Utilization of Agricultural Materials to Enhance Microbial Degradation of Polycyclic Aromatic Hydrocarbons in Soil

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The ability of agricultural waste materials to enhance bioremediation of soil contaminated with polycyclic aromatic hydrocarbons (PAHs) was investigated. Sandy soil spiked with phenanthrene, fluoranthene and pyrene was mixed with each of the following agricultural material: rice straw, peanut shells and rain tree leaves, at the ratio of 9:1. Bioremediation was performed in a small screw-capped vial used as a 2-gram microcosm with the moisture content of the soil mixtures adjusted to 60% of the water holding capacity of the soil and incubated at 30 ^{o}C in the dark. HPLC analyses revealed that addition of peanut shells and rain tree leaves could facilitate the decrease in concentrations to undetectable levels of phenanthrene within 24 days and fluoranthene and pyrene within 42 days, while rice straw did not produce any enhanced bioremediation effect. No decrease in PAH concentrations were observed in the soil mixtures without added agricultural materials or in the sterilized soil being used as a control. The results indicated that some biotic factors from the peanut shells and the rain tree leaves might play an important role in PAH degradation. Phenanthrene-degrading bacteria, representative PAH degraders, were detected by clear zone-forming around the colonies grown on carbon free mineral medium (CFMM) agar plate sprayed with phenanthrene solution. Numerous phenanthrene-degrading bacteria were found in mixtures of soil with non-sterilized peanut shells or rain tree leaves. Furthermore, bioavailability of PAHs in the soil mixtures was also determined via the dichloromethane extractability of the respective PAHs, indicating that the rain tree leaves provided more PAH availability than the peanut shells.

Key words: bioremediation, polycyclic aromatic hydrocarbons, agricultural waste materials.

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การใช้วัสดุจากการเกษตรเร่งการย่อยสลายสารพอลิไซคลิกอะโรมาติกไฮโดรคาร์บอนโดยจุลินทรีย์ในดิน

นารีรัตน์ เจริญช่าง ไพเราะ ปิ่นพานิชการ กอบชัย ภัทรกุลวณิชย์ สุเทพ ธนียวัน และ กาญจณา จันทองจีน (2546)
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งานวิจัยนี้ได้ศึกษาความสามารถของวัสดุเหลือใช้ทางการเกษตรในการเร่งการบำบัดดินที่ปนเปื้อนสารประกอบพอลิไซคลิกอะโรมาติกไฮโดรคาร์บอน โดยใช้ดินทรายที่ทำให้ปนเปื้อนด้วย ฟีแนนทรีน ฟลูออแรนธีน และไพรีนผสมกับวัสดุการเกษตรแต่ละชนิดได้แก่ ฟางข้าว เปลือกถั่วลิสง และใบจามจุรีในอัตราส่วน 9:1 ในหลอดแก้วที่มีฝาเกลียวปิดและทำให้เป็นระบบนิเวศน์จำลองที่มีดิน 2 กรัม โดยปรับความชื้นที่ 60% ของความจุในการอุ้มน้ำของดินและบ่มที่อุณหภูมิ 30°ซ ในที่มืด ผลการวิเคราะห์ HPLC แสดงให้เห็นว่าการเติมเปลือกถั่วและใบจามจุรีช่วยทำให้ปริมาณ ฟีแนนทรีนลดลงจนตรวจไม่พบภายใน 24 วัน ส่วนฟลูออแรนธีนและไพรีนลดลงจนตรวจไม่พบ ภายใน 42 วัน ในขณะที่ฟางข้าวไม่มีผลช่วยการย่อยสลาย ปริมาณ PAHs ไม่ลดลงในดินที่ไม่เติมวัสดุทางการเกษตรและในชุดควบคุมที่ใช้ดินปลอดเชื้อ ผลแสดงให้เห็นว่ามีปัจจัยทางชีวภาพบางชนิดในเปลือกถั่วและใบจามจุรีอาจมีบทบาทสำคัญในการย่อยสลายสารพอลิไซคลิกอะโรมาติกไฮโดรคาร์บอน ได้ตรวจหาแบคทีเรียที่ย่อยสลาย ฟีแนนทรีนซึ่งใช้เป็นตัวแทนของแบคทีเรียที่ย่อยสลายสารนี้ โดยการตรวจหาบริเวณใสรอบโคโลนีของแบคทีเรียบนอาหารเลี้ยงเชื้อ CFMM ซึ่ง ฉีดพ่นด้วยสารละลายฟีแนนทรีน พบแบคทีเรียที่ย่อยสลายฟีแนนทรีนจำนวนมากทั้งในดินที่ผสมเปลือกถั่วและดินที่ผสมใบจามจุรี นอกจากนี้ได้หาชีวปริมาณออกฤทธิ์ของ PAHs (ค่าของปริมาณสารนี้ที่สิ่งมีชีวิตในดินสามารถนำไปใช้ในการเจริญได้) โดยดูจากความสามารถในการสกัดสารนี้ด้วยไดคลอโรมีเทน พบว่าใบจามจุรีจะทำให้ค่าชีวปริมาณออกฤทธิ์ในดินของสารประกอบนี้ สูงกว่าในเปลือกถั่ว

