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INFLUENCE OF BIOAUGMENTATION IN BIODEGRADATION OF PAHs-CONTAMINATED SOIL IN BIO-SLURRY PHASE REACTOR

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ABSTRACT

Polycyclic Aromatic Hydrocarbons (PAHS) are important pollutants which have toxic, carcinogenic and mutagenic properties and are considered as a serious hazard to human health and environment. Bioremediation of PAHs contaminated soil was studied in the soil slurry phase bioreactor. For enhancement of biodegradation, bioaugmentation (which is the process of adding microorganisms with the potential of pollution biodegradation to the bio-slurry reactor) was applied. Phenanthrene ($C_{14}H_{10}$), a three-benzene ring PAHs, was added in concentration of 100mg/kg soil. Two isolated species and consortium of bacteria were inoculated to the medium in density of 7×10^7 CFU/mL. The analysis of variance (ANOVA) was used for finding of optimum levels of type of bacterial culture and presence effect of endogenous factors. The base of the bacteria was petroleum-contaminated soil from around Tehran petroleum Refinery. Control reactor (killed bacteria) showed 5% loss of phenanthrene and biodegradation in the non-augmented reactor (endogenous microorganisms) in a slurry bioreactor was about 17%. In the case of bioaugmentation with *Pseudomonas.spp, Pseudomonas aeruginosa* and consortium, phenanthrene degradation efficiency were 87.8%, 85.5% and 92.8% ,respectively, presenting the positive effect of biodegradation in consortium augmented compared to the isolated one. Colony forming units (CFUs) variation showed good conformity and agreement with the performance of the reactors with respect to phenanthrene degradation. Hence, the results of this experiment show that bioaugmentation may be considered as an effective method to enhance the bioremediation in removal of PAHs from contaminated soils.

Key words: Polycyclic aromatic hydrocarbons; Phenanthrene; Bioremediation; Bioaugmentation; Native microorganism

INTRODUCTION

Polycyclic Aromatic Hydrocarbons (PAHS) are important pollutants which are introduced into the environment through different ways such as anthropogenic activities, combustion, undesirable discharging of oil tankers, spills around petroleum refineries and gas plant facilities (Liebeg and Cutright, 1999; Leblond *et al.,* 2001; Mahvi and Mardani, 2005; Venkata Mohan *et al.,* 2008; Zhao *et al.,* 2008).

These compounds have toxic, carcinogenic and mutagenic properties (Mrozik *et al.,* 2003 ; Ganjidoust and Naghizadeh, 2005; Samimi *et al*., 2009) and are considered as a serious hazard to human health and environment (Karimi-Lotfabad *et al.,* 1996; Yerushalmi and Guiot, 2001; Delnavaz *et al*., 2008; Venkata Mohan *et al.,* 2008; Anastasi *et al.,* 2009; Larsen *et al.,* 2009; Muckian *et al.,* 2009;). PAHs are categorized as priority environmental pollutant by United State Environmental Protection Agency (USEPA) and European commission (EU).

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Bioremediation in which microorganisms are used to metabolize petroleum compounds is a cost – effective alternative for cleaning up PAHs-contaminated soils (Akhavan *et al.,* 2008; Wong, *et al.,* 2002). Three important factors affect the effectiveness of bioremediation: environmental, physical and chemical factors. The most important physical factor is availability of contaminant for the microorganisms. Availability is a complicated concept involving affinity of contaminants for the solid and gas phases and presence of the necessary microbial communities. PAHs are nonpolar and tend to partition onto the solid phase, which results in very low liquid phase contaminant concentration. In bioremediation, the most important chemical factors are the molecular structure and biodegradability (Ewies *et al.,* 1998; Rittman and McCarty, 2001). Because of low bioavailability, hydrophobicity, toxicity and complex structures of PAHs derivatives, bioremediation of these contaminants are complex (Venkata Mohan *et al.,* 2008).

