ORIGINAL ARTICLE

# Biodegradation of PAHs in petroleum-contaminated soil using tamarind leaves as microbial inoculums

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## Abstract

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**Biodegradation of PAHs in petroleum-contaminated soil using tamarind leaves as microbial inoculums**

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**Petroleum-contaminated soil contains various hazardous materials such as aromatic hydrocarbons and polycyclic aromatic hydrocarbons (PAHs). This study focused on PAHs since they are potentially toxic, mutagenic, and carcinogenic. Bioremediation of PAHs was carried out by adding tamarind leaf inoculums into petroleum-contaminated soil. Tamarind and other leguminous leaves have been reported to contained several PAH-degrading microorganisms. To minimize the amount of leaves added, the preparation of tamarind leaf inoculums was developed by incubating tamarind leaves with a sub-sample of contaminated**

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**soil for 49 days. After that, the efficiency of tamarind leaf inoculums was tested with two soil samples collected from a navy dockyard and railway station in Samutprakarn and Bangkok, respectively. These soil samples had different levels of petroleum contamination. Bioaugmentation treatment was carried out by mixing contaminated soil with the inoculum at the ratio of 9:1. For navy dockyard soil, the concentration of phenanthrene was decreased gradually and reached the undetectable concentration within 56 days in the inoculated soil; meanwhile 70-80% of fluoranthene and pyrene were remained at the end of treatment. For railway station soil, which had lower petroleum contamination, PAH degradation was more rapid, for example, the concentration of phenanthrene was below detection limit after 28 days. Besides PAHs, the amounts of several hydrocarbons were also reduced after treatment. At the same time, numerous phenanthrene-degrading bacteria, which were used as representatives of PAH degraders, could be observed in both inoculated soils. However, higher numbers of bacteria were found in railway station soil, which corresponded with the lower amount of PAHs and higher amount of soil nutrients. The results showed that inoculum prepared from tamarind leaves could be used to degrade PAHs as well as clean-up petroleum contaminated soil.**

**Key words :** PAHs, petroleum, bioaugmentation, bioremediation

## บทคัดย่อ

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**การย่อยสลายสารพีเอเอชในดินปนเปื้อนน้ำมันปิโตรเลียมโดยใช้ใบมะขามเป็นหัวเชื้อ**

**ว. สงขลานครินทร์ วทท. 2550 29(2) : 515-527**

ดินปนเปื้อนน้ำมันปิโตรเลียมประกอบด้วยสารอันตรายหลายชนิด เช่น สารอะโรมาติกไฮโดรคาร์บอน และสารโพลีอะโรมาติกไฮโดรคาร์บอน (พีเอเอช) การศึกษานี้เจาะจงไปที่สารพีเอเอชเนื่องจากเป็นสารที่มีแนวโน้มจะก่อพิษ ก่อให้เกิดการเปลี่ยนแปลงสารพันธุกรรมและก่อมะเร็ง การบำบัดทางชีวภาพของสารพีเอเอชได้ดำเนินการโดยเติมหัวเชื้อใบมะขามลงในดินปนเปื้อนน้ำมันปิโตรเลียม ทั้งนี้ได้มีรายงานก่อนหน้านี้ว่าใบมะขามและใบพืชตระกูลถั่วอื่น ๆ มีจุลินทรีย์ย่อยสลายสารพีเอเอช เพื่อเป็นการลดปริมาณของใบไม้ที่ใช้เติม จึงได้พัฒนาวิธีเตรียมหัวเชื้อใบมะขามจากการบ่มใบมะขามกับดินที่ปนเปื้อนส่วนหนึ่งเป็นเวลา 49 วัน หลังจากนั้นทดสอบประสิทธิภาพของหัวเชื้อใบมะขามโดยใช้ดินจากบริเวณอู่เรือของกองทัพเรือและสถานีรถไฟในจังหวัดสมุทรปราการและกรุงเทพมหานครตามลำดับ ดินเหล่านี้มีระดับของการปนเปื้อนน้ำมันปิโตรเลียมที่แตกต่างกัน การบำบัดโดยวิธีเติมเชื้อจุลินทรีย์ทำโดยผสมดินปนเปื้อนและหัวเชื้อในอัตราส่วน 9:1 สำหรับดินจากอู่เรือของกองทัพเรือ พบว่า ความเข้มข้นของฟีแนนทรีนในดินที่เติมเชื้อค่อย ๆ ลดลงจนถึงระดับที่ไม่สามารถตรวจสอบได้ภายในเวลา 56 วัน อย่างไรก็ตามเมื่อสิ้นสุดการทดลองยังมีฟลูออแรนทีนและไพรีนเหลืออยู่ 70-80% สำหรับดินจากสถานีรถไฟซึ่งมีการปนเปื้อนสารพีเอเอชน้อยกว่า พบว่าการย่อยสลายของสารพีเอเอชเกิดขึ้นเร็วกว่า เช่น ความเข้มข้นของฟีแนนทรีนต่ำกว่าระดับที่สามารถตรวจสอบได้หลังจาก 28 วัน นอกจากสารพีเอเอชแล้ว พบว่าสารไฮโดรคาร์บอนหลายชนิดมีปริมาณลดลงหลังจากการบำบัด ในขณะเดียวกัน พบว่ามีแบคทีเรียย่อยสลายฟีแนนทรีนซึ่งเป็นตัวแทนของผู้ย่อยสลายสารพีเอเอชอยู่จำนวนมากในดินที่เติมหัวเชื้อทั้งสองชนิด อย่างไรก็ดี ดินจากสถานีรถไฟมีจำนวนแบคทีเรียสูงกว่า ซึ่งสอดคล้องกับปริมาณสารพีเอเอชที่ต่ำกว่าและปริมาณสารอาหารในดินที่สูงกว่า ผลการทดลองแสดงว่าหัวเชื้อที่เตรียมจากใบมะขามสามารถใช้ในการย่อยสลายสารพีเอเอช พร้อมทั้งสามารถบำบัดดินที่ปนเปื้อนน้ำมันปิโตรเลียม