คำสำคัญ การบำบัดทางชีวภาพ พอลิไซคลิกอะโรมาติกไฮโดรคาร์บอน วัสดุเหลือใช้ทางการเกษตร

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are the main contaminant class in soils of former coal gasification sites, tar oil distillation plants or wood-preserving industries. Different biological technologies have been used in the treatment of such contaminated soils.[1] Soil remediation on an industrial scale is mostly conducted in soil systems that are treated by composting using agricultural wastes. Complex additives like fertilizer, bark chips, shredded plant materials or compost are utilized.[2] Additions of these materials enhance biodegradation by improving soil texture and oxygen transfer, and providing an energy source to rapidly establish a large microbial population.[3] Wagner and Zablotowicz[4] proposed that agricultural materials like corn meal, rye grass and poultry litter can be utilized for remediation of cyanazine and fluometuron in soil. In Thailand agricultural waste materials are generated in large amounts from agricultural production in almost every part of the country. The waste including rice straw, peanut shells and fallen leaves as used in this research, is employed in composting ingredients for soil improvement, increasing the contents of organic matter and elements for economic plant growth. Composting to treat many kinds of wastes, i.e., yard waste, municipal waste water sludge and municipal solid wastes, has been performed for decades. Composting to treat hazardous waste contaminants in soil is still emerging in ex-situ bioremediation.[5] Biodegradation of explosives and polycyclic aromatic hydrocarbons (PAHs) in soils have been reported to be effective by composting during full-scale applications;[6] however, the waste materials above have never been clarified as sources of suitable media for PAH bioremediation in soil.

In this paper, we investigate the role of agricultural wastes in Thailand (rice straw, peanut shells and rain tree leaves) to enhance biodegradation of phenanthrene, fluoranthene and pyrene in the soil, since they may be sources of nutrients and supply a great variety of microorganisms to improve recalcitrant PAH degradation in the soil.

MATERIALS AND METHODS

Soil

The soil used in this study was required to be free of PAH contamination. It was collected from a hill area at Suan Pueng district, Ratchaburi Province and analyzed for any PAH contamination by the methods for analyses of PAHs in the soil described below. It was then sieved to a particle size of 1.18 mm and the characterization of its physical properties and chemical composition was performed at the Agriculture Chemistry Division, Department of Agriculture, Ministry of Agriculture.

Agricultural waste materials

Rice straw was collected from a rice field in Ratchaburi Province. Peanut shells were purchased from a plant and fertilizer section available in several supermarkets in Thailand. Rain tree leaves were collected from the area surrounding the football field at Chulalongkorn University and ground with a blender and then sieved to a size of less than 0.84 mm.

