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Degradation of pyrene by immobilized microorganisms in saline-alkaline soil

Shanxian Wang[1,2], Xiaojun Li[1], Wan Liu[1,*], Peijun Li[1], Lingxue Kong[3], Wenjie Ren[1,2], Haiyan Wu[1,2], Ying Tu[4]

1. Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang 110016, China. E-mail: wsx_wsy@163.com
2. Graduate University of Chinese Academy of Sciences, Beijing 100049, China
3. Centre for Material and Fiber Innovation, Institute for Technology and Research Innovation, Deakin University, Geelong Vic 3217, Australia
4. College of Resource and Environmental Engineering, Liaoning Technical University, Fuxin 123000, China

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Abstract

Biodegradation of polycyclic aromatic hydrocarbons (PAHs) is very difficult in saline-alkaline soil due to the inhibition of microbial growth under saline-alkaline stress. The microorganisms that can most effectively degrade PAHs were screened by introducing microorganisms immobilized on farm byproducts and assessing the validity of the immobilizing technique for PAHs degradation in pyrene-contaminated saline-alkaline soil. Among the microorganisms examined, it was found that *Mycobacterium* sp. B2 is the best, and can degrade 82.2% and 83.2% of pyrene for free and immobilized cells after 30 days of incubation. The immobilization technique could increase the degradation of pyrene significantly, especially for fungi. The degradation of pyrene by the immobilized microorganisms *Mucor* sp. F2, fungal consortium MF and co-cultures of MB+MF was increased by 161.7% ($P < 0.05$), 60.1% ($P < 0.05$) and 59.6% ($P < 0.05$) after 30 days, respectively, when compared with free F2, MF and MB+MF. Scanning electron micrographs of the immobilized microstructure proved the positive effects of the immobilized microbial technique on pyrene remediation in saline-alkaline soil, as the interspace of the carrier material structure was relatively large, providing enough space for cell growth. Co-cultures of different bacterial and fungal species showed different abilities to degrade PAHs. The present study suggests that *Mycobacterium* sp. B2 can be employed for *in situ* bioremediation of PAHs in saline-alkaline soil, and immobilization of fungi on farm byproducts and nutrients as carriers will enhance fungus PAH-degradation ability in saline-alkaline soil.

Key words: saline-alkaline soil; immobilization; PAHs-degrading microorganisms; biodegradation characteristics; *Mycobacterium*
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Introduction

Saline-alkaline soils are widely distributed on the earth and the total global area of salt-affected soils including saline-alkaline soils is 8.31×10^9 ha (Martinez-Beltran and Manzur, 2005). Saline-alkaline soils contaminated with creosote, coal tar, and crude oil have an ubiquitous distribution of polycyclic aromatic hydrocarbons (PAHs) which are toxic xenobiotic fused-ring aromatic compounds and recalcitrant to different types of degradation (Sun et al., 2010b; Haritash and Kaushik, 2009; Gao et al., 2006). Salt-affected soils represent approximately 40% of the land in the world and PAHs are ubiquitous in terrestrial ecosystems (Betancur-Galvis et al., 2006). For instance, contamination of soil with PAHs occurred at the former Lake Texcoco, which covers an area of 5000 ha saline-alkali soils (Betancur-Galvis et al., 2006). In China, oilfields such as Shengli Oilfield, Daqing Oilfield and Dagang Oilfield are typical saline-alkaline regions contaminated with PAHs due to oil spillage that occurred during transportation and storage of petroleum-related products (Nie et al., 2009; Tang et al., 2010).

