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EFFECT OF HUMIC COMPOUNDS ON BACTERIAL GROWTH IN BIOREMEDIATION OF PAHS

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ABSTRACT

Polycyclic Aromatic Hydrocarbons (PAHs) which are introduced into environment are potentially carcinogenic, mutagenic and toxic contaminants. The effect of extractable humic substances (EHS) on bacterial density in bioremediation of anthracene in liquid systems was investigated. The ratio of EHS to anthracene were in two concentrations of 0.35 and 1.05 g dry EHS (with 30% organic matter) per one mg anthracene. In the tests with EHS, an increase in bacterial density even by 8 fold of magnitude was seen in 12-15 days. Then a fast decrease was occurred and prolonged till the end of the test time for the tests that had EHS without anthracene. In the tests which anthracene was the only substrate increasing in bacterial population was not seen. The results showed that up to 21 days the system was free from degradation. So the first increasing in bacterial population showed that EHS might be used as a readily substrate for PAH degraders. The presence of EHS (fulvic and humic acid) can stimulate bacterial community and activity that caused enhancement in anthracene bioremediation.

Key words: Anthracene, bioremediation, humic substance, microbial population

INTRODUCTION

Polycyclic Aromatic Hydrocarbons (PAHs) are introduced into the environment through natural and anthropogenic combustion processes causing them to be ubiquitous in environment (Menzi *et al*., 1992; Juhasz and Naidu, 2000 and Nieman *et al*., 2001). These compounds are potentially carcinogenic, mutagenic and toxic contaminants (Yerushalmi and Guiot, 2001; Verrhiest *et al*., 2002; Samanta *et al*., 2002 and Zhang *et al*., 2005). At present, employing the biochemical abilities of microorganisms is the most popular strategy for the biological treatment of contaminated soils and waters (Semple *et al*., 2001). The biological strategy is dependent on the catabolic activities of the microflora, optimizing the conditions for growth and biodegradation (Semple *et al*., 2001). In this manner, bioremediation is one of the best technologies for removing pollutants from the environment (Ewies *et al*., 1998 and Ramirez *et al*., 2001). Bioavailability of organic contaminants has been identified as a major constraint for complete bioremediation (Semple *et al*., 2001 and Park *et al*., 2002). Researches have been designed to explain and overcome problems of PAHs poor availability (Laor *et al*., 1996) by using humic substances (HSs). Several mechanisms about the effect of HSs on bioremediation are studied during in recent years and are described as: covalent and noncovalent bounding, adsorption solubility enhancement, microbial activity enhancement, increasing in microbial population and help enhancing production of useful enzymes in biodegradation. Two major technologies using HS are phytoremediation and compost which could stimulate microbial population ability in bioremediation (Alkorta and Garbiso, 2001; Richnow *et al*., 2000; Gramms *et al*., 1999 and Van Elsas *et al*., 1998), but it is not clear whether increased dissipation of PAHs in the system containing humic substances is due to a specific

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stimulation of PAH degraders in the medium. Hence it is desirable to optimize the activity of degradative microorganisms (Allard and Nielson, 1997). Substrate utilization patterns were shown to vary depending on the both composition and density of the inoculums that is used (Van Elsas *et al*., 1998).

The goal of this study was to test the hypotheses of using of humic substances as the stimulating factor of bacterial community and their activity for remediation of PAHs. Anthracene, a three ring PAH, may be found in relatively high concentrations in coal tar contaminated sites (Yaghmaei, 1999), wood preserving, gas work sites (Juhasz and Naidu, 2000) and petroleum refining (Blakely *et al*., 2002).

MATERIALS AND METHODS

Contaminated soil for preparing PAH microbial adapted culture was taken from surroundings of Tehran Refinery. Uncontaminated soil from the same place was passed through sieves for grading, and then it was analyzed by XRD defractometer for mineral texture. More than 92.88% of soil passed through of the sieve by mesh #200 (<50 μm), so based on graining size it was lean clay. By XRD defractometer and separation of mineral in the range of clay, the soil was consisting of quarts, calcite, clinochlore, muscovite and montmorillonite, respectively of plentiful.