For soils in which the microorganisms do not have the ability of biodegradation of these compounds, bioaugmentation is recommended as a process for enhancement of the ratio of bioremediation. Bioaugmentation is the addition of endogenous or genetically engineered microorganisms (GEM) with the potential of pollution biodegradation (Vogel, 1996; Limbergen *et al.,* 1998; Zhang *et al.,* 2000 ; ; Venkata Mohan *et al.,* 2005; Venkata Mohan *et al.,* 2006; Venkata Mohan *et al.,* 2008).

The use of native microflora for bioaugmentation is prefered because these microorganisms have more ability for adaptation to particular pollutant than non-endogenous microorganisms (Silva *et al.,* 2009) and using GEMs may be lead to gene transfer which is not desirable (Urgun-Demirtas *et al.,* 2006). Jacques reported that Kästner by using PAH degrader bacteria could achieve to about six and ten fold increasing in biodegradation of pyrene and anthracene respectively (Jacques *et al.,* 2008).

Phenanteren a three-ring angular PAH was used as a model of PAHs (Samanta *et al.,* 1999; Vossoughi *et al.,* 2002). The bioslurry reactor is a recent area of interest to environmentalists in bioremediation studies because the bacteria and hazardous materials are in a solid-liquid medium under predefined optimized control system (Venkata Mohan *et al.,* 2004). Mixing in slurry-phase results in agitation and surface scrubbing that can result in the release of some of the adsorbed contaminants.

The objective of the present study was to investigate the effect of bioaugmentation in an ex-situ slurry phase by inoculation of isolated and consortium of high potential PAHs biodegrader bacteria.

MATERIALS AND METHODS

Chemical agents

Phenanthrene with purity of 98% and acetonitrile in HPLC grade were purchased from Merck Company. Nutrient agar and R2A agar were purchased form Hi Media. Chemical materials for mineral salt medium (MSM) were purchased from Merck, Sigma and Aldrich Chemical Companies.

Adaptation

Contaminated soil for preparing PAHs microbial culture was taken from surrounding of Tehran refinery because of long time contact and suitable properties(Alimohammadi *et al*., 2005). Bacteria were grown in an aqueous MSM contained per litre: 0.8 g K_2HPO4, 0.2 g KH_2PO4 1 g KNO_3, 0.2 g $MgSO_4.7H_2O$, 0.1 g $CaCl_2.2H_2O$, 0.1 g NaCl, 0.01 g $FeCl_3.6H_2O$ and 1 mL trace element solution. The trace element solution contained per liter:23 mg $MnCl_2.2H_2O$, 30 mg $MnCl_4$, 32 mg H_3BO_3, 39 mg $CoCl_2.2H_2O$, 50 mg $ZnCl_2$, 30 mg $NaMnO_4.2H_2O$ and 20 mg $NiCl_2$ (Ressler *et al.,* 1999).

For preparing microbial culture 100 mL MSM was added to 10 g of contaminated soil (10% w:v). The mixture was stirred for 24 hours using a magnetic stirrer. After 20 minutes for settling, one mL of supernatant was added to 50mL sterile MSM plus phenanthrene. The MSM was freshened every week to prevent any deficiency of nutrients and carbon source. After one month, the adapted microorganisms were brought on nutrient agar for supplying later experiments (Rezaei Kalantary and Badkoubi, 2004).

Experimental methods

Two isolated cultures and a consortium of bacteria which had the potential of PAHs biodegradation were used for inoculation in bioagmentation. Soil was passed through 2mm sieve, then was spiked with the phenanthrene at a ratio of 100 mg/kg soil.

The soil was inoculated with bacterial culture which was prepared in MSM. The optical density of the culture at 630 nm was 1 (OD at $E_{630\ nm}$=1) (Ressler *et al.,* 1999). Experiments were conducted in 1L flasks each containing 40g soil and 400mL MSM.