Although biodegradation using microorganisms has proved to be the most cost-effective and practical method of treating wastes containing PAHs (EI-Naas et al., 2009) among available technologies, the biodegradation of organic contaminants in soils can be intensively affected by environmental factors such as salinity and alkaline pH (Fernández-Luqueño et al., 2008), which limits its wide application. It is reported that salinity inhibits rapid removal of hydrocarbons from soil by impeding microbial activity (Margesin and Schinner, 2001), as increased salinity in soil decreases the number and types of substrates that can be utilized by microorganisms (Riis et al., 2003). In addition, the biodegradability of PAHs by microorganisms is likely to be impaired under alkaline pH conditions (Norris, 1993). However, if the microorganisms are in an immobilized form, this will promote degradation as immobilization is known to offer protection from extremes of pH and toxic compounds in the contaminated soil (Su et al., 2006). Of all the immobilization methods, physical adsorption on farm byproducts was found to be the optimum method owing to its properties of being highly

* Corresponding author. E-mail: liuwan63@hotmail.com

granular, absorbent, biodegradable and inexpensive (Xu and Lu, 2010).

In this study, different microorganisms were immobilized to remove pyrene from saline-alkaline soil. All the microorganisms had been previously proved to have the ability to biodegrade PAHs (mainly phenanthrene and pyrene) in a liquid medium. Farm byproducts and nutrients were used as carrier materials and bulking agents to improve oxygen diffusion and to immobilize a greater quantity of microorganism cells. The effects of pyrene degradation by interacting bacteria and fungi as co-cultures were also investigated. This study was carried out in a fully controlled environment (e.g., spiked soil, climate-controlled incubation chambers) in order to (1) screen PAH-degrading microorganisms which were effective in saline-alkaline soil during *in-situ* bioremediation; (2) assess the effect of the immobilization technique on PAH biodegradation in saline-alkaline soil.

1 Materials and methods

1.1 PAH

Pyrene, a four-ring PAH, was used as the model PAH compound due to its structure being found in most carcinogenic PAHs (Sarma and Pakshirajan, 2011). Pyrene (97% purity), was purchased from Fluka Co. (Germany). The molecular weight of pyrene (high-molecular-weight PAH) is 202.26 g/mol and its octanol-water partition coefficient ($\log K_{ow}$) is 4.48 (Yaws, 1999).

1.2 Soil preparation

Saline-alkaline soil samples without any detectable PAHs were collected from the 0–20 cm depth zone of a saline-alkaline region in Dagang Oilfield (38°42′12″N, 117°29′23″E), in May 2009. The soil samples were air-dried in the dark, passed through a 2-mm sieve, and then stored in a dark chamber before use. The basic physicochemical properties of soil are as follows: pH 8.75, salt content 2.28%, Cl^- 0.23 mg/g, total organic matter 0.73%, total N 0.056%, total P 0.065%, total K 2.711%, clay 10.2%, silt 79.0% and sand 10.8%. The pyrene with a target concentration (100 mg/kg) was spiked into the soil using the method described by Brinch et al. (2002). The soil samples were stored in a refrigerator at 4°C. Initial concentrations of pyrene in the spiked soil were analyzed before the incubation of microorganisms.

1.3 Substrate

1.3.1 Liquid medium preparation

The medium contained 0.05% yeast extract, 0.01% NaCl, 0.1% NH_4NO_3, 0.1% K_2HPO_4, 0.1% KH_2PO_4, 0.02% $MgSO_4{\cdot}7H_2O$, 0.01% $CaCl_2$ and 0.002% $FeCl_3$ (Li et al., 2008). The medium of 100 mL was added to a 300 mL Erlenmeyer flask and underwent sterilization in an autoclave at 121°C for 30 min. pH was adjusted to 7.2–7.5 for bacteria and 6.0 for fungi.

1.3.2 Immobilization carrier preparation

Fresh corn cobs were air-dried and mechanically ground to obtain particles with a size of approximately 0.5–1.5 mm. They were pretreated by soaking in calcium hydroxide for 24 hr and then mixed with other accessories (wheat bran, soybean flour, and sucrose) in the proportion of the formula and stirred to constitute the microorganism immobilization composite carrier. The water content of the carrier mixture was maintained between 60%–70%, while pH was adjusted to 7.2. The carrier mixture was then put into polyethylene bags resistant to high voltage and high temperature with 500 g in each bag, and sterilized by autoclaving twice at 121°C, 1×10^5 pa for 90 min before use. Table 1 outlines the formula for the immobilization composite carrier substrate.