Chemicals

Fluoranthene and pyrene were products of Kanto Chemical Co., Inc. (Tokyo, Japan), and phenanthrene was from Sigma Co., Inc. (St. Louis, USA). All chemicals used were of analytical grade.

Enhancement of PAH degradation in the soil by agricultural materials

Sieved soil, 1.8 g, was packed into a 23x85-mm screw cap vial. The soil in each vial was spiked with 3 PAHs, phenanthrene, fluoranthene and pyrene, prepared as stock solutions in acetone, each at concentration of 0.1 mg/g soil, by adding each compound dropwise. After standing overnight at room temperature for acetone removal, the soil samples were individually mixed with agricultural materials, rice straw, peanut shells or rain tree leaves, at the ratio of 9:1 (soil : material). The moisture content of the soil mixtures was adjusted to 60% of the water holding capacity of the soil by adding sterile distilled water. Incubation was carried out at 30°C in the dark for 42 days. A set of similar samples was also prepared for each treatment for sampling at 14-day intervals to observe the growth of bacterial degraders. The sterilized soils or agricultural materials to be used as controls free of microorganisms were prepared by autoclaving at 121°C, 15 lbs/m^2 for 20 min, and then allowed to stand overnight. The autoclaving treatment was repeated twice in a similar manner. All treatments were performed in duplicate.

Extraction and analyses of PAHs in the soil

An amount of 2 g of anhydrous sodium sulphate was added to the soil mixture or soil sample (2.0 g) and extracted with 3 ml of dichloromethane by continuous mixing in a vortex mixer for 2 min, followed by 15 min of ultrasonication (Decan Ultrasonics, model FS400, England). The sample was centrifuged at 1,000 rpm for 5 min and the dichloromethane phase containing PAHs was recovered. Extraction was repeated twice and the extracts were combined and evaporated to dryness by a rotary vacuum evaporator (model N, Rikakikai, Japan). The residue was redissolved in 500 μl of methanol for further analysis. The PAH concentration in the sample was quantified by high performance liquid chromatography (HPLC) using a reversed phase column (Inersil ODS-3, 4.6 mm x 150 mm, GL Science, Japan) at 40°C with 80% aqueous methanol as a mobile phase at a flow rate of 1.0 ml min^{-1}. Detection of PAHs was monitored at 275 nm using commercial phenanthrene, fluoranthene and pyrene as standards.

Detection of phenanthrene-degrading bacteria

Phenanthrene-degrading bacteria, representatives for PAH degraders, were determined by adding 18 ml of sterile 0.85% NaCl solution into the 2 g soil mixture and mixing for 2 min in a vortex mixer. The soil suspension was subjected to ten-fold serial dilutions and 100 μl of each dilution was spread on a carbon-free mineral medium (CFMM) agar plate and sprayed with 20 mg/ml phenanthrene solution in diethylether. The plates were incubated at 30°C for up to 14 days and observed for colonies with surrounding clear zones.

Bioavailability of PAHs in the soil and the soil mixtures

Bioavailability of PAHs in the soil or soil mixtures containing agricultural materials (peanut shells and rain tree leaves) was determined from the extractability of each compound from the sterilized soil or soil mixture using the organic solvent dichloromethane as described by Verstraete and Devliegher.[7] Two grams of sterilized soil or soil mixture (1.8 g soil, 0.2 g agricultural material) were individually mixed with 0.2 mg phenanthrene, fluoranthene or pyrene and aged in the dark for various periods of time as indicated in the results. The extractable amount of each PAH was analyzed by HPLC.

Extractability of PAHs in the peanut shells and rain tree leaves

Extractability of phenanthrene, fluoranthene and pyrene in the peanut shells and the rain tree leaves by dichloromethane was determined. Ground peanut shells or rain tree leaves (0.2 g) were mixed individually with 0.2 mg of the three PAHs, under sterile conditions and aged in the dark for a given period of time as specified in the results. The extractable amount of each PAH was analysed by HPLC.