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Polycyclic aromatic hydrocarbons (PAHs) are priority pollutants from their potential toxicity, mutagenicity and carcinogenicity. PAH-contaminated soil and groundwater are widespread and originate from petroleum-related activities, coal-refining process, and petrochemical industries. Low-molecular weight PAHs (containing less than four benzene rings) are acutely toxic, which affect the reproduction and mortality of aquatic animals and most high-molecular weight PAHs (containing four or more benzene rings) are mutagenic and carcinogenic (Samantha *et al*., 2002). Due to their hydrophobic nature, most PAHs in aquatic and terrestrial ecosystems bind to particulates in soil and sediments, which results in low bioavailability for biological uptake (Kastner and Mahro, 1996).

Microbial degradation represents the major route responsible for the ecological recovery of PAH-contaminated sites (Johnsen *et al*., 2005). Many species of bacteria have been shown in recent years to have the abilities to use PAH up to four-ring compounds as sole source of carbon and energy (Bouchez *et al*., 1995). PAH bioremediation techniques have been carried out either by adding nutrients (biostimulation) or degrading microorganisms (bioaugmentation) to the contaminated soil (Samantha *et al*., 2002). Bioaugmentation may be carried out by adding either pure culture or microbial consortium. The introduction of microbial consortium has the advantage over pure culture because the consortium can resist wide variations in natural environment (Forsyth *et al*.,1995). However, the isolation and maintenance of microbial consortium is difficult.

The study was interested in applying microbial consortium adherent to plant leaves, which contains diverse microbial species, for bioaugmentation of PAH contaminated soil. Phyllophere microorganisms are able to decompose diverse plant polymers and have been reported to degrade pollutants, for example, microorganisms colonizing spruce needles decreased the amount of trichloroacetic acid (TCA) in air samples (Forczek et al., 2004). Laine and Jorgensen (1996, 1997) used straw compost to enrich pentachlorophenol-degrading bacteria before applying to soil microcosms and later to a pilot scale study. They also found that straw compost offers a good nutrient source and attachment material for bacteria, thus ensuring better competition ability and protection against predation and toxic effects in the soil environment.

In Thailand, Charoenchang *et al*. (2003) found a potent consortium of phenanthrene-, fluoranthene- and pyrene-degrading microorganisms on rain tree leaves and used these bacteria for bioaugmentation of PAH-contaminated soil. They suggested that tree leaves and their inherent bacteria may be used directly as inoculum for bioremediation. In fact, leguminous leaves (from rain tree, tamarind, and yellow flame tree) can enhance phenanthrene and pyrene degradation in artificially PAH-contaminated soil (Siriwarasin, 2002). However, the application of plant materials on a large scale bioremediation may pose some practical problems from acquiring sufficient amount of material (Laine and Jorgensen, 1996).

The objective of this study was to minimize the amount of added leaves by developing a leaf inoculum preparation technique. Tamarind leaves were selected as sources of PAH-degrading microorganisms. Leaf inoculum was prepared by incubating tamarind leaves with sub-sample of petroleum-contaminated soil. The pre-incubation was necessary for the enrichment of phyllosphere microorganisms that could degrade PAHs as well as tolerate other pollutants in the soil. In this study, pre-incubation period was determined and the efficiency of inoculum was tested in soil microcosms. Three types of PAHs, including phenanthrene, fluoranthene, and pyrene were monitored during bioaugmentation as modeled PAHs. Since the inoculum was prepared from small amounts of tamarind leaves, this approach could be effectively applied for clean-up of a large contaminated area. In addition, inoculum for bioaugmentation of other pollutants may be developed using a similar approach.

## Materials and methods

### 1. Chemicals

Phenanthrene was purchased from Sigma Chemical (USA), while fluoranthene and pyrene were purchased from Kanto Chemical (Japan). All chemicals used were of analytical grade.