In order to find the optimum levels of type of bacterial culture and presence effect of endogenous factors, full-factorial design was applied. Statistical method was analysis of variance (ANOVA).

The sample had the similar blanks; B_1 was the sterile soil and B_2 was the nonsterile soil (with endogenous microflora). CB_1, CB_2 and CB_3 were the sterile soils, inoculated with the same culture as samples. All of these soils, were spiked before inoculation with the same concentration of phenantherene with their similar samples. MB_1, MB_2 and MB_3 were the sterile soil with the same inoculation and without spike of phenanthrene (Table 1). Samples and blanks were put in the shaker at the velocity of 180 rpm in room temperature for two months.

Table 1: Summary of experimental tests

Run	Test name	Microorganisms		Phenanthrene concentration
		Endogenous	culture	
1	S_1	+	Mix	100
2	S_2	+	*Pseudomonas*.spp	100
3	S_3	+	*Pseudomonas aeruginosa*	100
4	B_1	-	-------	100
5	B_2	+	-------	100
6	CB_1	-	Mix	100
7	CB_2	-	*Pseudomonas*.spp	100
8	CB_3	-	*Pseudomonas*	100
9	MB_1	-	Mix	----
10	MB_2	-	*Pseudomonas*.spp	----
11	MB_3	-	*Pseudomonas aeruginosa*	----

Extraction and Analysis

Phenanthrene was extracted from the soil according to EPA 3550B, by using ultrasonic (RK31 h, Bandeling Electronic Sonorex). Samples were analyzed by HPLC (KNAUER Company). The column of HPLC was C18 and the solvent was 95% acetonitril and 5% DDW. It was detected at 254nm. Microbial population was measured by using plate count agar or HPC (heterothrophic plate count media)(Arbabi *et al.,* 2004).

RESULTS

After enrichment and isolation, four spices were isolated: *Pseudomonas.spp, Bacillus, Pseudomonas aeruginosa* and *Acinetobacter*. The selections of *Pseudomonas.spp* and *Pseudomonas aeruginosa* for developing the bacterial inoculation were based on their demonstrated success for PAHs biodegradation (Nourieh *et al.,* 2010). Hence these two cultures and consortium of bacteria were used for bioaugmentation investigation.

An important decrease in the phenanthrene concentration was observed in all systems (augmented and non-augmented), except for abiotic control, during the first 3 weeks (Fig. 1). Bioaugmentation in all cases showed lower values of residual phenanthrene, but this amount in consortium inoculation was more than the others (Figs. 2-4), however it was not significant (Fig. 1).

The trend of phenanthrene removal in

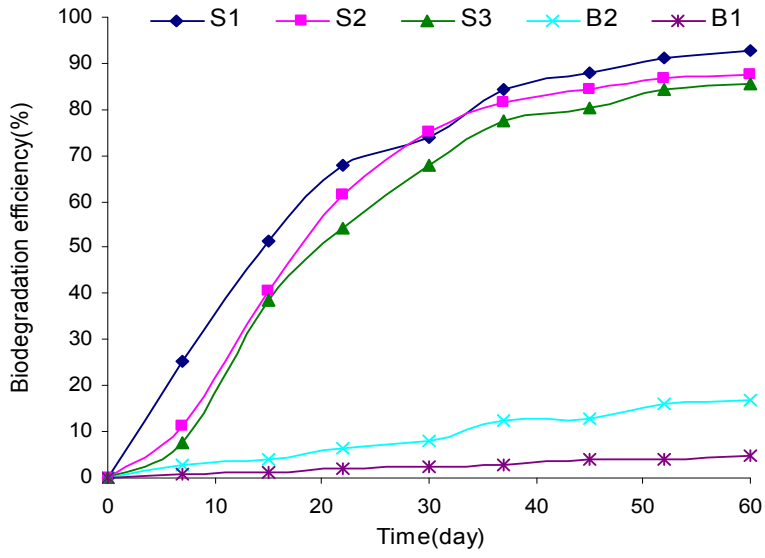

Fig 1: Phenanthrene degradation profile during augmented and non-augmented operations