1.4 Microorganisms

The microorganisms that were evaluated for PAH degradation in saline-alkaline soil included bacteria: B1 (*Pantoea* sp.), B2 (*Mycobacterium* sp.) and bacterial consortium MB (*Pseudomonas* sp., *Gordonia* sp., *Bacillus* sp., *Ochrobactrum* sp. and *Mycobacterium* sp.); fungus: F1 (*Penicillium* sp.), F2 (*Mucor* sp.) and fungal consortium MF (*Mucor* sp., *Phaeosphaeria* sp., *Fusarium* sp., *Aspergillus niger*, *Trichoderma* sp. and *Cunninghamella* sp.); bacterial and fungal co-cultures: B1+F1 and MB+MF. In this study, B1 and F1 were isolated from saline-alkaline soil of Dagang Oilfield through enrichment and culture in a pure mineral plate in which pyrene was added as a sole carbon and energy source (pyrene concentration 50 mg/kg, initial plate salinity 2%, pH 8.6); B2, MB, F2 and MF were isolated from heavily contaminated soil of a Shenfu sewage irrigated area using the method of enrichment and culture in a mineral plate where PAHs were added as sole carbon and energy source (total PAHs concentration 200 mg/L, in which phenanthrene: anthracene:pyrene:chrysene:benzo(a)pyrene = 2:2:2:1:1 ($m/m/m/m/m$), pH 7.0 for bacterium and pH 6.0 for fungus).

1.5 Experimental design

1.5.1 Preparation of free and immobilized microorganisms

Cultures of microorganisms were grown on nutrient broth agar (NBA) slants for bacteria and potato dextrose agar (PDA) slants for fungi at 0–4°C. The liquid medium was

Table 1 Formula of immobilization carrier substrate

Immobilization carrier component	Dosage	
	Bacteria (%)	Fungi (%)
Rice chaff	60	20
Wheat bran	20	15
Corn cob	16	80
Soybean flour	1.0	2.0
Sucrose	1.0	2.0
Gypsum	0	1
Calcium oxide	1.5	1.17
KH_2PO_4	0.06	0
$(NH_4)_2HPO_4$	0.2	0.5
$MgSO_4{\cdot}7H_2O$	0.026	0.15

inoculated with the cultures, and incubated at 28°C with a reciprocal shaker (HZQ-C, Dongming Medical Instrument Plant in Harbin, China) at 150 r/min until the microorganisms in the medium reached the late exponential phase and finally formed 1-level liquid seed.

After cooling, the treated composite carrier was inoculated with 1-level liquid seed as mentioned above at 10% (*V*/*W*), stirred uniformly and incubated in an incubator (HHB12-420, the Five Seven Electric Equipment Factory of Forty Fifth Middle School in Shenyang, China) at 28°C for 5–10 days. The ultimate product was called 2-level solid seed.

1.5.2 PAH degradation

To study the biodegradation of a single PAH in saline-alkaline soil by free and immobilized microorganisms, 250 g pyrene-spiked saline-alkaline soil was collected into a 400-mL beaker and inoculated. Inoculation of free microorganisms consisted of 1-level liquid seed (10%, *V*/*W*) and inoculation of immobilized microorganisms consisted of 2-level solid seed (10%, *m*/*m*). The microbial reagents were then added and mixed with the soil. The beakers were covered with sterilized tier gauze to prevent contamination with other microorganisms and fast evaporation of water. All the beakers were stored in a dark incubator (HPG-280HX, HDL, China) at 28°C. Distilled water of known weight was supplied every three days. All the experiments were performed in triplicate. Sampling dates were 0, 5, 10, 20 and 30 days from the beginning of incubation. The samples were stored at –20°C and cryodesiccated using a freeze dryer (FD-1C-50, BYK, China) before PAH analysis. A summary of treatments and control beakers is presented in Table 2.

1.6 Chemical analysis

Pyrene in saline-alkaline soil samples was extracted using a previously described method (Dong et al., 2010).

