Bioremediation of PAHs from Contaminated Soils by *Festuca aroundiacea* in the Presence of *Bacillus licheniformis* and *Bacillus mojavensis*

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ABSTRACT: Bioremediation of PAHs from oil-contaminated soils was investigated. *Bacillus licheniformis* and *Bacillus mojavensis*, were inoculated in rhizospheric soil with *Festuca aroundiacea* in a greenhouse study. The concentration of PAHs in the rhizosphere of soil inoculated with both bacteria were significantly (*p* < 0.05) reduced compared to the non-inoculated soil. PAH concentrations in shoots were largely independent of those in soil, roots, or rhizosphere soil. In the soil inoculated with both bacteria, the concentration of PAHs in roots and shoots of the plant was less than for other treatments. Results showed that the plant-promoted biodegradation was the predominant contribution to remediation of soil PAHs.

INTRODUCTION

PAHs are aromatic compounds with two or more benzene rings. Usually, they are formed during the thermal degradation of organic molecules and similar compounds [1]. The common sources of these compounds in the environment can be human activities, forest and grassland fires, oil spills, volcanoes and industries [2]. The PAHs compounds are known as toxic, mutagenic and carcinogenic pollutants [3]. They are not easily eliminated from the environment under normal circumstances. If the molecular weight increases, their resistance to degradation will increase. Due to their widespread presence in the air, soil and sediments, they have attracted attention. These compounds may be eliminated from the environment in one of the following ways: evaporation, light oxidation, chemical oxidation, superficial adsorption of soil particles and leaching [4].

These methods are usually expensive, ineffectual, costly and time consuming and transmission of contamination from one phase to another. Among all the studied methods used for the elimination of such compounds from the environment, bioremediation and phytoremediation are processes that requires the least amount of energy and chemical substance. They are degrading the dangerous pollutants to less dangerous ones [5].

Maiti *et al.* (2013) isolated and identified one strain of *Bacillus* from oil contaminated soil in India and found that it could mineralize anthracene, fluoranthene, pyrene and benzo (a) pyrene [6].

Toledo (2006) isolated fifteen bacterial strains from solid waste oil samples for their capacity to grow in the presence of naphthalene, phenanthrene, fluoranthene, or pyrene as the sole carbon sources. The isolates were identified by 16S rDNA sequence, and results showed that the strains belonged to the genera *Bacillus, Bacillus pumilus* (eight strains), and *Bacillus subtilis* (two strains) [7].

In fact, phytoremediation is a technology that acts based on the natural activities occurring in the soil. These activities include symbiotic relationships between plants, microorganisms and the environment. Phytoremediation has advantages such as plant’s com-
plex root system that occupies a large amount of the soil and supports a large population of bacteria in the rhizosphere. Its secretions can directly affect the activity of the bacterial population in the rhizosphere and eliminate pollution. In fact, one of the best methods for cleansing the oil-contaminated soils is phytoremediation. For this purpose, plants should be used which are more compatible and flexible in harsh conditions caused by hydrocarbons [8].

Research has shown that plant roots have a great effect on PAH decomposition in such a way that a high level of decomposition happens in the vicinity of the plant roots [9].

Although abiotic factors such as pH, moisture, oxygen and available nutrients can affect the decomposition of PAHs, microbial processes notably have a greater share in the decomposition of these compounds [10]. Petroleum hydrocarbons absorb specific populations of decomposing bacteria and cause the expansion of decomposing bacterial populations [1].

Banks et al. (2003) stated that a large range of grasses and legume herbs with their symbiotic bacteria have shown increased removal of oil pollutants from the soil [8].

Some studies have also shown that plant affect uptake of lipophilic contaminants from soils, and results show that the plant lipids are the major factor for the differences in plant lipophilic compounds uptake such as dieldrin, heptachlor, aldrin and heptachlor epoxide [11].

However, there is still a lake of direct evidence to evaluate the plant uptake, accumulation and subsequent translocation of PAHs. Although, several studies have revealed the uptake and accumulation of PAHs from the atmosphere as a result of the deposition PAHs particle bound compounds on the leaf cuticle [11], uptake and accumulation of PAHs by plant in oil-contaminated soils are still under study, and information is scant on correlations between plant PAH concentrations and PAHs translocation in plants.

In this study, Festuca arundinacea was inoculated with two indigenous bacteria isolated from oil-contaminated soil. The species of bacteria for inoculation were Bacillus licheniformis ATHE9 and Bacillus mojavensis ATHE13 and their effect on the removal of hydrocarbons from the plant’s rhizosphere was studied.