### 2. Soil and tamarind leaves

Soil samples were collected from two petroleum contaminated sites, a navy dockyard in Phra Pradaeng district, Samut Prakarn and a railway station in Khet Bangkok Noi district, Bangkok. Navy dockyard site was highly contaminated with used diesel oil from ship engines, which was dumped into the soil once or twice a week. The contamination of railway station soil was lower and caused by fuel leakage and spill during train operation. All debris was removed from soil samples and then the soil was air dried for 16 hr. Dried soil was ground and sieved to a particle size of 2.36 mm. Characterization of their physical and chemical properties was performed at the Agriculture Chemistry Division, Department of Agriculture, Ministry of Agriculture (Table 1). Fallen tamarind leaves were collected from a garden in Chonburi Province and air dried prior to grinding and sieving to a particle size of 2.36 mm. Yard waste was collected from a golf course and air dried. Background concentrations of PAHs and four selected hydrocarbons in soil, tamarind leaves, and yard waste were analyzed after collection (Table 2).

### 3. Preparation of tamarind leaf inoculum

Tamarind leaf inoculum was prepared by packing 1.8 g navy dockyard soil into a 24 ml screw cap vial. The soil was further spiked with 200 µl of 1,000 ppm phenanthrene, fluoranthene and pyrene in acetone solution to increase the amount of PAHs in soil. The final concentration of each PAH was around 115-120 ppm. After standing overnight for acetone evaporation, 0.2 g tamarind leaf powder was added to each vial with the ratio of soil to leaves equal to 9:1. Moisture content of the soil mixture was adjusted to 70% of its water holding capacity. The vials were incubated at 35°C in the dark until the amounts of PAHs were decreased.

**Table 1. Properties of petroleum contaminated soil collected from Navy Dockyard and Railway Station.**

| Parameters | Navy Dockyard Soil | Railway Station Soil |
|---|---|---|
| Soil texture | Sandy soil | Loamy sand soil |
| Sand (%) | 95 | 82 |
| Silt (%) | 4 | 16 |
| Clay (%) | 1 | 2 |
| Organic matter (%) | 10.80 | 20.60 |
| Organic carbon (%) | 6.24 | 11.98 |
| Total-nitrogen (%) | 0.03 | 1.21 |
| Phosphorus (ppm) | 2 | 541 |
| C:N:P ratio | 31,200 : 150 : 1 | 221 : 22 : 1 |
| Potassium (ppm) | 20 | 165 |
| Calcium (ppm) | 92 | 4,848 |
| Magnesium (ppm) | 7 | 14 |
| Moisture (%) | 89.36 | 22.63 |
| EC (electrical conductivity) (mS/cm) | 1.48 | 0.16 |
| pH | 4.1 | 7.8 |
| Water holding capacity (%) | 10.31 | 35.30 |

**Table 2. Background concentrations of phenanthrene, fluoranthene, pyrene, and selected hydrocarbons in tamarind leaves, yard waste and soil samples. The table also showed retention time (RT) of each compound on the GC chromatogram.**

| Compoundsa | RT (min) | Concentrations (ppm)[b] | | | |
|---|---|---|---|---|---|
| | | Navy Dockyard soil | Railway Station soil | Tamarind Leaves | Yard waste |
| Phenanthrene | 13.86 | 14.8 | 6.0 | 5.0 | 3.9 |
| HC 1 | 16.68 | 43 | 7 | ND | ND |
| HC 2 | 19.57 | 54 | 7 | ND | ND |
| Fluoranthene | 21.53 | 17.5 | 7.5 | ND | ND |
| Pyrene | 23.01 | 19.2 | 10.0 | 8.6 | ND |
| HC 3 | 25.41 | 42 | 8 | ND | ND |
| HC 4 | 31.06 | 34 | 4 | ND | ND |

[a] **Four hydrocarbons (HC 1-4) detected in both soil samples were selected for the study based on their relatively high concentrations and the retention time that closed to other PAHs.**
[b] **Values are average from triplicate samples. ND, not detected.**

## 4. Preparation of soil microcosms

Twenty-gram soil microcosms were prepared in 225 ml glass bottles. Similar to the preparation of tamarind leaf inoculum, petroleum-contaminated soil samples were further spiked with 2 ml of 1,000 ppm phenanthrene, fluoranthene and pyrene in acetone solution. There were two treatments, namely inoculated soil and inoculated soil supplemented with yard waste materials. The first inoculated microcosms contained 18 g of soil and 2 g of tamarind leaf inoculum. In the second treatment, 16.2 g of soil was mixed with 2 g of tamarind leaf inoculum and supplemented with 1.8 g of yard waste materials. Control treatments were uninoculated soil microcosms, which contained 20 g of soil. Moreover, a sterilized soil microcosm was made to determine the loss of PAHs and hydrocarbons by non-biological activities. Sterilization was done by autoclaving the soil samples at 121°C, 15 lbs/$m^2$ for 20 min, and then allowing them to stand overnight. The autoclaving treatment was repeated twice in a similar manner and then cyclohexamide was added to prevent fungal contamination. All treatments were performed in triplicates. Water content of the soil mixtures was adjusted to 70% of water holding capacity and they were then incubated at 35°C in the dark. The microcosms were sacrificed every 14 days to analyze for the remaining PAHs and hydrocarbons.

## 5. Detection of PAH degraders

Number of phenanthrene-degrading bacteria, representatives of PAH degraders, was determined by plate count technique (Charoenchang *et al*., 2003). Briefly, ten-fold serial dilutions of soil samples were plated on a carbon free mineral medium (CFMM) agar plate and sprayed with 20 mg/ml phenanthrene solution in diethylether. After incubation at 30°C for up to 14 days, the numbers of colonies with clear zone were counted.