Soils for preparing of humic substance were taken from the forests around Sari in north of Iran. It was taken from a depth of 7-15 cm. The extractable humic substances (EHS) consisted of FA and HA fractions were obtained according to Mecozzi *et al*., (2002). The dry amount of EHS and its organic compound fraction were determined as 0.35mg in 1ml and 30% respectively according to Standard Methods (1995). Anthracene and naphthalene were purchased from Merck Chemical Co. with purities of 96% and 98%, respectively. Methylene chloride, acetone, acetonitrile (for calibration curve of HPLC) in HPLC grade, sulfuric acid, sodium chloride, sodium hydroxide and HCl were purchased from Merck Chemical Co. Nutrient agar and nutrient broth were purchased from Hi media and Difco Chemical companies, respectively. Chemical materials for mineral medium were purchased from Sigma and Aldrich Chemical companies.

Adaptation

Bacteria were grown in an aqueous mineral medium, which are referred to as "sufficient" mineral medium. This medium contained (g/L): KH_2PO_4, 3.4g; K_2HPO_4, 4.3g; $(NH_4)_2SO_4$, 2g; $MgSO_4$, 0.2 g; $CaCl_2$, 0.04g; $FeSO_4$, 0.03g and 25 mL(per 1 L medium)of the trace element solution of the following composition (mg/L): $MnCl_2$, 40; Na_2MoO_4, 80; $CuSO_4$, 6; $ZnSO_4$, 60; H_3BO_3, 13 (Kozliak *et al*., 2000). The pH of the solution was 6.8. After autoclaving, "sufficient" mineral medium carbon sources were added. The culture was enriched using Naphthalene (2 rings) and Anthracene (3 rings) separately as the sole source of carbon for bacterial growth in salt and trace elements medium. A mixed microbial culture was derived using soil from Tehran Refinery site. 100 mL sterile DW was added to 10 grams of soil (10% w:v). The mixture was stirred for 24 hours on magnetic stirrer. After 20 minutes for settling, one mL of supernatant was added to 50mL medium containing salt, trace element and carbon source. The mineral medium was freshened every five days to prevent any deficiency of nutrients and carbon source. This procedure continued for seventy days. Then the adapted bacteria were brought on nutrient agar for supplying later experiments.

Investigation of tests (or experimental methods)

For investigation the bacterial population changes in liquid medium, at first 15mL of sufficient mineral medium was poured into vials. 20mL of sufficient mineral medium was poured to each vial, as blank. The diameter and height of vials were 22 and 180 mm, respectively. After sterilization, 2 mL of stock solution with the concentration of 1000mg anthracene/L acetone was added to each vial, which was necessary according to Table 1. The concentration of anthracene in medium was 10mg/L (Rezaei Kalantary *et al*., 2004).

EHS solution was added to liquid in two concentrations of 3.5 and 10.5 g dry EHS which supplied OM in concentration of 1 (Van Stempvoort

et al., 2002) and 3 g per 1L of medium. The EHS solution was sodium salt in NaOH; so by adding it to the vials, the pH of medium increased and was adjusted on 6.8-7 by using H_2SO_4. Then the medium was inoculated with mixture of bacteria. Mixture of bacteria from agar plates was added to 500mL sterile sufficient mineral medium, after mixing it on magnetic stirrer, 5mL of suspension was added to each vial. At the end the liquid volume in each vial was 20 mL.

Taguchi method was applied in order to save time and to have minimum error (Qualitek-4). According to this method, 9 experimental tests were used (Table 1) and all of them were triplicate.