The concentration of PAH was analyzed using a gas chromatograph (GC 6890N, Agilent, USA) equipped with a flame ionization detector (GC-FID) and a DB-5 capillary column (30 m × 0.32 mm i.d. × 0.25 μm film thickness). The initial oven temperature was 90°C (holding time 1 min), increased to 295°C at 18°C/min and held for 2 min. The total runtime was 14.39 min. Nitrogen gas was used as the carrier gas (1.5 mL/min) and makeup gas (35 mL/min). A 1.0 μL aliquot of the extract was injected using an injector which was held at 250°C and detector at 300°C in the splitless mode. The recovery of the PAH pyrene was determined in triplicate samples at 1 level (100 mg/kg) for the spiked saline-alkaline soil.

Table 2 Experimental setup for treatments of pyrene-spiked saline-alkaline soil

Set name	Treatment	Dosage
CK1	Sterilized soil	10%, *V*/*W*
CK2	Soil + liquid medium with no cells	(CK1, CK2)
CK3	Soil + bacteria carrier with no cells	10%, *m*/*m*
CK4	Soil + fungus carrier with no cells	(CK3, CK4)
T1	Soil + free B1	10%, *V*/*W*
T2	Soil + free F1	(T1–T8)
T3	Soil + free (B1+F1)	
T4	Soil + free B2	
T5	Soil + free F2	
T6	Soil + free MB	
T7	Soil + free solution	
T8	Soil + free (MB+MF)	
T9	Soil + immobilized B1	10%, *m*/*m*
T10	Soil + immobilized F1	(T9–T16)
T11	Soil + immobilized (B1+F1)	
T12	Soil + immobilized B2	
T13	Soil + immobilized F2	
T14	Soil + immobilized MB	
T15	Soil + immobilized MF	
T16	Soil + immobilized (MB+MF)	

1.7 SEM examination of immobilized system

The carrier materials and farm byproducts immobilized with *Mycobacterium* sp. B2 were fixed in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer for 3 hr, rinsed twice for 10–15 min each in a phosphate buffer and post-fixed in 1% osmium tetraoxide buffer for 1–2 hr at 4°C. The corn cob was then washed twice in a phosphate buffer for 10–15 min each time and dehydrated with a series of ethanol-water solutions (ethanol 30%, 50%, 70%, 80%, 90% and 100%) at room temperature. The corn cob was dried with the CO_2 critical point technique (Critical Point Dryer-HCP2, Hitachi, Japan) and sputter-coated with gold (IB-5 Ion Coater, Eiko, Japan). Observations were done using a scanning electron micrographs (SEM, JSM-T300, JEOL, Japan).

1.8 Statistical analyses

The degradation of PAH was calculated with the following equation:

$$R_d = (C_0 - C_n) / C_0 \times 100\%$$

where, R_d (%) represents degradation rate, C_0 (mg/kg) represents the initial concentration of PAH and C_n (mg/kg) represents the remaining concentration of PAH after incubation for n days (Sun et al., 2010).

Treatment effects were compared using analysis of variance (ANOVA) and the means of data were compared by Duncan's test. Means between treatments at a given time and between periods for a given treatment with $P \leqslant 0.05$ were considered statistically different. All the statistical analyses were carried out using SPSS 16.0.

2 Results

2.1 Pyrene degradation

2.1.1 Pyrene degradation of CK

The percentage of PAH removals from the spiked saline-alkaline soils of CK after 5, 10, 20 and 30 days of incubation is presented in Fig. 1a. During the treatment period, the removal of pyrene in CK2, CK3 and CK4 was significantly higher than that in CK1 ($P < 0.05$). At the end of 30 days, the degradation of pyrene in CK3 and CK4 added with carrier materials was 27.3% and 28.3%, respectively, which is 17.6% and 22.2% higher than that in CK2. However, no significant difference in pyrene