RESULTS AND DISCUSSION

Characterization of the soil and the agricultural materials

As each soil sample to be used in the present study had to be free of PAH contamination, this was first checked by HPLC analysis. No PAH contamination was observed (data not shown). The properties of the soil and of the agricultural materials were then determined as shown in Tables 1 and 2, respectively. The C:N:P ratio of the soil sample was 123:10:2, which was close to that of 100:8:2 reported by Hupe et al.[3] was suitable for PAH bioremediation. However, the C:N ratios of the agricultural materials shown in Table 2 were in the range of 51:1 to 12:1, so the addition of those materials to the soil sample may affect the C:N:P ratio of the soil mixture.

Table 1. Characteristics of the soil.

Component	Characteristics
Soil texture	sandy soil
Sand	73%
Silt	19%
Clay	8%
Organic matter	2.12%
Organic carbon	1.23%
Organic nitrogen	0.10%
C / N	12:1
Phosphate	0.02%
pH	6.5
Cation exchange capacity (meq/100 g)	5.2
Water holding capacity	30.73%

Table 2. Description of agricultural materials.

Component	Rice straw	Peanut shells	Rain tree leaves
pH	6.9	5.2	6.6
Organic carbon	45.08%	42.88%	36.42%
Nitrogen	0.89%	0.87%	3.10%
Phosphate	0.39%	0.39%	0.49%
Potassium	1.98%	0.75%	0.90%
C / N	51:1	42:1	12:1
Moisture content at 70°C 20h	7.76%	10.87%	13.46%

Effects of the agricultural materials on the enhancement of PAH degradation in the soil

The experiments on the enhancement of the agricultural materials on the PAH degradation in the soil were performed as described in the Materials and Methods section. As shown in Figure 1, phenanthrene could not be detected in the soil sample mixed with peanut shells and rain tree leaves after 28 days of incubation, while fluoranthene and pyrene were undetectable after 42 days of incubation in the presence of the same agricultural materials. However, rice straw showed no enhancing effect on PAH degradation; therefore, peanut shells and rain tree leaves were selected for further study. Addition of the peanut shells or rain tree leaves to the soil may change the C:N:P ratio of the soil as mentioned earlier, but these materials showed enhancing effects on bioremediation. Therefore, the change in the C:N:P ratio may not be over the limiting range of the requirement of soil microorganisms for growth and physiological functions. Kastner and Mahro[2] reported that addition of compost materials to soil was not only used as a source of nutrient and organic matter but also served as a source of microorganisms that might play a major role in biodegradation.

Effects of sterilized or non-sterilized peanut shells or rain tree leaves on PAH degradation in sterilized or non-sterilized soil samples

To determine whether organic matter or microorganisms in the soil sample or the agricultural materials play a role in PAH depletion, sterilized or non-sterilized peanut shells or rain tree leaves were added to sterilized or non-sterilized soil samples spiked with PAHs. The results in Figure 2 showed no difference in PAH concentrations in the sterilized or non-sterilized soils within a 42 day-period of incubation, indicating that indigenous microorganisms in the soil played no important role in PAH degradation. However, the slight depletion of PAH concentrations observed might be due to some physical factors such as volatilization or sorption to the soil matrix.[2] Similar results were found with addition of sterilized peanut shells or rain tree leaves to sterilized or non-sterilized soil, indicating that organic matter in the agricultural materials had no effect on bioremediation. In contrast, addition of non-sterilized peanut shells and rain tree leaves to the soil significantly decreased the PAH concentrations, which indicated that the enhancing effect on PAH degradation may be due to the microorganisms inherent in these materials. The presence of phenanthrene degrading bacteria, representative PAH degraders, which were detected in the soil mixtures containing non-sterilized peanut shells (Figure 3A) or non-sterilized rain tree leaves (Figure 3B), was the evidence that supporting the above interpretation.

