarbon, phenanthrene, based on modification of USEPA methods #3550B, #8310 and

Table 1: Specification of prepared mineral salt mediums (MSM) for different concentrations of phenanthrene

Compounds	MSM_{100} [a]	MSM_{500} [b]	MSM_{1000} [c]
HK_2PO_4 (g/L)	0.11	0.55	1.1
NH_4Cl (g/L)	0.18	0.9	1.8
NKO_3 (g/L)	0.35	1.75	3.5
Na_2SO_4 (g/L)	1	2	2
$MgSO_4.7H_2O$ (g/L)	0.2	0.3	0.3
TES[d] (mL)	1	1	1
pH	6.5-7	6.5-7	6.5-7

(a) MSM_{100}= 100 mg/kg of phenanthrene
(b) MSM_{500}= 500 mg/kg of phenanthrene
(c) MSM_{1000} = 1000 mg/kg of phenanthrene
(d) Trace = Trace element solution

NIOSH #5506 by HPLC with UV detection [19, 20]. Phenanthrene concentration was measured by HPLC with chromatographic conditions as follows:

- Analytical column: C_{18} Ultra Sep ES PAH QC Speica, 60 × 2 mm ID
- Flow rate: 0.5 mL/min
- Injection rate: 50 µl
- Elute: Acetonitrile/water: (40-100) %
- UV detector wavelength: 254 nm

According to pre-test results the optimum elute condition for phenanthrene was determined at 60/40 (acetonitrile/water) [21].

In this study, extraction was conducted by ultrasonication method using ultrasonic instrument (RK31H, Bandelin Electronic, Sonorex, and 35 kHz, made in Germany). This method is an efficient extraction process compared with other extraction methods such as: Soxhlet Extraction, Supercritical Fluid Extraction using CO_2 and Microwave Assisted Extraction [7].

In our modified procedure 1g of contaminated soil was dried at room temperature and after sieving (using sieve #50), was suspended in 10 mL of acetonitrile and extracted by ultrasonic bath at 40-45 ºC for 60 minutes. Extracts were settled for 10 minutes and centrifuged at 6000 rpm for 15 minutes. Elutes were filtered by 25 mm, 2 µm pore size, PTFE membrane filter and submitted to HPLC-UVD analysis [9].

Pilot plant design and operation

Bioventing technique, a kind of bioremediation technologies using artificial air flow, was selected for

Table 2: Microbial and Physico-chemical specifications of petroleum contaminated soil samples.

Properties	Sample Location	
	Oil refinery of Tehran	Bushehr Oil Zone
Initial Phenanthrene Conc. (mg/kg)	60	12
Microbial population (CFU)	8.5×10^4	3×10^4
Gravel (%)	0	1.24
Sand (%)	90	91.86
Silt (%)	--	6.9
Clay (%)	10	--
Moisture (%)	6.4	1
pH	6.8	7.1
Organic carbon (%)	7.89	7.312
Bulk density (g/mL)	0.7	0.6
Uniformity coefficient (Uc)	3.70	1.79
Coefficient of curvature (Cc)	0.49	0.73
Effective size (mm)	0.075	0.12
Type of soil *	SP-SC	SP-SM

**SP-SC: Clayey-Sand, poorly graded sand-clay mixture*
SP-SM: Silty-Sand. Poorly graded sand-silt mixture

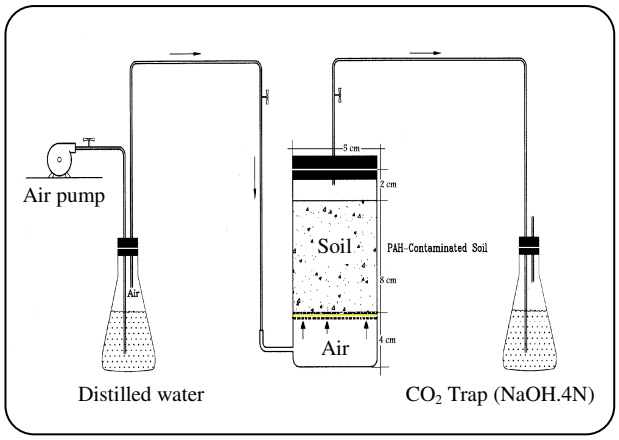

Fig. 1: Schematic diagram of pilot plant for phenanthrene contaminated soil bioremediation.