MATERIALS AND METHODS

Sampling

Soil samples were collected from contaminated soils around the tanks of the Isfahan Oil and Gas Refinery that occasionally collected and were piled up to reduce the risk of ignition and occurrence of fire with pH 7 and 4.7% organic matter. The 10 following polycyclic aromatic hydrocarbons were studied: naphthalene (NAP), acenaphthene (ANA), acenaphthylene (ANY), phenanthrene (PHE), anthracene (ANT), benzo[a]anthracene (BaA), chrysene (CHR), benzo[a]pyrene (BaP), dibenzo[a,h]anthracene (DBahA), benzo[ghi]perylene (BPY). Before transporting to the greenhouse, to ensure the homogeneity of the treatments, soils were sieved through 3 mm mesh and mixed.

Isolation and Identification of PAHs Degradating Bacteria

The enrichment, isolation, morphological and physiological characteristics, analysis of 16S rDNA sequence and evaluation PAHs-degradation potential of degrading bacteria in oil-contaminated soil samples were conducted using Zhang et al. (2009) methods [12]. Then two bacterial isolates with more optical density and degradation ability of PAHs were selected for further studies.

Seed Inoculation with Bacteria

From two selected bacteria (Bacillus licheniformis and Bacillus mojavensis) an inoculum liquid with the concentration of McFarland standard No. 3 was prepared and seeds were placed in inoculum liquid plates for 5 to 6 hours before they cultured. McFarland standards were used as a reference to adjust turbidity of bacterial suspensions so the number of bacteria will be within a given range for standardized microbial testing. After that in the seedling stages, one mL of the same inoculum liquid were inoculated to the plant after 3 weeks [13].

Experimental Procedure

An equal amount of contaminated soil was added to each pot (7 kg) and twenty seeds of Festuca arundinacea inoculated with bacteria were planted in the pots. This study was designed as a completely randomized block factorial with three replications. The plants were grown under controlled conditions for 3 months. They were exposed to light for 18 hours with a temperature range of 2–30°C. The relative humidity was fixed at 70%. After 3 months the rhizosphere soil around the roots was gently collected and transferred to the labo-
Bioremediation of PAHs from Contaminated Soils by Festuca aroundiacea

PAHs Analyses of Soil and Plant Samples

In order to investigate the PAH concentration, 5 g of soil and plant samples were passed through a 2 mm sieve and 5 g of active sodium sulfate was added. They were then extracted by a mixture of 150 ml of acetone and dichloromethane with a volume ratio of 1:1 using a soxhlet apparatus. After its volume was reduced with rotary evaporation and then dried with nitrogen gas, they were kept in the freezer at –20°C until injection time [14].

At the time of injection of the soil samples to GC-FID (Agilent 7890 A), certain concentrations of m-Terphenyl as internal standard were added to all samples (Rodrigo et al. 2009). To measure PAHs with a GC-FID device, a HP-5 column (30 m × 0.25 mm I.D. 0.25 μm film thickness, Agilent) was used. GC-FID device was programmed with inlet temperature of 260°C and N₂ carrier gas flow of 1.5 ml min⁻¹.

The initial temperature of 70°C reached 290°C with the gradient of 5°C. Subsequently, it reached 305°C with the gradient of 1°C min⁻¹. Detector temperature was 270°C in this program.

Analysis of polycyclic aromatic hydrocarbons in plant samples were carried out by an Agilent GC Model 6890 coupled to a quadrupole mass spectrometer (5975 C). The Phenantren d10 was added in specific concentration as an internal standard to all samples. The system was operated in electron impact mode (EI, 70 eV). A HP-5 MS column (30 m × 0.25 mm I.D. 0.25 μm film thickness, Agilent) and a temperature programming were used in order to get the proper GC separation. The temperature started from 80°C with a 5 min hold time, it was then increased to 150°C at a rate of 10°C min⁻¹. It was then increased to 300°C at a rate of 5°C min⁻¹, keeping the final temperature for 5 min. Injection was performed in the split less mode. Helium gas was used as a carrier gas at a constant flow rate of 1.5 ml min⁻¹.

The injector and transfer line temperatures were 250 and 280°C, respectively. Ions were selected after considering the total ion chromatogram of solution of compounds. The ions were divided into four groups. Peak detection and integration were carried out using Chemstation software and AMDIS (automated mass spectral deconvolution and identification system).