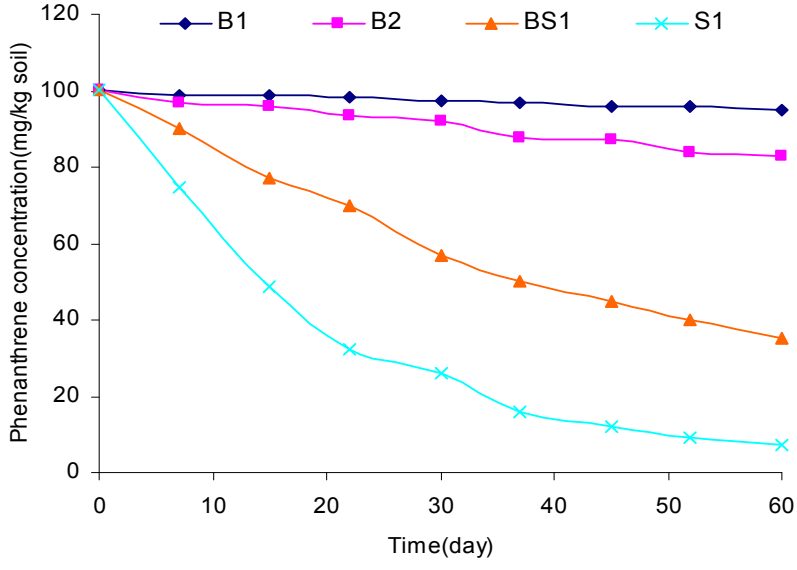

Fig 2: Trend of phenanthrene removal in bioaugmentation with consortium

bioaugmentation with consortium is shown in Fig. 2. The abiotic control (B1) shows 5% loss in pollutant and the endogenous microorganisms degraded about 17% of phenanthrene in 2 months (Fig. 2). According to the analysis of variance (ANOVA), the F-ratio for inoculated culture and endogenous microorganisms were about 88.3 and 21.7, respectively (Table 2).

The bacterial population after inoculation to

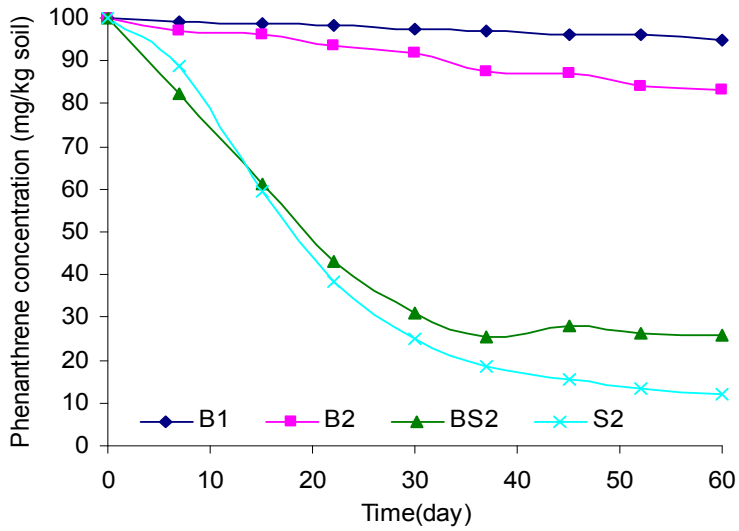

Fig 3: Trend of phenanthrene removal in bioaugmentation with *Pseudomonas*.spp

Table 2: Summary of analysis of variance

Source	Sum of squares	DF	Mean squares	F ratio	P value	PE%
Culture	7020.62	3	2340.21	88.34	0.0020	80.28
Endogenous	574.94	1	574.94	21.70	0.0187	19.72
Residual	79.47	3	26.49			
Core total	7675.04	7				