Table 1: Summary of experimental tests according to Taguchi method

Test number	Mix culture	Humic concentration (w:w)	Anthracene concentration (mg/L)
1	N[(a)]	10.5	10
2	A[(b)]	10.5	0
3	B[(c)]	10.5	10
4	N	3.5	10
5	A	3.5	10
6	B	3.5	0
7	N	0	0
8	A	0	10
9	B	0	10

(a) Mix culture of bacteria that their carbon source was naphthalene
(b) Mix culture of bacteria that their carbon source was anthracene
(c) Without mix culture

Extraction and analysis

Anthracene was extracted according to the standard methods for liquid-liquid extraction (APHA, 1995). Methylene chloride was used as a solvent. Samples were analyzed by HPLC (Waters Company). The column of HPLC was ODS (Octa Desil Silyl) and the solvent was 90% methanol and 10% DDW. Helium was used for sparging in HPLC.

Microbial population test

The most probable number (MPN) was used to measure the population of inoculated (Taylor, 1962 and Juhasz *et al*., 2000). The suspension of bacteria in ringer solution (8.5 gr NaCl /L DW) was diluted ten fold to 10^{-10}. The medium in MPN was sterile Nutrient Broth in five replicates and in 10 series.

RESULTS

After adaptation, two mix cultures of microorganisms were separated as mixA and mixN, with the carbon sources of anthracene and naphthalene respectively. These two mix cultures were consisting of six types of bacteria. These bacteria were *microbacterium Lacticum, Bacillus mycoides, Sphingomonas parapaucimobilis, Acidovorax (pseudomonas) facilis, Capnocytophaga presumably ochracea* and *Actinomyces Spp.*

The bacterial population changes of the samples and blanks are shown in the Fig.1 and 2. The tests 1, 4 and 7 were inoculated with mixN. The test 7 was not spiked with the contaminant, and humic substances were not added to it and it was considered as a blank.

The tests 2, 5 and 8 were inoculated with mixA. The test 2 was not spiked with the contaminant, and humic substances were not added to test 8. Tests 2 and 8 also considered as blank. In this research the minimum level of base factor in Taguchi method was used as the best results; so by this method the level 2 of mix culture (mixA) showed the better result.