In an earlier experiment, we found that rice straw had no enhancing effect on PAH degradation, perhaps because it lacked microorganisms with PAH degrading ability. There have been reports on the degradation of some PAHs by co-metabolism in the presence of a PAH with simple structure such as phenanthrene, a tricyclic PAH, by many soil micro-organisms,[8] including *Sphingomonas* sp. isolated from lubricant-contaminated soil in Thailand.[9] The present work showed that degradation of fluoranthene and pyrene, both tetracyclic PAHs, also occurred in the presence of phenanthrene; therefore, it remains for further investigation to show whether co-metabolism is involved or not.

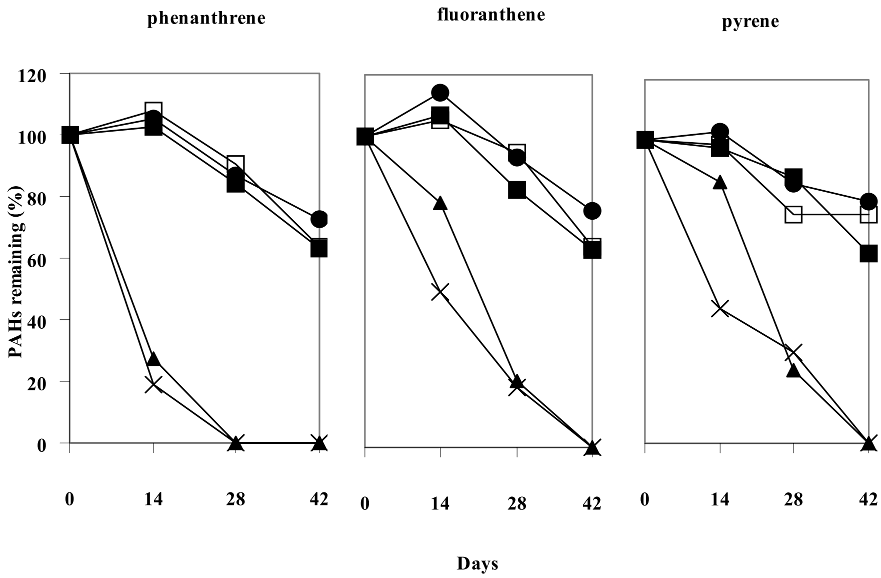
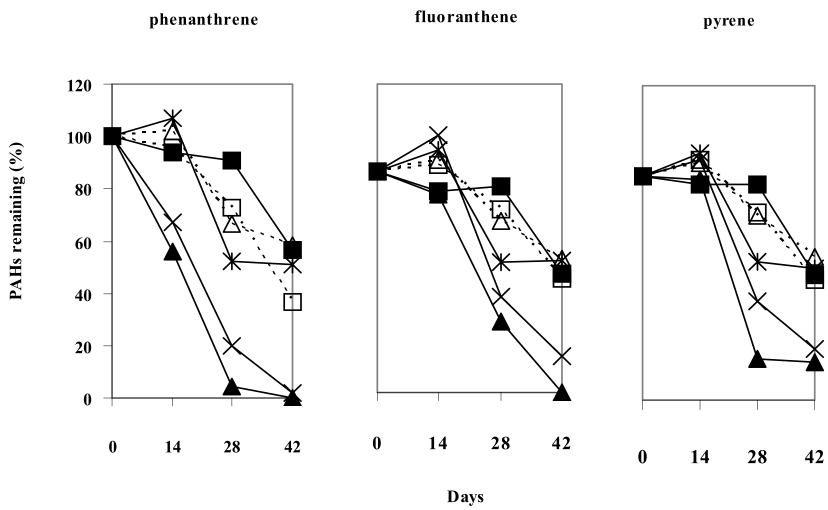
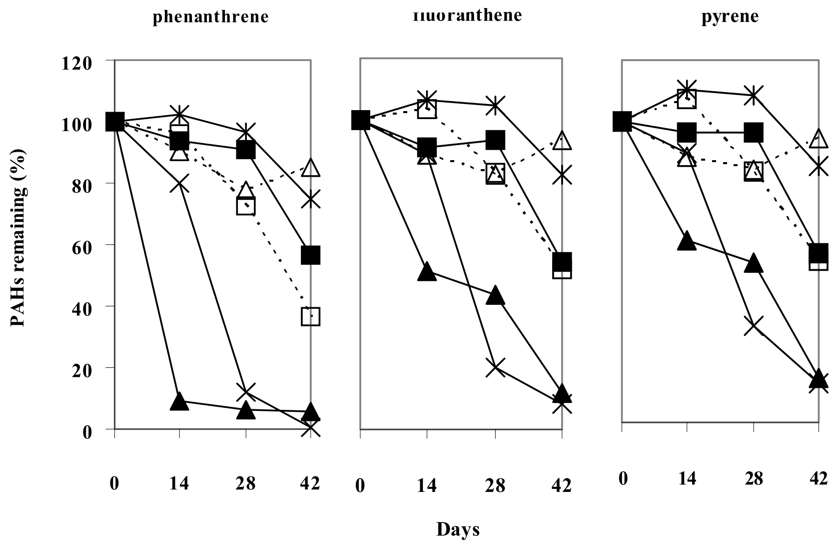