## 6. Extraction and analyses of PAHs

PAHs and other hydrocarbons were extracted by adding 2 soil volumes of n-hexane and 3/4 soil volumes of 15% Triton-x 100 solution directly to the microcosm. The samples were shaken by orbital rotary shaker at 250 rpm for 6 hours before frozen to solidify the aqueous layer. Solvent fraction was passed through an anhydrous sodium sulfate filled Pasteur pipette to clean-up the sample as well as eliminate water. PAHs dissolved in solvent fraction were transferred to autosampling vial for gas chromatography (GC) analysis. The

extraction technique was developed in the laboratory and found to have extraction efficiency more than 95%.

GC analysis was performed with a Hewlett-Packard 6890 equipped with flame inonization detector (GC-FID) and a HP-5 (5% Phenyl Methyl Siloxane) fused-silica capillary column (30 m x 0.32 mm ID; thickness, 0.25 µm). The following operating conditions were used: injector temperature 280°C, detector temperature 250°C, initial column temperature 80°C then, programmed at 80°C to 160°C at a rate of 25°C/min hold for 3 minutes and 160°C to 240°C at a rate of 3°C/min and hold for 3 minutes and 240°C to 300°C at a rate of 40°C/min and hold for 8 minutes. Carrier gas was helium (average linear volume of 13.3 ml/min) and make-up gas was nitrogen at 60 ml/min. Split ratio was kept at 5:1. Under these conditions, the retention time of standard phenanthrene, fluoranthene and pyrene are 13.86±0.5 min, 21.53 ±0.5 min, and 23.01±0.5 min, respectively. Each sample was analyzed by comparing the amount of PAHs recovered from soil to a standard curve of PAHs.

## Results

### 1. PAH degradation in tamarind leaf inoculum

The appropriate incubation time for preparing tamarind leaf inoculum was determined by comparing the amount of PAHs in 2 g navy dockyard soil with and without tamarind leaves after incubation. Although, the soil sample contained some background PAHs as well as hydrocarbons (Table 2), it was further spiked with 100 ppm each of phenanthrene, fluoranthene, and pyrene to increase the amount of soil PAHs and consequently enrich for PAH-degrading bacteria. The amounts of all PAHs in soil without tamarind leaves were nearly 100% of the initial amount throughout the study (Figure 1). Meanwhile, the amounts of PAHs, especially phenanthrene and fluoranthene, in tamarind leaves added soil were around 80% of their initial concentration and significantly lower than in soil without tamarind leaves at day 56. The results indicated that tamarind leaves were required for the decreasing of soil PAHs. At the beginning, the number of PAH-degrading bacteria on tamarind leaves was probably minimal, however these bacteria were growing as the amount of PAHs were decreasing at the end of study. Longer incubation time would result

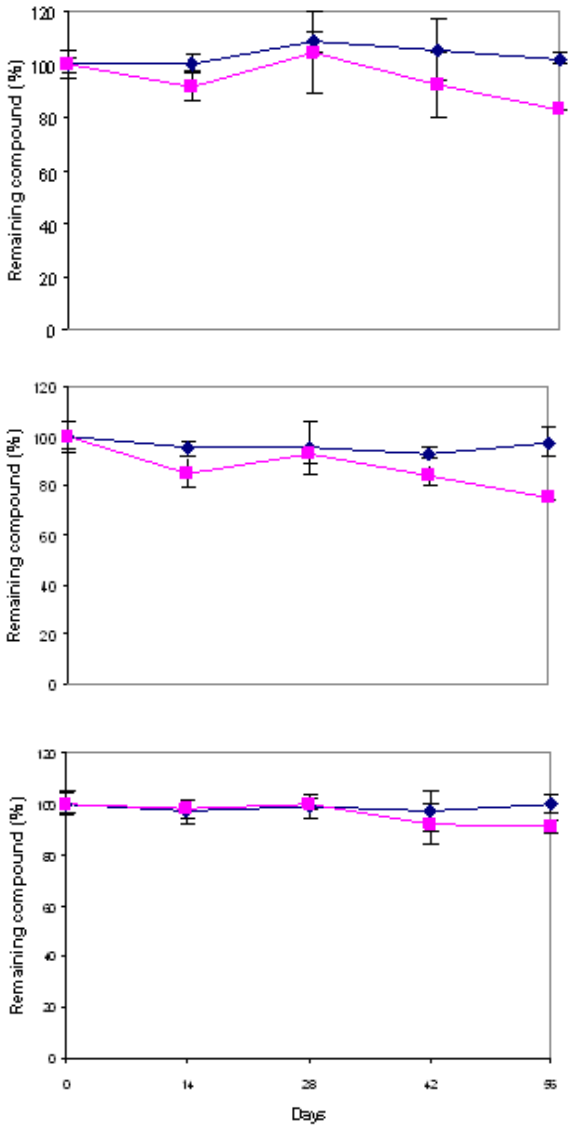


**Figure 1. Percentage of remaining phenanthrene (A), fluoranthene (B) and pyrene (C) in the tamarind inoculum (Navy Dockyard soil mixed with tamarind leaves; ( ■ ) compared to the control soil, which had no tamarind leaves ( ◆ ).**

in higher number of PAH-degrading bacteria and consequently higher amounts of PAH removal. However, the long incubation period would delay PAH bioremediation process. We therefore selected 49 days to prepare tamarind leaf inoculum for the following soil microcosm experiment.