Statistical Analysis

Statistical analyses were performed using SAS software and the comparison of means was done with Duncan test at 5% level. The Festuca inoculated with Bacillus licheniformis ATHE9 (F1), Festuca inoculated with Bacillus mojavensis ATHE13 (F8), Festuca inoculated with both bacteria (F1,8) and Festuca without inoculation (F0) as control treatment.

RESULTS AND DISCUSSION

The isolated bacterial strains of ATHE9 and ATHE13 were identified as Bacillus licheniformis and Bacillus mojavensis according to its 16S rDNA sequence as well as biochemical characteristics. They are gram-positive short rods, motile and spore forming, aerobic, oxidase positive, catalase positive, manitol positive, citrate positive.

Bacillus licheniformis strain ATHE9 and Bacillus mojavensis strain ATHE13 could utilize naphthalene, phenanthrene, anthracene, pyrene and other PAHs as sole carbon sources. The degradation efficiencies were examined by GC-Mass and the results showed that these isolates could remove PAHs (the initial concentration of 12.8 mg L⁻¹) in 6 days at 30°C and pH 7.3, accordingly these two isolates were selected to inoculate the plant. The Accession No. of Bacillus licheniformis ATHE9 and Bacillus mojavensis ATHE13 in gene bank are KC329470.1 and KC469987.1 respectively.

Concentrations of PAHs in Soil

Before transferring the soil in to the pots and applying treatments, the hydrocarbon concentrations of the soil were measured. The chemical and physical properties of soil and concentrations of PAHs were showed in Table 1.

The concentrations of these hydrocarbons in the soil are much higher than the standard range of 50 to 1100 µg kg⁻¹ [15]. Therefore, these results confirm the needs for cleanup the soil from hydrocarbons. Furthermore, leaving this soil in non-isolated conditions causes contamination of groundwater and air pollution because these compounds spread in water and air based on their solubility coefficient and steam pressure.

After 3 months, the concentrations of 10 PAHs in soil decreased significantly (p < 0.05). More than 77, 82, 68, 45, 11, 21, 0.6, 32, 4 and 12 percent of naphthalene, acenaphthene, acenaphthylene, phenanthrene, anthracene, benzo[a]anthracene, chry-
sene, benzo[a]pyrene, dibenzo[a,h]anthracene and benzo[ghi]perylene, respectively, were removed from the non- inoculated soil (F0 in Figure 1) as the treatment with lowest reduction during the experimental period. The effectiveness of each treatment in reducing the hydrocarbon concentrations in rhizosphere are shown in Figures 1 and 2.

As seen in Figures 1 and 2, the concentration of hydrocarbons in the rhizosphere of the treated samples greatly decreased in comparison with the initial contaminated soil (Table 1). In the rhizosphere, hydrocarbons with 2 or 3 benzene rings were more degraded compared to heavier hydrocarbons with more rings. Heavy hydrocarbons were less exposed to microorganisms degradation because of their lower solubility in aqueous phase and higher adsorption on soil surfaces [16].

Figure 1 shows that the decomposition of 2 and 3-ring hydrocarbons in Festuca and Bacillus mojavensis ATHE13 (F8) treatment was much higher than other samples.

Given these facts, it can be stated that the Bacillus mojavensis ATHE13 has high ability for improving the conditions of plant roots and it causes higher decomposition of hydrocarbons with less rings and eliminates them from the rhizosphere. Furthermore, the difference in behavior of the bacteria in degenerating various hydrocarbons (in terms of rings number) is because of the unique differences of each bacterium. Each bacterium based on its specific enzyme systems acts specifically in the decomposition of some types of hydrocarbons [17].