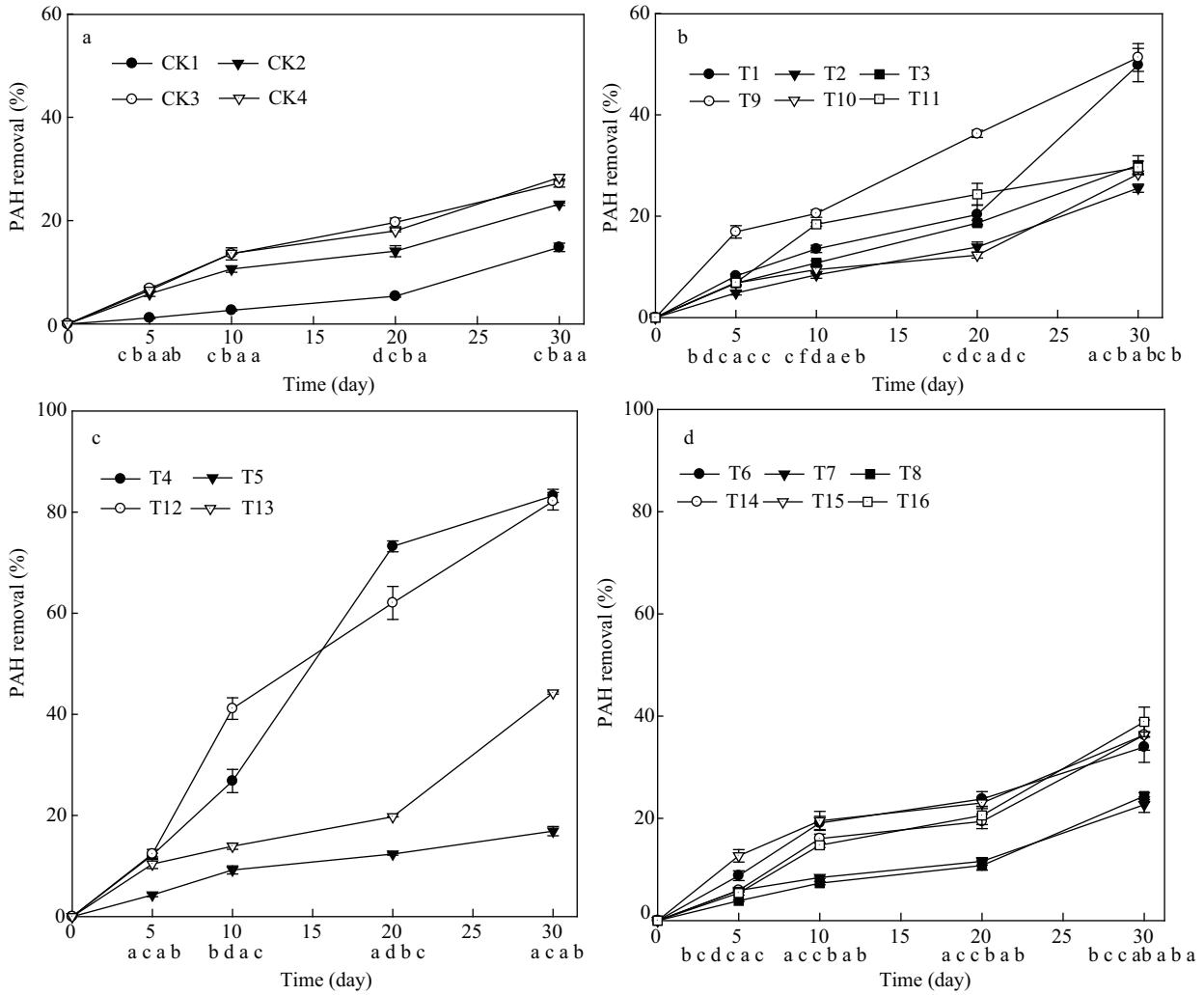

Fig. 1 Results of controls (a), treatments with single microbes isolated from Dagang Oilfield soil and their bacterial-fungal co-cultures (b), treatments with single microbes isolated from heavily contaminated soil of Shenfu sewage irrigated area and their bacterial-fungal co-cultures (c), and treatments with the mixed microbial consortium isolated from heavily contaminated soil of Shenfu sewage irrigated area and their bacterial-fungal co-cultures (d). Sample numbers $n = 3$, error bar = 1 standard deviation. Different lower-case letters indicate significances between treatments, and the sequence of the letters represents the sequence of corresponding legend ($P < 0.05$).

degradation between CK3 and CK4 was observed ($P > 0.05$) except at day 20 ($P < 0.05$).