**2. PAH degradation in soil microcosms**

Two types of soil, navy dockyard and railway station soils, were used to determine the efficiency of tamarind leaf inoculum on degrading PAHs. The inoculated soil microcosms contained 20 g contaminated soil and 49-day old tamarind leaf inoculum at the ratio of 9:1. PAH degradation in the inoculated soil microcosms (Figure 2 and 3) was more effective than in tamarind leaf inoculum (Figure 1). For example, almost 100% of phenanthrene was remained in the tamarind leaf inoculum at day 14, while there was only 25% of phenanthrene left in the inoculated railway station soil microcosms at the same period. The results indicated that tamarind leaf inoculum promoted the reduction of lag period and increased the extent of PAH degradation in inoculated soil microcosms.

When compared between treated microcosms, it was also found that tamarind leaf inoculum enhanced the efficiency of biodegradation process in both soil types. For inoculated navy dockyard soil, the concentration of phenanthrene was decreased gradually and reached the undetectable concentration within 56 days; meanwhile 70-80% of fluoranthene and pyrene were remained at the end of treatment (Figure 2). On the other hand, the percentage of all PAH degradation was insignificant in both sterilized and uninoculated soil. The activity of tamarind leaf inoculum was reduced when yard waste materials were added to the soil. We found that higher amounts of PAHs (around 80-90%) remained in inoculated soil supplemented with yard waste. However, the amount of PAHs was still lower than in uninoculated soil (100%) at day 56.

The results from railway station soil were similar to navy dockyard soil, which showed higher degradation of PAHs in inoculated soil than in uninoculated soil. Rapid degradation of all PAHs was observed within 14 days of incubation in inoculated soil microcosms (Figure 3). The concentration of phenanthrene was decreased rapidly and reached the undetectable concentration within 28 days; meanwhile around 60% of fluoranthene and pyrene remained in the soil at the same period. In railway station soil, the effects of yard waste on PAH degradation were marginal. Consequently, the supplementation of yard waste was not necessary in this bioremediation approach.

**3. Number of PAH degraders in soil microcosms**

Number of phenanthrene degraders was used to represent PAH-degrading bacteria. In both soil types, the results showed that tamarind leaf inoculum increased the number of soil phenan-

**Table 3. Number of phenanthrene-degrading bacteria in treated soil microcosms.**

| Type of soil | Treatments | Phenanthrene-degrading bacteria (CFU/ g soil x $10^2$) | | | | |
|---|---|---|---|---|---|---|
| | | 0 day | 14 days | 28 days | 42 days | 56 days |
| Navy Dockyard | Sterilized | <10 | <10 | <10 | <10 | <10 |
| | No inoculum | <10 | <10 | <10 | <10 | <10 |
| | Soil + Inoculum | 2.2 | 6900 | 8200 | 10000 | 15800 |
| | Soil + Inoculum + Yard waste | 1.4 | 5.5 | 10 | 70 | 92 |
| Railway Station | Sterilized | <10 | <10 | <10 | <10 | <10 |
| | No inoculum | 0.12 | 0.42 | 0.7 | 0.9 | 1.2 |
| | Soil + Inoculum | 10.6 | 11800 | 54700 | 780000 | 950000 |
| | Soil + Inoculum + Yard waste | 9.2 | 120 | 4700 | 91000 | 107300 |