Figure 1. The profile of remaining PAHs in the sterilized soil (□), non-sterilized soil (■), soil/rice straw mixture (●), soil/peanut shells mixture (X) and soil/rain tree leaves mixture (▲) incubated for a 42 day-period. All mixtures were non-sterilized.

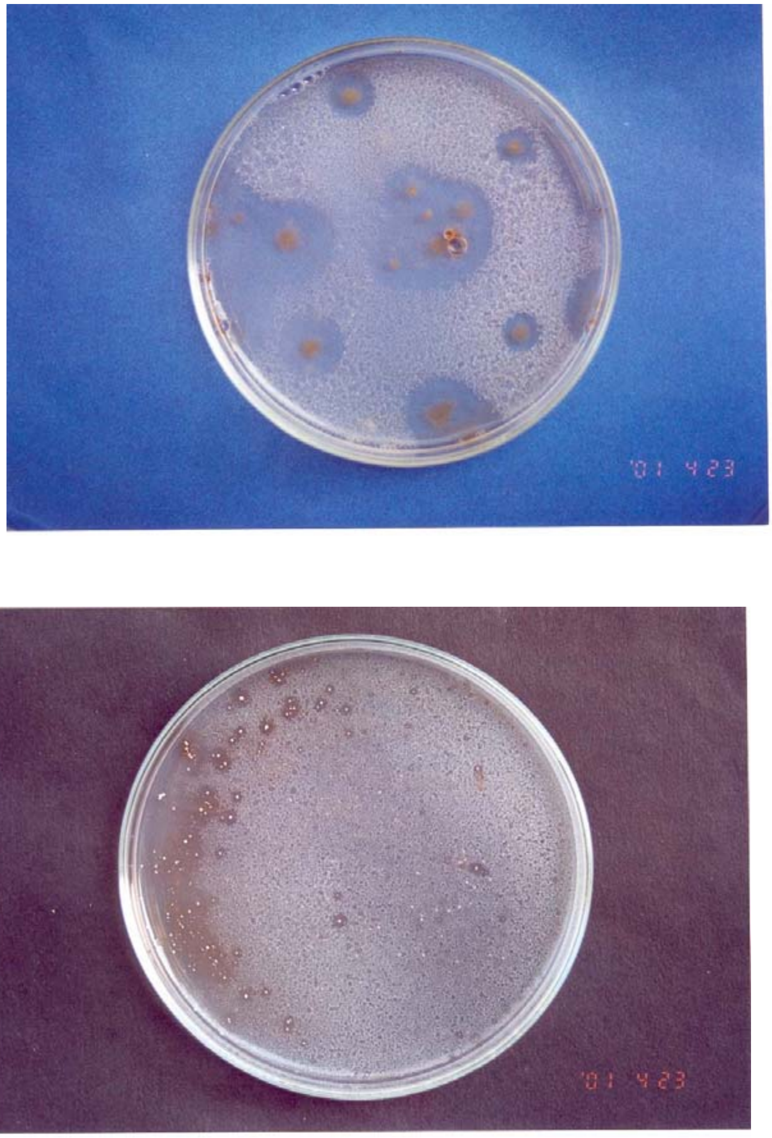


(A)