2.1.2 Biodegradation of pyrene by microbes isolated from saline-alkaline soil

The biodegradation of pyrene by free and immobilized *Pantoea* sp. B1, *Penicillium* sp. F1 and B1+F1 in saline-alkaline soil is presented in Fig. 1b. T9 exhibited a high pyrene removal ability with 88.2%–146.4% increase when compared with CK3 during the entire incubation period ($P < 0.05$). The degradation in T9 was 20.5%–73.4% higher than that in T11 ($P < 0.05$), while T11 removed pyrene in saline-alkaline soil 4.6%–94.6% more than T10 did. The differences in pyrene degradation among the three free microbes T1, T2, T3 were similar to those among the corresponding immobilized microbes T9, T10, T11. After 30 days, free and immobilized B1 exhibited the best pyrene removal ability, which was 49.8% and 51.2%, respectively. In T2, T3, T10 and T11, pyrene was degraded only by 25.5%–29.6%. Compared with free B1, immobilized B1 significantly increased pyrene degradation by 69.3%–106.1% except at 30-day incubation ($P < 0.05$). No significant differences in pyrene degradation rate between T2 and T10, T3 and T11 were observed for 20- and 30-day incubation.

2.1.3 Biodegradation of pyrene by microbes isolated from soil of Shenfu sewage irrigated area

The percentage of PAH removals from the spiked saline-alkaline soils by free and immobilized *Mycobacterium* sp. B2 and *Mucor* sp. F2 biodegradation is presented in Fig. 1c. Among all the treatments of the saline-alkaline soil, biodegradation with bacterium B2, showed the most effective pyrene removal efficiency, with a range of 12.2%–83.2% in T4 and 12.4%–82.2% in T12. Pyrene degradation in T12 increased sharply between 5 and 10 days, several days earlier than that in T4, in which the sharp increase in pyrene degradation rate occurred between day 10 and 20. The pyrene removal rate in T5 was not as high as in CK2 ($P < 0.05$) (Fig. 1a). There were no significant differences between the pyrene biodegradation ability in T4 and T12 until 30 days ($P > 0.05$). However, the immobilization of fungi F2 clearly enhanced the pyrene degradation rate, as the pyrene degradation of T13 was 50.9%–161.7% higher

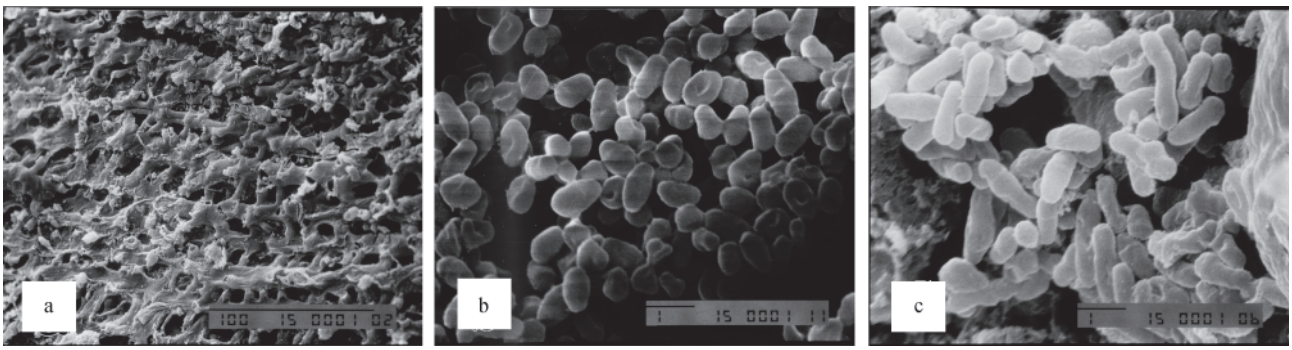

Fig. 2 Scanning electronic microscope (SEM) micrographs of immobilization matrix ×500 (a), free B2 ×15000 (b), immobilized B2 on corn cobs ×15000 (c).

than that of T5 ($P < 0.05$).