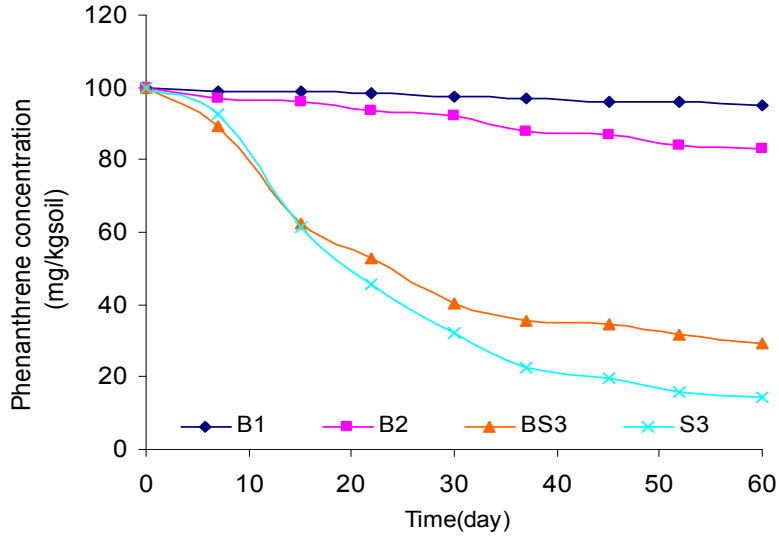

Fig 4: Trend of phenanthrene removal in bioaugmentation with *Pseudomonas aeruginosa*

sterile and non-sterile systems was about 7×10^7 and 5×10^8 CFU m/L respectively (Figs. 5-6). After initial decreasing about 20-300 fold of magnitude in the first week, it increased in 4-6 fold of magnitude in two months (Fig. 6). The population decrease in the blanks continued till the end of the tests (Figs. 5 and 7).

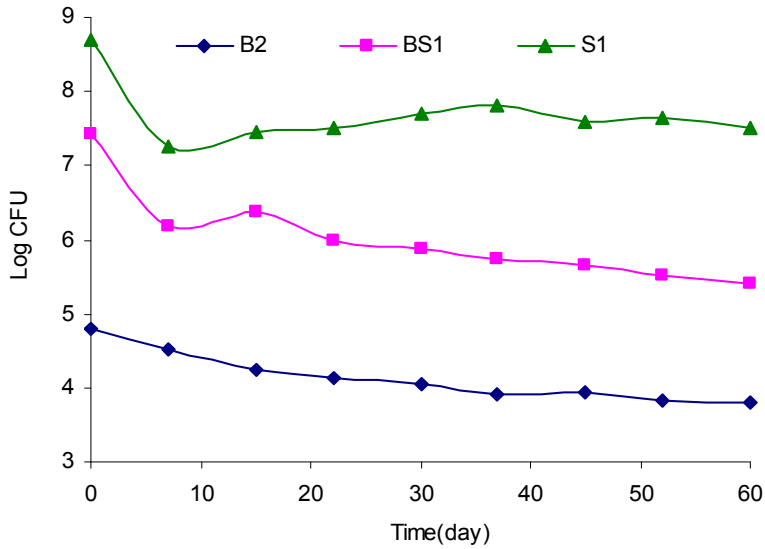

Fig 5: CFU variation in mix culture augmented, non-augmented and endogenous during bio-slurry phase reactor operation