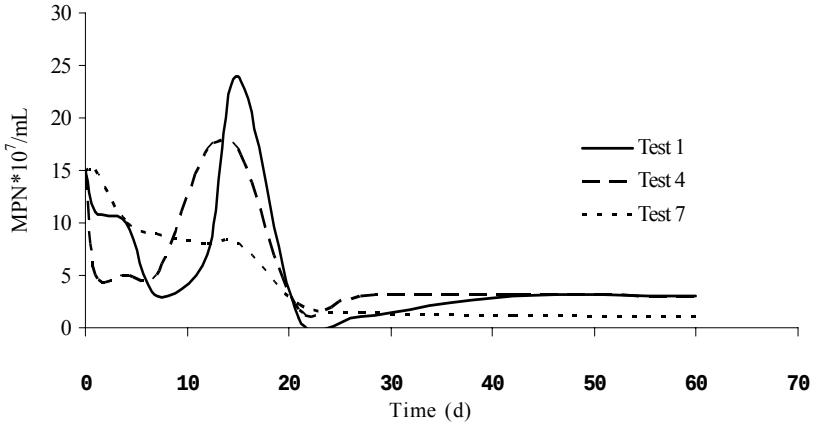

Fig. 1: Bacterial population changes of mixN in tests1, 4 and 7

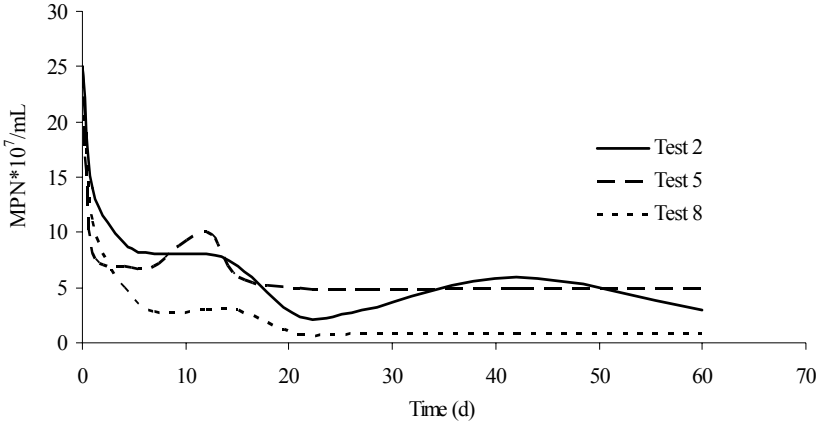

Fig. 2: Bacterial population changes of mixA in tests2, 5 and 8

But the two mix cultures did not show essential difference in decreasing of ECA (Fig. 3). The numbers of population of two mix cultures in the inoculation were different; so the accurate comparisons between them were not possible. Level of 1, 2 and 3 of variable are mixN, mixA and B (without inoculation).

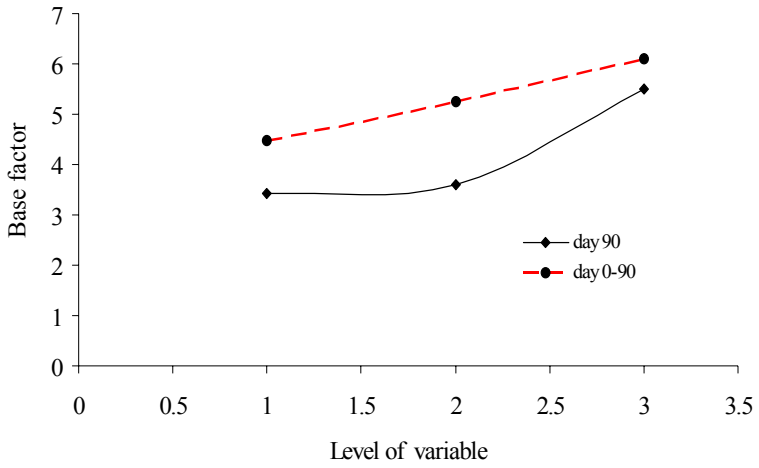

Fig. 3: Results of experimental tests according to Taguchi method

DISCUSSION

As it is shown in Fig. 1 in test1, an initial reduction in bacterial population was seen in 7 days, and then it increased. At day 15 it was 8 times as much as day 7. Then it dropped from 24×10^7 MPN/100mL to 0.9×10^7 MPN/100mL (about 1/27) in 6 days. After that a little increase was seen in bacterial population till day 42. The trend of bacterial population changes in tests 4 and 5 showed that this change was similar as test 1, but different in tests 7 and 8. In test 7 (without any substrate) and test 8 (the only substrate was anthracene) no increase in bacterial population during 60 days was seen (Fig. 1). In these two tests, the trend of initial decrease continued during the test time. The trend of bacterial population changes in test 5 was similar to test 4 with a minor difference.