2.1.4 Biodegradation of pyrene using mixed microbial consortium

The percentage of PAH removal rate from the spiked saline-alkaline soils by free and immobilized MB, MF and MB+MF biodegradation during the incubation is shown in Fig. 1d. During the whole treatment, the range of pyrene degradation in T6 was 8.8%–34.0%, which is 45.6%–62.5% higher than that of CK2 ($P < 0.05$). There was no significant difference between the pyrene degradation in T7 and CK2 until day 30 ($P > 0.05$) (Fig. 1a). Compared with the results of T6, the pyrene degradation in T8 decreased significantly by 28.3%–63.8% ($P < 0.05$). No significant difference between the pyrene biodegradation in T7 and T8 was observed ($P > 0.05$). After 30-day incubation, the pyrene removal in T14 was 33.1% higher than that in CK3 ($P < 0.05$), and the degradation in T15 was 28.1% higher than that in CK4 ($P < 0.05$). No significant difference in pyrene biodegradation was observed among T14, T15 and T16. Compared with the free MF and free MB+MF, pyrene degradation in T15 and T16 increased as much as 60.1%–114.4% and 39.2%–101.3% ($P < 0.05$), respectively. However, no significant difference between the pyrene biodegradation in T6 and T14 was observed after incubation of 30 days.

2.2 Scanning electron micrographs

The immobilized microstructures examined under the scanning electron microscope (SEM) are shown in Fig. 2. The SEM of the matrix structure of the carrier material is shown in Fig. 2a, indicating that the interspace of the carrier material structure is relatively large, providing enough space for cell growth. The SEM of free B2 is shown in Fig. 2b, exhibiting good growth in the liquid medium. The SEM of the adsorption of B2 on the surface of the immobilizing carrier is showed in Fig. 2c, indicating that a good growth of B2 after immobilization on the corn cobs can be confirmed by its widespread distribution.

3 Discussion

Although some microbial species that are able to enhance PAHs degradation in soils have been well documented (Gan et al., 2009; Haritash and Kaushik, 2009; Fernández-Luqueño et al., 2011), few studies have been carried out on the efficiency of PAHs degradation by microorganisms in saline-alkaline soil, due to the inhibitory catabolic activity and survival ability of introduced microorganisms under saline-alkaline stresses (Sijm et al., 2000). In this study, the microorganisms isolated from saline-alkaline soil of Dagang Oilfield and heavily PAH-contaminated soil of a Shenfu sewage irrigated area were used to evaluate the bioaugmentation efficiency of introduced microbes in saline-alkaline soil with high PAH concentration, to select effective microbes and optimize their cultivation using an immobilization method.

3.1 Degradation of pyrene by microbial cultures in saline-alkaline soil

Among all the microorganisms in the present study, the bacteria generally had a better pyrene degradation ability than the fungi did. In general, fungi have a lower PAH degradation ability in marsh soil than in forest soil, suggesting some intolerance to saline conditions (Leitão, 2009). Unlike bacteria, fungi cannot assimilate PAHs as a single source of carbon and energy. Therefore, fungi need an additional carbon source to support their growth, usually a lignocellulosic material such as sawdust, woodchips or straw (Leitão, 2009). Thus, the saline-alkaline soil used in this study belongs to clay soil which is lacking in organic matter and nutrients and not suitable for the growth of fungal hypha. Other factors affecting the colonization of non-autoclaved soil by fungi are that the fungi grow more slowly than bacteria, and the presence of indigenous soil microorganisms that may compete with the fungi for sources of carbon and nitrogen, disables the fungal growth (Acevedo et al., 2011). It was reported by Wick et al. (2007) that unlike bacteria, the hyphal mode of growth permits fungi to access less-available contaminants or even mobilize bacteria to access pollutants in the soil. Furthermore, it was shown in previous study that oxygen concentrations have a significant effect on phenanthrene removal (Meléndez-Estrada et al., 2006). As the saline-alkaline soil represented clay soil in which the oxygen level between soil particles is relatively low, fungi in saline-alkaline soil do not perform as well as bacteria do.