Figure 2 show that sample inoculated with both bacteria has lower concentration of hydrocarbons with 4, 5 and 6 rings. These results suggest that the presence of both bacteria together is effective in the decomposition of heavier hydrocarbons with higher number of rings.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Amount</th>
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<tbody>
<tr>
<td>pH</td>
<td>7.24 ± 0.04</td>
</tr>
<tr>
<td>EC (dS.m⁻¹)</td>
<td>3.4 ± 0.25</td>
</tr>
<tr>
<td>CEC (meq.100 g⁻¹)</td>
<td>7 ± 1</td>
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<tr>
<td>OM (%)</td>
<td>4.6</td>
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<tr>
<td>Clay (%)</td>
<td>16.6</td>
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<tr>
<td>Sand (%)</td>
<td>75</td>
</tr>
<tr>
<td>Silt (%)</td>
<td>8.4</td>
</tr>
<tr>
<td>FC water content (%)</td>
<td>22.4</td>
</tr>
<tr>
<td>Naphthalene (mg.kg⁻¹)</td>
<td>16 ± 6</td>
</tr>
<tr>
<td>Acenaphthene (mg.kg⁻¹)</td>
<td>18 ± 4</td>
</tr>
<tr>
<td>Benzo[a]anthracene (mg.kg⁻¹)</td>
<td>22 ± 8</td>
</tr>
<tr>
<td>Benzo[a]pyrene (mg.kg⁻¹)</td>
<td>5 ± 1/3</td>
</tr>
<tr>
<td>Benzo[ghi]perylene (mg.kg⁻¹)</td>
<td>2/5 ± 1</td>
</tr>
<tr>
<td>Acenaphthylene (mg.kg⁻¹)</td>
<td>32 ± 9</td>
</tr>
<tr>
<td>Anthracene (mg.kg⁻¹)</td>
<td>4/5 ± 2</td>
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<tr>
<td>Phenanthrene (mg.kg⁻¹)</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>Chrysene (mg.kg⁻¹)</td>
<td>16 ± 6</td>
</tr>
<tr>
<td>Dibenz[a,h]anthracene (mg.kg⁻¹)</td>
<td>8 ± 2</td>
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Figure 1. The concentration of polycyclic (2 and 3 rings) aromatic hydrocarbons in the treated rhizosphere. Festuca inoculated with Bacillus licheniformis ATHE9 (F1), Festuca inoculated with Bacillus mojavensis ATHE13 (F8), Festuca inoculated with both bacteria (F1,8) and Festuca without inoculation (F0).
benzene rings. Haritash and Kaushik in 2009 reported similar results [10].

Additionally, the results also showed that the concentrations of hydrocarbons in the treatment of Festuca without inoculation and inoculation with Bacillus licheniformis ATHE9 were significantly higher than other treatments ($p < 0.05$) so Bacillus mojavensis ATHE13(F8) and also both bacteria (F1,8) will make the pollution condition more tolerable for the plants and the rhizosphere of the plant is able to degrade more PAHs. Su and Zhu in 2007 also presented such results [18]. According to the results obtained in this study, it can be stated that the mechanism of phytoremediation, which involves the plant and its dependent microbial activities in the rhizosphere, is greatly capable of decomposing and eliminating PAHs from soil environments.

**Concentrations of PAHs in Plants**

Concentrations of Dibenzo[a,h]anthracene and Benzo[ghi]perylene in roots and shoots were not detectable. But concentrations of other PAHs in roots were higher than shoots (Table 2). Acenaphthylene, Acenaphthene, Phenanthrene, Anthracene and Benzo[a]anthracene concentrations in roots increased proportionally with those in rhizosphere soils. With increasing log $K_{ow}$ values of the compounds the PAH concentrations in roots decreased. Similar results for other contaminants in plant roots were reported. Root

![Figure 2. Comparison of the concentration of polycyclic (4, 5 and 6 rings) aromatic hydrocarbons in the treated rhizosphere. Festuca inoculated with Bacillus licheniformis ATHE9 (F1), Festuca inoculated with Bacillus mojavensis ATHE13 (F8), Festuca inoculated with both bacteria (F1,8) and Festuca without inoculation (F0).](image_url)