decontamination of PAH- contaminated soils. Soil samples with different particle size distribution (i.e., clayey-sand and silty-sand) and different phenanthrene concentrations (e.g., 100, 500, and 1000 mg/kg) in the amount of 200 g after adding of microbial mixed culture, MSM containing P & N (phosphorus and nitrogen) and moisture (around 20 %), were filled in to the bioreactor in pilot plant. To prevent reduction of water content of the soil samples, the supplied air was passed through the distilled water to compensate the moisture reduction in soil samples. Also to prevent distribution of off-gas resulting from phenanthrene decomposition in laboratory space, a 4N NaOH solution was used as an absorbent. Schematic diagram of the pilot plant used in this experimental study is shown in Fig. 1. This pilot plant was operated for about 5 months under laboratory conditions (20-25 ^oC). Additionally blanks were analyzed for abiotic control. During this research soil samples were collected and analyzed for phenanthrene concentration, CFU, pH, and water content, every 7 days.

RESULTS

Initial analysis of soils for fungi determination did not show any growth on solid media. Bacterial population analysis as CFU/g for the two different types of contaminated soils is shown in table 2. The soil particle size distribution analysis was carried out using the stack of sieves from 4 to 200 meshes opening size. Also the percentages of gravel, sand, silt and clay and also the types of soils (based on USCS) were measured. In addition other necessary properties of collected contaminated soils e.g., phenanthrene concentration, pH, moisture content, organic carbon were analyzed. Results of these analyses are presented in table 2.

Extraction of phenanthrene with different concentrations in two kinds of selected soil samples using sonication method, showed a 90 percent extraction rate on the average.

Bacterial population changes over time in clayey contaminated soils using consortium BMTRS and BMBOZ are shown in Figs. 2 and 3, respectively.

Results of biodegradation efficiency of phenanthrene with concentrations of 100, 500 and 1000 mg/kg, in CS soil (using consortium BMTRS) and SS soil (using consortium BMBOZ) over time are depicted in Figs. 4 and 5, respectively.

Bacterial analyses using confirmative series tests and API 20E kit test showed that the ominant bacterial populations in the BMTRS consortium (with clayey-sand soil mixture) were *Pseudomonas* and *Serratia* and in the BMBOZ consortium (with silty-sand soil mixture) were *Bacillus, Micrococcus and Serratia* strains.

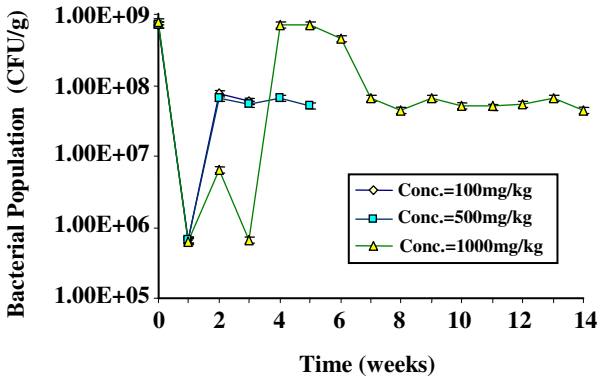

Fig. 2: Bacterial population changes over time in clayey contaminated soils using consortium BMTRS.

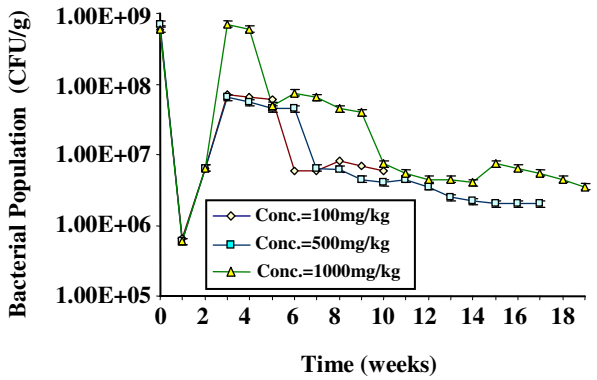

Fig. 3: Changes in bacterial population over time in silty contaminated soils using consortium BMBOZ.

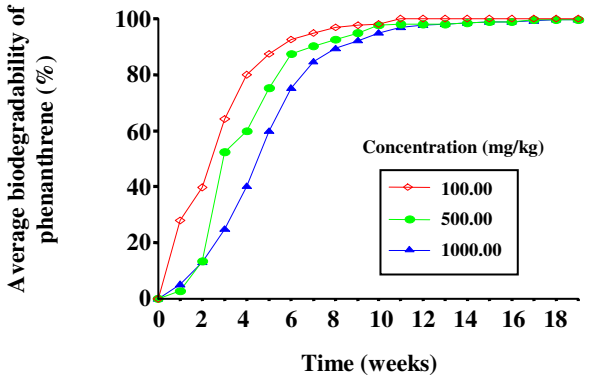

Fig. 4: Removal efficiency of phenanothrene over time in clayey soil containing BMTRS consortium.