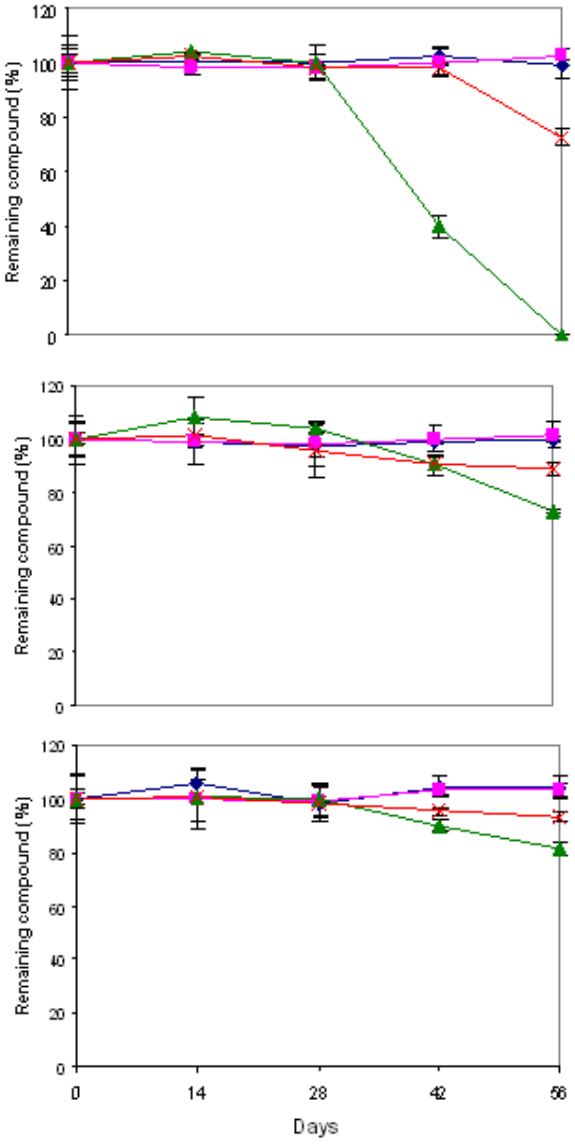


**Figure 2. Percentage of remaining phenanthrene (A), fluoranthene (B) and pyrene (C) in soil from Navy Dockyard after treatment; sterilized soil (◆), uninoculated soil (■), inoculated soil (▲), and inoculated soil supplemented with yard waste (×).**

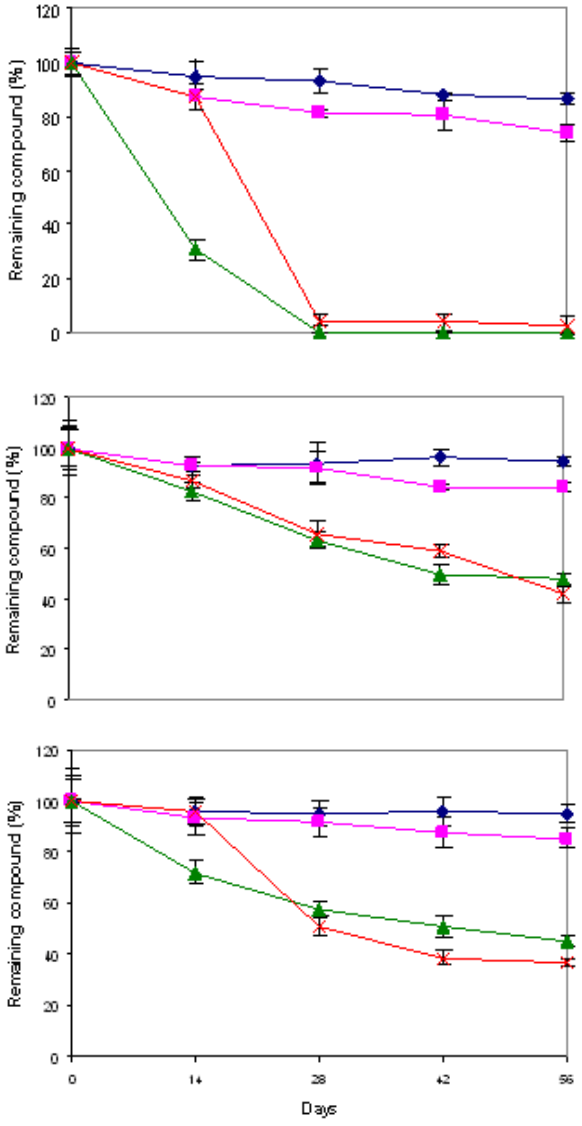


**Figure 3. Percentage of remaining phenanthrene (A), fluoranthene (B) and pyrene (C) in soil from Railway Station after treatment; sterilized soil (◆), uninoculated soil (■), inoculated soil (▲), and inoculated soil supplemented with yard waste (×).**

threne-degrading bacteria by 10 folds at the beginning of study (Table 3). The number of phenanthrene-degrading bacteria ranged from 1.4 x $10^2$ to 10.6 x $10^2$ CFU/g in the inoculated soil, while there were less than 10 CFU/g of the bacteria in the uninoculated soil. The results suggested that tamarind leaf inoculum contained high number of phenanthrene degraders. After

incubation, the number of phenanthrene degraders increased considerably in all inoculated soil but not in uninoculated soil, which contained < 10 to 120 CFU/g. At day 56, there were 1.58 x $10^6$ CFU/g and 9.5 x $10^7$ CFU/g of phenanthrene degraders in the inoculated navy dockyard and railway station soil, respectively. The results suggested that bacteria from the inoculum were able to survive and grow in the contaminated soil.

When comparing between soil types, it was found that more phenanthrene degraders were detected in railway station soil regardless of treatments. The results indicated that the soil from railway station was probably more conducive to bacterial growth than navy dockyard soil. The number of phenanthrene-degrading bacteria in the inoculated soil with yard waste materials was lower than in the inoculated soil without yard waste (Table 3). This phenomenon was found in both soil types. Therefore, the supplementation of yard waste probably had adverse effects on phananthrene-degrading bacteria. These results also supported the earlier PAH data, which indicated that the supplementation of yard waste could not enhance PAH degradation in the inoculated soil microcosms.

**4. Degradation of other hydrocarbons in soil microcosms**

Besides PAHs, soil samples from both locations contained several hydrocarbons, which were showed by various peaks on GC chromatogram (Figure 4). The identification of these hydrocarbons has not yet been done; however, we could monitor their degradation along with other PAHs by comparing the areas of each hydrocarbon peak before and after treatment. Four hydrocarbon compounds (or peaks) were selected as model compounds based on their relatively high concentrations and their retention times that were close to studied PAHs. The retention times of these hydrocarbon compounds were 16.68, 19.57, 25.41, and 31.06 min.