**Table 2. Root and Shoot PAH Concentrations (mg kg$^{-1}$, dry weight).**

<table>
<thead>
<tr>
<th></th>
<th>NAP</th>
<th>ANY</th>
<th>ANA</th>
<th>PHE</th>
<th>ANT</th>
<th>BaA</th>
<th>CHR</th>
<th>BaP</th>
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<tbody>
<tr>
<td><strong>Roots</strong></td>
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<td></td>
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<tr>
<td>F1</td>
<td>0.2 ± 0.02</td>
<td>0.1 ± 0.04</td>
<td>0.06 ± 0.01</td>
<td>0.10 ± 0.01</td>
<td>0.09 ± 0.004</td>
<td>0.03 ± 0.004</td>
<td>0.03 ± 0.004</td>
<td>0.04 ± 0.003</td>
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<tr>
<td>F8</td>
<td>0.16 ± 0.03</td>
<td>0.11 ± 0.04</td>
<td>0.06 ± 0.008</td>
<td>0.11 ± 0.09</td>
<td>0.09 ± 0.002</td>
<td>0.03 ± 0.003</td>
<td>0.03 ± 0.008</td>
<td>0.02 ± 0.009</td>
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<tr>
<td>F1,8</td>
<td>0.14 ± 0.07</td>
<td>0.08 ± 0.07</td>
<td>0.04 ± 0.01</td>
<td>0.08 ± 0.02</td>
<td>0.06 ± 0.005</td>
<td>0.02 ± 0.007</td>
<td>0.05 ± 0.002</td>
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<tr>
<td>F0</td>
<td>0.20 ± 0.05</td>
<td>0.12 ± 0.08</td>
<td>0.09 ± 0.008</td>
<td>0.12 ± 0.08</td>
<td>0.18 ± 0.08</td>
<td>0.06 ± 0.009</td>
<td>0.09 ± 0.006</td>
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<tr>
<td>Analysis of variance</td>
<td>NS</td>
<td>*</td>
<td>**</td>
<td>**</td>
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<tr>
<td><strong>Shoots</strong></td>
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<tr>
<td>F1</td>
<td>0.19 ± 0.01</td>
<td>0.11 ± 0.03</td>
<td>0.06 ± 0.005</td>
<td>0.11 ± 0.05</td>
<td>0.09 ± 0.03</td>
<td>0.10 ± 0.008</td>
<td>0.03 ± 0.005</td>
<td>0.03 ± 0.007</td>
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<tr>
<td>F8</td>
<td>0.19 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.04 ± 0.005</td>
<td>0.09 ± 0.06</td>
<td>0.06 ± 0.006</td>
<td>0.07 ± 0.009</td>
<td>0.02 ± 0.009</td>
<td>0.02 ± 0.003</td>
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<tr>
<td>F1,8</td>
<td>0.1 ± 0.02</td>
<td>0.07 ± 0.004</td>
<td>0.04 ± 0.01</td>
<td>0.07 ± 0.04</td>
<td>0.08 ± 0.007</td>
<td>0.02 ± 0.006</td>
<td>0.02 ± 0.009</td>
<td>0.02 ± 0.003</td>
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<tr>
<td>F0</td>
<td>0.20 ± 0.07</td>
<td>0.11 ± 0.03</td>
<td>0.15 ± 0.08</td>
<td>0.10 ± 0.03</td>
<td>0.05 ± 0.002</td>
<td>0.15 ± 0.005</td>
<td>0.10 ± 0.001</td>
<td>0.06 ± 0.005</td>
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<tr>
<td>Analysis of variance</td>
<td>NS</td>
<td>NS</td>
<td>**</td>
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**Note:** $**p < 0.01; *p < 0.05; NS, not significant.**
accumulations of these PAHs increased with their concentrations in soil. By contrast, the lipids in roots were usually the major reservoir for highly water insoluble contaminants [19].

Results indicated that inoculation treatments had more effect on removal of PAHs. In the treatment inoculated with both bacteria, accumulation of PAHs in the shoots and roots was less than others. This shows that bacteria have reduced the hydrocarbon concentrations in the rhizosphere that is why plants uptake them less (Figure 1 and 2).

However, the concentrations of the ten PAHs in Festuca shoots showed significant differences with various treatments and were correlated with those in the roots. Wild et al., (2005) indicated that phenanthrene and anthracene translocate slowly into plant roots, only up to 1500 µm in length over a 56-day period [20]. Although there is a lack of information on root uptake of PAHs, this study showed accumulation of PAHs in roots. Several studies suggested that the root uptake of lipophilic organic compounds can be in correlation with root lipid contents [21].

Shoot uptake of PAHs also was enhanced along with the increase of their concentrations in root. The concentrations of PAHs in shoots were statistically less than in roots. Howsam et al., (2001) suggested that uptake of lipophilic organic compounds from the atmosphere by shoots is dependent on the plant lipid contents and plant surface area [22].

CONCLUSION

Phytoremediation is one of the best ways to eliminate PAHs from the soil. In this study, Festuca was inoculated with two types of Bacillus. The results of the study indicate that phytoremediation can be effective in the removal of PAH contamination from soil. Moreover, the results showed that inoculation of bacteria to plants could play an important role in the rate of elimination of hydrocarbons. There were different rates of PAHs uptake to roots or shoots from soil. PAHs may be taken up by roots via passive processes to subsequently accumulate into the root organic matter. The accumulation of PAHs was well correlated with PAH concentrations in rhizosphere soils. Because of the low PAH mass transport rates in soil, it was difficult for PAHs to translocate from rhizosphere soils to roots. Therefore, for soils contaminated with PAHs, both the plant uptake capacity and the PAH mass transport rate must be taken into account to assess the phytoremediation efficiency. Results from the present study indicated that contributions of plant uptake and rhizosphere effect to remove PAHs from soils are significant and rhizosphere effect and plant uptake have important role to remove PAHs from contaminated soils.

REFERENCES