As far as bacteria were separated from soil and humic substance was also extracted from soil, there is a probability that bacteria might use a fraction of humic carbon which degrades simply as a substrate. The bacterial population in the tests with humic compounds had a sharp increase in 7 days, which shows using of readily degradable compounds in humic substances. In comparison between tests 1, 4 and 7 (all of them were inoculated with mixN), there was no increase in bacterial population in test 7, but increasing were seen in test 1and 4 between days 12-15. As there was not any carbon source in test 7, the decrease may be related to the carbon sources. In test 4, the trend of population change was similar to test 1, but the maximums in test 1and 4 were 2410^7 and 1710^7 MPN/100mL, respectively. The higher amount of bacterial population in test 1 may be for the concentration of EHS, which in test 1 was three fold of test 4(Klavins and Serzane, 2000 and Van Stempvoort *et al*., 2002). In both tests (1and4) the population dropped from day 15-21, and at day 24, the minimum of the mix culture population was seen which may be for the decreasing effect of EHS(Van Stempvoort *et al*., 2002; Klavins and Serzane, 2000). After day 21, little increase in bacterial population was seen. In this case the extractable concentration of anthracene (ECA) was reduced; hence, the bacterial density increase may be due to the application of anthracene. As far as anthracene is not a readily substrate (Laor *et al*., 1999; Juhasz and Naidu, 2000) the less amount of increase in bacterial community in tests with anthracene and EHS (tests 1,4 and 5) comparing with the test with EHS only (test 2), may be as a result of the presence of anthracene. So anthracene may serve as an inhibitor substrate (Boopathy, 2000) comparing with EHS. But the first increase in bacterial population in the test with only anthracene as substrate (test 8) and the second increase in the test with EHS and

anthracene, showed that anthracene can serve as a substrate but not as easily as EHS. There is a significant difference between ECA in test 1 and 4 with test 3 (P value is 0.026 and 0.011, respectively) which may be related to the presence of bacteria in the tests l and 4, with more than 95 % reliability. In tests 1, 2, 4 and 5 the trend of decreasing in bacterial community was very fast. Therefore, it is the other reason for confirmation the role of EHS as a substrate. No correlation was found between the number of bacteria enumerated by MPN technique and the degradation rates for anthracene, confirming results of Kao and Borden (1997).

Scientists suggested that the presence of the microorganisms capable of degrading natural humic substances were responsible for the co-metabolic degradation of PAHs (Qiu and McFarland, 1991; Wischmann and Steinhart, 1997; Kästner, 2000; Semple *et al*., 2001 and Reid *et al*., 2002). Thus co-metabolism of EHS (HA and FA) provides an important substrate for bacteria in the medium and potential for enzyme transformation reaction that would not occur in a system without humic substances. Many observations have indicated that microorganisms do not employ single compounds for growth, but rather a wide range of natural or anthrophic carbon sources (Martins and Mermoud, 1998). Hence the presence of EHS had been preferred by microorganisms. Changes in bacterial community and results of anthracene removal in humic systems confirms this hypothesis and confirms the results of Kästner and Mahro (1996). Also Binet *et al*., (2000) reported that modification of microbial composition or activity might increase the transformation rate of toxicants. So the increase in anthracene removal in humic substance medium is due to modification of bacterial community under the effect of the presence of EHS (FA and HA) which in phytoremediation processes was also seen (Gunther *et al*., 1996; Klavins and Serzane, 2000; Alkorta and Garbisso, 2001).

Extracellular enzymes like peroxidase and laccase can be stabilized by adsorption to humic compounds which this stabilization support the enzyme. Then the extracellular enzyme can catalyze the HA and FA oxidants and produce active couple of combination between organic contaminants and humic substance. The presence of stabilized extra cellular enzymes attached to humic compounds is useful for supplying nutrient for microorganisms (Gramss *et al*., 1999). Nutrient supplying leads to stimulation in bacterial activity. On the other hand, bacteria produce extracellular polymers, which are hetropolysacaride. Liu *et al* showed that the presence of these polymers caused to release phenanthrene (Liu *et al*., 2001). So increasing of bacterial activity caused releasing in PAHs and increasing in bioremediation. On the other hand, a growth substrate can initiate growth of organisms and induce the producing of catabolic enzymes (Juhasz *et al*., 2000). In this research, EHS play the role of growth substrate that could induce the initiation growth and cause the increase in bacterial population.

HA can act as terminal electron acceptor in anoxic medium (Kästner, 2000), which caused increasing in bacterial population. Enhancement of bacterial community in the tests which had humic compounds, even though without anthracene, is related to the presence of humic compounds (Kästner, 2000).

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