Degradation of these hydrocarbons was significantly increased after incubating the soil samples with tamarind leaf inoculum. After 56 days, 40-51% of all hydrocarbons were degraded in the inoculated navy dockyard soil mirocosms, while there was no degradation in the uninoculated soil (Table 4). Similar results were found from railway station soil but to a higher extent. In the inoculated railway station soil, all monitored hydrocarbons were completely degraded after 56 days, while only 10-18% of such hydrocarbons were degraded in the uninoculated soil. The degradation of hydrocarbons in navy dockyard soil was enhanced by yard waste supplementation, in which percent hydrocarbon degradation was significantly increased to 55-100%. The result was different from PAH degradation data, in which yard waste supplementation reduced the extent of PAH degradation.

## Discussion

The results showed that tamarind leaf inoculum enhanced PAH and hydrocarbons degradation in petroleum-contaminated soil microcosms. When compared with uninoculated soil, bacteria in inoculated microcosms degraded PAHs quicker and at higher extent. Most of PAH-degrading populations in the treated microcosm came from tamarind leaf inoculum since higher amounts of phenanthrene-degrading bacteria were found in the inoculated soil. By mixing tamarind leaves with petroleum-contaminated soil, phyllosphere bacteria that able to utilize petroleum compounds (e.g. PAHs and hydrocarbons) as well as their degrading-intermediates were enriched. In addition, bacteria in tamarind leaf inoculum were pre-adapted to the contaminated soil environment, thus they were active and proliferated well in the microcosms after application.

Pre-adapted bacteria have been used as inocula for the bioremediation of chlorophenol- and benzene-contaminated soil, although the processes of inoculum preparation were different from that used in this study. For example, Laine and Jorgensen (1996) used percolator enrichment system for selection of chlorophenol degrading microorganisms in straw compost and remediated soil; while Semple *et al*. (1998) incubated spent

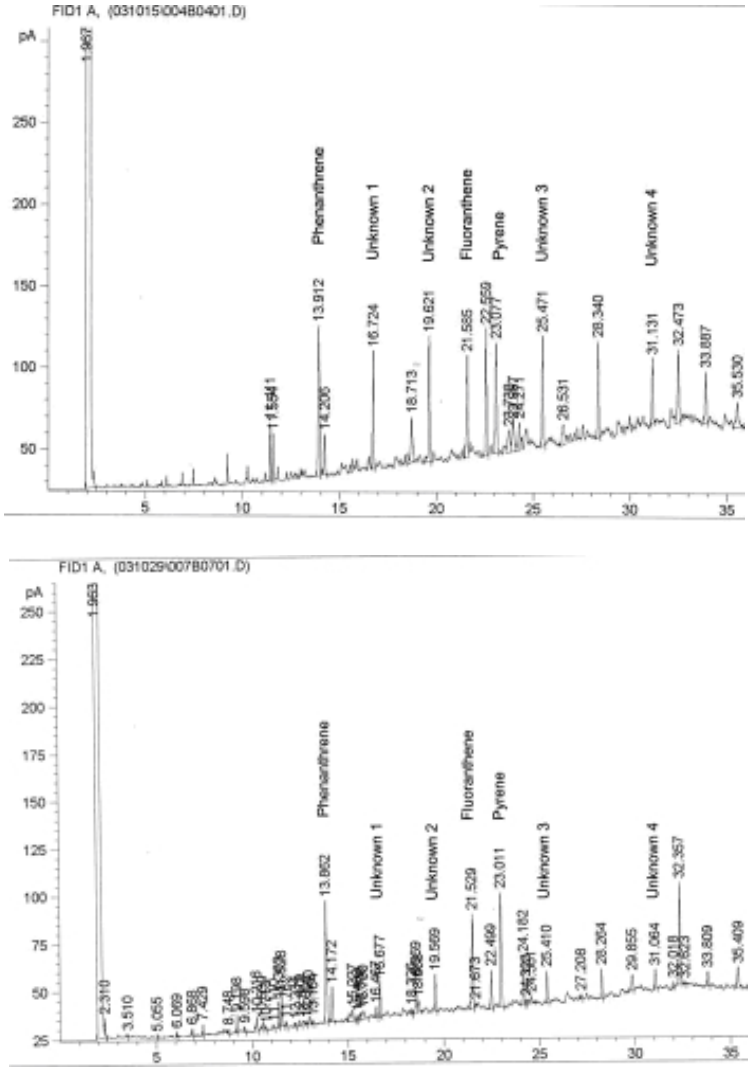


**Figure 4. Gas chromatograms of petroleum-contaminated soil collected from navy dockyard (A) and railway station (B) before treatment. The GC peaks showing phenanthrene, fluoranthene, pyrene, and four selected hydrocarbons (labeled as unknown 1-4).**

mushroom substrate with benzene for 3 months before apply for the remediation. Carlstrom and Tuovinen (2003) also found that pre-incubation of yard-waste compost subsamples with phenanthrene prior to placing the compost into biometers accelerated the onset of mineralization and increased the relative amount of mineralized phenanthrene.