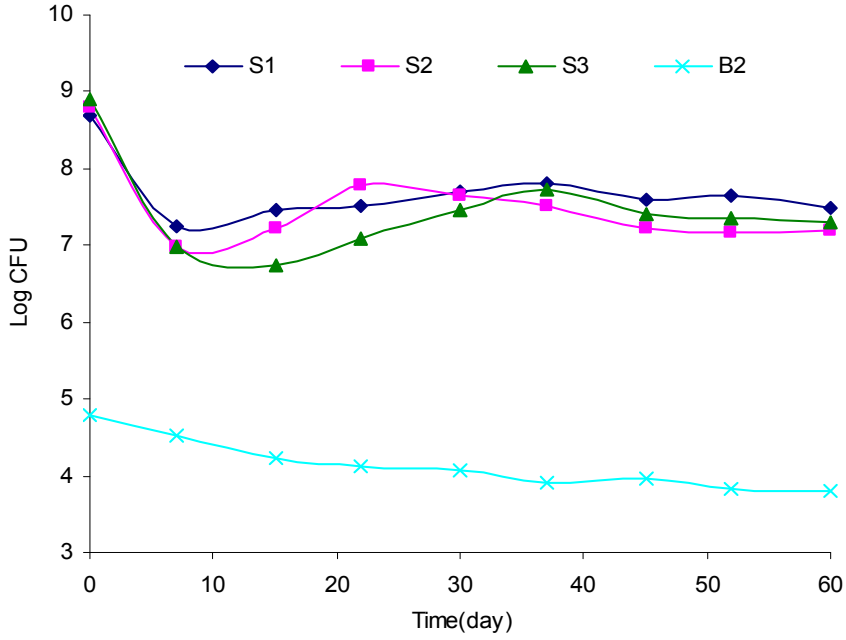

Fig 6: CFU variation in augmented samples and endogenous during bio-slurry phase reactor operation

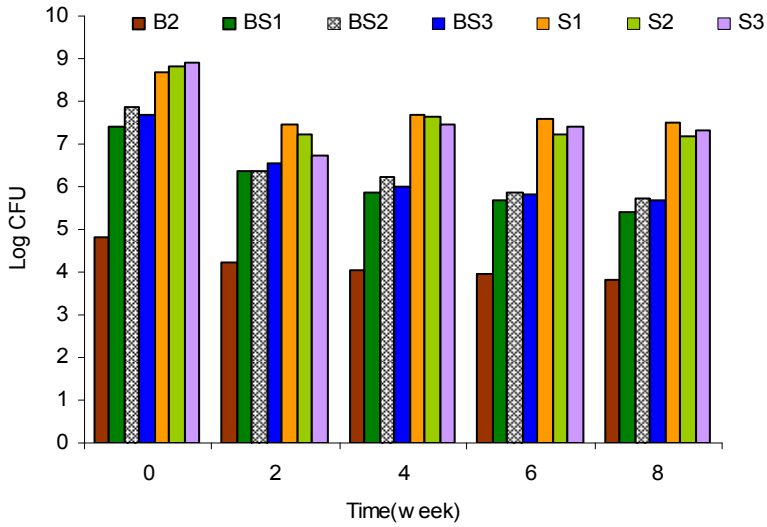

Fig 7: CFU variation in the blanks and samples during 8 weeks

DISCUSSION

The two cultures with high potential for bioremediation which were used for bioaugmentation investigation were gram negative. Mrozik *et al* (2003) had reported that most of PAHs degradable bacteria are gram negative (Mrozik *et al.,* 2003).

The phenanthrene concentration on the 60th day in S_1, B_1, B_2 and CB_1 (Table 1) were 7.25, 95, 83and 35 mg/kg soil respectively. The reduction of 5% in B_1 which had not any microorganisms may be related to the bounding between phenanthrene and soil texture and aging after two month (Hwang and Cutright, 2002) or may be lost. Ruberto *et al,* (2003) in their research, in spite of poisoning the culture, obserred 30% loss in control (Ruberto *et al.,* 2003).

Comparison between the other tests shows that the biodegradation in B_2 (with the endogenous microflora) was less than 20%. Hence the endogenous microflora in a bio-slurry system had a weak efficiency (Jacques *et al.,* 2008) and it was about 75% and 50% less than the system with inoculated of consortium to non-sterile and sterile systems, respectively. The F-ratio would also confirm it.