(B)

Figure 2. The profiles of remaining PAHs in the soil or the mixtures with the peanut shells (A) or the rain tree leaves (B): sterilized soil (□), non-sterilized soil (■), sterilized soil / sterilized peanut shells or rain tree leaves mixtures (△), sterilized soil / non-sterilized peanut shells or rain tree leaves mixtures (X), non-sterilized soil / sterilized peanut shells or rain tree leaves mixtures (*) and non-sterilized soil / non sterilized peanut shells or rain tree leaves mixtures (▲).



(B)

Figure 3. Phenanthrene-degrading bacteria on CFMM agar plate sprayed with phenanthrene solution in diethylether. The bacteria were isolated from the soil mixtures containing non-sterilized peanut shells (A) or rain tree leaves (B).

Bioavailability of PAHs in the soil mixtures

For a long period of time there have been reports on soil contaminant PAHs having limited extractability by organic solvents due to the sorption of the compounds into the soil particles, causing low bioavailability for microorganisms.(2, 7, 10) The present work, therefore, determined the bioavailability of PAHs from various sterilized samples spiked with PAHs, which included the soil, the soil/peanut shells mixture and the soil/rain tree leaves mixture, by extracting with dichloromethane. As shown in Table 3, the soil/rain tree leaves mixture had highest PAHs extractability. Moreover, the PAHs extractabilities of the rain tree leaves and the peanut shells spiked with PAHs were also determined. It was found that the rain tree leaves had higher extractability than that of the peanut shells (Table 4). This might indicate that the PAHs were more deeply sorbed into the peanut shells than the rain tree leaves.

Table 3. Dichloromethane extraction of PAHs (phenanthrene, fluoranthene and pyrene) from soil, soil/peanut shells mixture and soil/rain tree leaves mixture after various periods of aging under sterile conditions (ND: not determined).

	Aging time (days)	Percentage of extractable PAHs		
		phenanthrene	fluoranthene	pyrene
Soil	0	100	100	100
	7	ND	ND	ND
	14	ND	ND	ND
	21	59.95	83.07	81.54
	40	49.56	57.26	59.63
	60	41.54	59.16	62.55
Soil/peanut shells	0	100	100	100
	7	70.23	73.2	74.83
	14	69.58	71.69	72.05
	21	67.16	65.66	65.8
	40	51.78	54.06	56.35
	60	36.62	43.52	47.03
Soil/rain tree leaves	0	100	100	100
	7	102.95	100.3	99.12
	14	91.03	86.77	85.25
	21	106.53	102	98.82
	40	73.57	82.72	85.1
	60	82.92	80.59	81.07

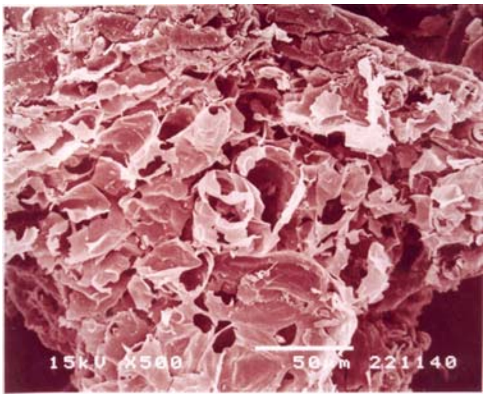
Table 4. Dichloromethane extraction of PAHs (phenanthrene, fluoranthene and pyrene) from peanut shells and rain tree leaves after various periods of aging under sterile conditions.