The presence of PAHs in soil has been shown to increase the degrader numbers but have no impact on overall bacterial, fungal, or actinomycete numbers (Gentry *et al*., 2003). Our study showed that tamarind leaf inoculum contained several kinds of active bacteria that could degrade many PAHs and hydrocarbons in petroleum-contaminated soil. In the environment, phenanthrene degraders are prevalent but bacteria that degrade high molecular weight PAHs are rare (Samanta *et al*., 2002). Johnsen *et al*. (2005) suggested that an efficient PAH degrader is char-

**Table 4. Percent degradation of hydrocarbon compounds in treated microcosms after 56-day incubation.**

| Type of soil | Treatments | Percent degradation (%)* | | | |
|---|---|---|---|---|---|
| | | HC1 | HC2 | HC3 | HC4 |
| Navy Dockyard | No inoculum | $0^{A}$ | $0^{A}$ | $0^{A}$ | $0^{A}$ |
| | Soil + Inoculum | $51^{B}$ | $44^{B}$ | $40^{B}$ | $47^{B}$ |
| | Soil + Inoculum + Yard waste | $100^{C}$ | $57^{C}$ | $55^{C}$ | $100^{C}$ |
| Railway Station | No inoculum | $12^{D}$ | $10^{D}$ | $12^{D}$ | $18^{D}$ |
| | Soil + Inoculum | $100^{C}$ | $100^{E}$ | $100^{E}$ | $100^{C}$ |
| | Soil + Inoculum + Yard waste | $88^{E}$ | $100^{E}$ | $100^{E}$ | $100^{C}$ |

*** Comparisons between treatments within each column (hydrocarbon, HC) are significantly different (LSD, P <0.05) if marked with different capital letters.**
**** Ratio of soil to tamarind leaves to yard waste.**

acterized by its intrinsic oligotrophy, a natural tendency to form biofilms on surfaces in oligotrophic environments and the ability to acquire and express degradative genes. From the rapid reduction of PAHs in our study, tamarind leaf inoculum probably contained various efficient PAH degraders, since these bacteria were originally from phyllosphere, an oligotrophic environment. In addition, the ability to degrade PAHs was distributed quickly throughout the soil microcosms as seen from the reduced lag period in the inoculated soil. Johnsen *et al*. (2005) suggested that the spreading of PAH-catabolic abilities among bacteria in polluted soil is due to the presence of PAH-degradative genes on mobile, genetic elements and conjugative gene-transfer.

The extent of PAHs and hydrocarbons degradation was also depended on characteristic of soil samples. We found that PAHs and hydrocarbons were degraded more in soil samples from railway station than from navy dockyard. This was probably due to the difference in C:N ratio and other soil physical and chemical properties between these samples. C:N ratio of railway station soil (10:1) was closer than that of the navy dockyard soil (208:1) to the appropriate C:N ratio (12.5:1) for bioremediation recommended by Hupe *et al*. (1996). Moreover, this soil had high amount of nutrients and pH near neutral, which would be suitable for growth of diverse bacterial populations. Breedveld and Karlsen (2000) also found that the presence of organic matter and asphaltic compounds in soil is associated with high PAH residual levels. The navy dockyard samples were highly contaminated, thus PAHs might associate with other contaminants and become unavailable for biodegradation. In addition, the high amount of contaminants may be toxic to soil microorganisms.

Nonetheless, tamarind leaf inoculum prepared from navy dockyard soil could be used to enhance PAH degradation in the railway station soil. The result showed that the preparation of inoculum could be done with soil from other locations. This would save cost and reduce incubation time for the bioremediation. Soil samples contained various PAHs and hydrocarbons were suitable for inoculum preparation since the intermediates from mineralization of certain PAH species. For example, products derived from pyrene transformation have the potential to accumulate in PAH-contaminated system and that such products can significantly influence the removal of other PAHs (Kazunga and Aitken, 2000).

The degradation of PAHs was decreased when yard waste materials were added to the inoculated soil. However, yard waste supplementation enhanced hydrocarbon degradation in the inoculated soil microcosms. The results suggest that PAH-degrading populations were different from hydrocarbon-degrading populations. In this

study, yard waste may selectively enhance the growth of hydrocarbon-degrading bacteria but not PAH-degrading bacteria. Carmichael and Pfaender (1997) also reported that many kinds of supplement materials can increase the densities of heterotrophic microorganisms, as measured by plate count technique but did not increase population of PAH-degrading microorganisms, as measured by the spray-plate technique.

In conclusion, the study was able to prepare tamarind leaf inoculum by incubating leaves with sub-sample of petroleum contaminated soil. The technique was quite simple and yet this inoculum was proved to be a good source of effective PAH degraders. Using this approach, the amount of added tamarind leaf for PAH bioremediation could be reduced. Meanwhile, the quality of inoculum produced from various sources of tamarind leaves may be different and needs to be further investigated. In addition, long-term storage of the tamarind leaf inoculum is not recommended. The inoculum contained undefined mixed bacterial culture and the storage may change the structure and activities of specific PAH-degrading populations. The knowledge from this research could be used for developing field-scale PAH iodegradation treatment as well as for bioremediation of other pollutants.

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