Hamdi *et al,* (2007) used bioaugmentation/ biostimulation for enhancement of PAHs biodegradation (Hamdi *et al.,* 2007). In our research the bioaugmentation had removal efficiency about 85-93%, while Ruberto *et al.* (2003) reported only about 75% in the best condition (Ruberto *et al*., 2003). The phenanthrene removal in co-cultivation of *Acinetobacter sp*. and rice was 87% (Gao *et al*., 2006). Comparison between CB_1 and S_1 shows that removal in an augmented system (S_1) was about 25% more than the other one. On the other hand, inoculation to a non-sterile system had more efficiency than inoculation to a sterile system, so augmentation could improve the rate of bioremediation (Venkata Mohan *et al.,* 2008), which does not agree with Silva *et al.,* 2009, who had reported that bioaugmentation had not significantly effect on bioremediation of low molecular weight PAHs (Silva *et al.,* 2009). Yu *et al,* (2005) did not observe any significant difference between natural attenuation and bioaugmentation in phenanthrene removal, but it was in pyrene elimination. They related it to negative interaction which may have occurred between inoculums and endogenous microbial community (Yu *et al.,* 2005).

The culturable microoganisms which can grow in the presence of phenanthrene can be represented by CFU (Venkata Mohan *et al.,* 2008) which was periodically monitored in the reactors during the experiment time. The bacterial population decreased in all testes in about 7 days; Ramirez *et al.,* 2002 had observed the same decrease in their research (Ramirez *et al.,* 2001). The decreasing in B_2 was prolonged till the end of the test which may be due to nonpotential of native soil microflora in phenanthrene biodegradation (Venkata Mohan *et al.,* 2008).

On the other hand, the bacteria were reproduced on a nutrient agar which is a ready medium for consumption. When these bacteria were inoculated to the soil in which the only carbon source is phenamnthrene, they were transferred from a banquet medium to a famine medium which makes the reduction in population and reproduction (Rittman and McCarty, 2001).

Also the interaction or competition between the autochthonous and the enriched microbes may result in the inhibitory effect in bioaugmentation in the initial days (Yu *et al.,* 2005) which may be the other reason for decreasing in bacterial population. The second decreasing in population may be related to substrate reduction or toxic intermediate production (Ruberto *et al.,* 2003; Gao *et al.,* 2006).

Comparison between the isolated and consortium inoculation (S_1, S_2 and S_3) shows that the phenanthrene biodegradation in augmented consortium was more than the isolated one. It may be due to the ability of certain strains in removal of intermediates produced by other members of consortium which facilites the phenanthrene removal. Leblond *et al* (2001) showed that a mixture of four different species was more effective than each single one in PAHs biodegradation (Leblond *et al.,* 2001).

Jacques *et al* (2008) reported that the significant synergistic promotion of PAHs mineralization was occurring by mixture of bacteria (Jacques *et al.,* 2008). Churchill *et al.* 1999 reported that use of isolated bacteria for biodegradation prolonged more than the time needed for mixulture(Churchill *et al.,* 1999). Fig. 1 shows that 50% reduction of pollution by isolated *Pseudomonas.SPP* and *Pseudomonas aeruginosa* occurred in 18 and 20 days, respectively, but it took 14 days for consortium. The phenanthrene removal by isolated *Pseudomonas.SPP*, *Pseudomonas aeruginosa* and consortium were 87.75%, 85.52% and 92.75%, respectively.

The trend of phenanthrene concentration changes shows after a lag phase in pure cases, decreasing was rapid till the 37th day; then it became slow. This is similar to Silva *et al,* (2009) who reported that the non-available fraction is due to physical interaction with soil matrix (Silva *et al.,* 2009). The rate of biodegradation in S_1, B_2 and CB_1 were 1.55, 0.3 and 1.3 mg/kg /day. The removal in augmented system was about 5 times higher than native microflora.

As a conclusion the results of our experiment show that bioaugmentation may be considered as a valuable method to enhance the bioremediation in removal of PAHs in contaminated soils.

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