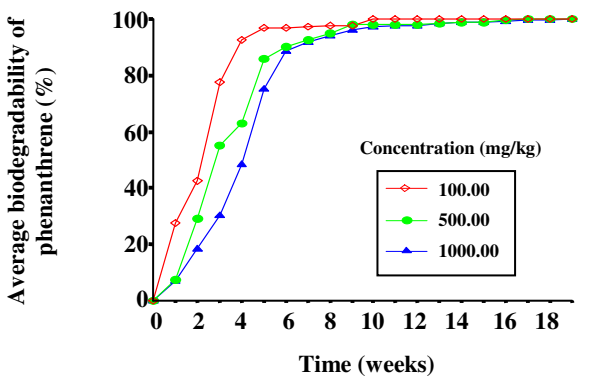

Fig. 5: Removal efficiency of phenanthrene over time in sily containing BMBOZ consortium.

DISCUSSION

According to Figs. 2 and 3, bacterial population growth over time in two different samples (BMTRS and BMBOZ) shows almost similar changes patterns. That means the bacterial population was reduced rapidly after contacting with oil polluted soils during first week due to lack of adapting to the new environment (Lag phase) and toxicity of phenanthrene, but after that bacterial growth was increased (Log phase) with using new substrate as a sole growth source and adapt to new environment (second, third and fourth weeks). After week four the bacterial growth was entered to the stationary phase and population changes was pretty constant over time of biotreatment.

Figs. 4 and 5 depict average biodegradability of phenanthrene during time of bioremediation. As indicated in these figures more than 80 % average biotreatment of phenanthrene for concentrations of 100, 500 and 1000 mg/kg in both soil samples has been took place in 5, 6, and 7 weeks, respectively. Therefore, the bioremediation of the lower concentrations of phenanthrene needs shorter time compared with the higher concentrations. Results from pilot studies showed that the bioremediation of phenanthrene up to non-detectable level (with concentrations of 100, 500 and 1000 mg/kg of clayey-sand soil using BMTRS consortium) was achieved within 3, 5, and 14 weeks, respectively. While in silty-sand soil composition using BMBOZ consortium, bioremediation was achieved within 10, 17 and 19 weeks, respectively.

With respect to the results, removal efficiency of phenanthrene in CS soil was higher than SS soil sample. Thus, biodegration abilities of BMTRS consortium in CS soil samples was more than of BMBOZ consortium in SS soil samples. In general, according to the results, removal of lower concentrations of phenanthrene, e.g., 100 mg/kg takes a shorter time compared with higher concentrations, e.g., 1000 mg/kg. Based on Young and Cernighlia studies on bioremediation of different concentrations of PAH

(i.e., 100, 500, and 1000 mg/kg) in contaminated soils, higher concentration of PAH takes a long time of biotreatment due to it's toxicity to bacterial population and reducing their growth during bioremediation [22].

It can be concluded that BMTRS consortium is more potent than BMBOZ consortium in biodegrading of phenanthrene with different concentrations especially in CS soil composition with sticky and impermeable structure.

According to the bacterial analysis and separation results the BMTRS consortium comprising *Pseudomonas* and *Serratia* species dominantly, and BMBOZ consortium consists of *Serratia, Micrococcus* and *Bacillus* strains. In general and according to the fast removal of different concentrations of phenanthrene in CS soil samples using BMTRS consortium containing *Pseudomonas* strains on one hand, and the main role of this group of bacteria in degrading PAHs compounds in the oil contaminated soils on the other hand, it may be concluded that the reason for more efficient removal of phenanthrene in this type of soil depends on the type of bacteria species, namely *Pseudomonas Pudita* .

Based on the literature review the most important bacteria in degrading the PAHs compounds in contaminated soils belong to *Pseudomonas* strains [3-5].

Contaminated soil microbiology (CFU analysis) showed that the contaminated soils contain higher population of bacteria (3×10^4 - 8.5×10^4 CFU). Hence, it can be concluded that the bioremediation of contaminated soils with enrichment of the bacterial population in contaminated soil (biostimulation) in Iran may be considered as a feasible practice.

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