Agricultural materials	Aging time (days)	Percentage of extractable PAHs		
		phenanthrene	fluoranthene	pyrene
Peanut shells	0	100	100	100
	20	80.06	83.07	81.54
	40	49.56	57.26	59.63
	60	43.5	59.67	66.65
	80	41.54	59.16	62.55
Rain tree leaves	0	100	100	100
	20	100.5	95.9	90.91
	40	84.05	77.94	76.62
	60	80.9	68.7	69.71
	80	77.41	62.5	64.67

Furthermore, the structures of both agricultural materials were examined under a scanning electron microscope (SEM), which revealed that the peanut shells had more complicated micropores than the rain tree leaves (Figure 4). Therefore, the structure of the agricultural materials may also play an important role in bioremediation, as Verstraete and Devliegher[7] reported that the soil microorganisms could directly and more easily act on the surface–sorbed compounds. However, as the agricultural materials had sorption ability to PAHs and higher extractability than the soil particles, they therefore may enhance soil bioremediation by increasing bioavailability of the PAHs to microorganisms.

In conclusion, rain tree leaves and peanut shells had enhancing effects on the bioremediation of phenanthrene, fluoranthene and pyrene in the soil contaminated with these compounds. Both agricultural materials were sources of microorganisms with PAH degrading ability. Wagner and Zablotowicz[4] reported that many agricultural materials were used as supplementing sources of nutrients and carbon for soil biotreatment but not many were reported as sources of PAH degrading microorganisms. This work reports the new finding of a potent consortium of phenanthrene-, fluoranthene- and pyrene-degrading microorganisms inherent in some agricultural materials. As pyrene is a four-ring recalcitrant PAH, finding microorganisms capable of degrading pyrene will therefore be useful for environmental treatments in the future.

(A)



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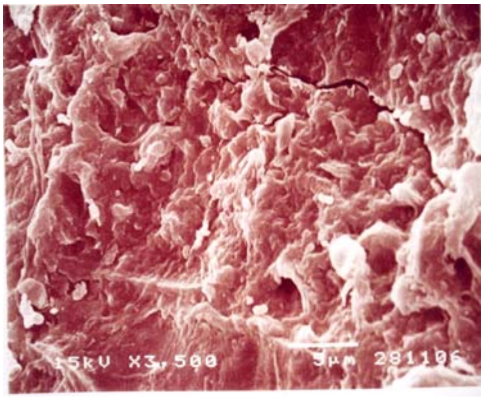

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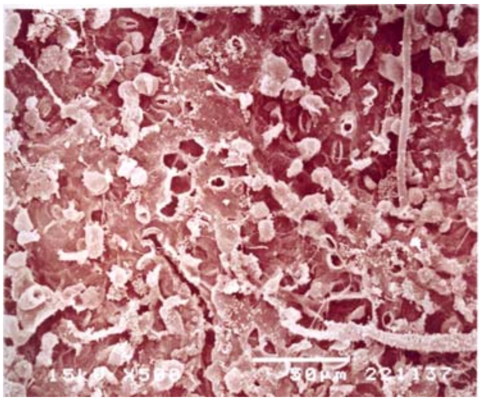

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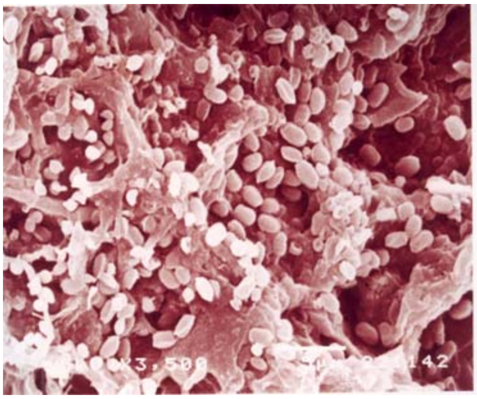

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Figure 4. Scanning electron micrographs of peanut shells (A) and rain tree leaves (B) at 500x and 3500x magnifications.

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