The indigenous microbes screened from polluted soils are usually more effective in metabolizing PAHs than organisms obtained from elsewhere in bioremediation (Li et al., 2008). The pyrene removal efficiency using B2 in this saline-alkaline soil was higher than B1 due to B1

being only isolated from saline-alkaline soil of Dagang Oilfield but not PAHs-contaminated saline-alkaline soil, while B2 was screened from aged heavily polluted soil. *Mycobacterium* is often isolated from PAH-contaminated soil as degraders of PAHs (Uyttebroek et al., 2006) and some of them can even degrade benzo[a]pyrene when some cometabolic substrates are supplied (Moody et al., 2004). Even though there is nutrient deficiency, the B2 bacterial strain is able to develop complete biosynthetic pathways and does not have complex nutrient requirements at high saline conditions. There was a sharp increase of pyrene degradation occurring between day 10 and 20 for free B2 in the present research, which is in agreement with the result reported by Zhou et al. (2008). Previous studies suggested that *Mycobacterium* play important roles in the fate of PAHs in the environment (Vila et al., 2001), while the present study shows that the bacterium *Mycobacterium* sp. is promising to degrade PAH in saline-alkaline soil.

3.2 Immobilization effects on pyrene biodegradation in saline-alkaline soil

Most of the studies on immobilization technology performed were aimed at degrading organic contaminants in water or loamy soil (Su et al., 2008). A new insight into its application in saline-alkaline soil PAH bioremediation has been shown in the present study. The pyrene biodegradation intensification by immobilized fungi used in this work was not as good as that using bacteria, which might be due to the fact that the saline-alkaline soil badly restrained the degradation ability of the free filamentous fungus, but the immobilizing microstructure could provide a suitable environment for quick growth of the fungi. It was reported that the PAHs degradation can be confirmed by the appearance of known metabolites from ligninolytic enzyme action (Acevedo et al., 2011). Bacterial populations can decompose lignocellulosic material and have the ability to use it as a carbon and nitrogen source (Acevedo et al., 2011). Substrate limitation is likely to occur due to competition with indigenous microorganisms in soil, inhibiting lignocellulosic material decomposition by the fungi using free cells (Šnajdr and Baldrian, 2006). Therefore, the added carrier material might provide the introduced fungi with enough extra lignocellulosic material to enhance its selectivity to support the introduced fungus growth in non-autoclaved soil. It was also found by Su et al. (2008) that immobilized *Mucor* sp. shows better removal efficiencies and quicker disappearance of benzo[a]pyrene (BaP) than free fungi. SEM of the immobilization matrix shows that the macroporous structure provides enough space and a better environment for the growth of microorganisms (Fig. 2a). B2 are well distributed in the surface of the matrix (Fig. 2c). In fact, compared with free cells, the immobilized cells have many positive effects on PAH degradation: (1) protection from harsh environmental conditions such as pH, temperature, organic solvent and toxic compounds; (2) the abundant microspores that immobilized carrier materials possessed aided immobilization of microorganisms and protected the enzymatic system of microorganisms, permitting excellent mass diffusion of substrate and oxygen; (3) relative ease of product separation; (4) increased cell density and (5) alleviated susceptibility to contamination by foreign microorganisms and accelerated mass transfer of water, oxygen, nutrients and hydrocarbons, and provision of nutrition for the microflora (Zhao et al., 2009; Takei et al., 2010, 2011; Xu and Lu, 2010).

3.3 Degradation of pyrene in saline-alkaline soil by bacterial-fungal co-cultures

There are fundamental problems with bacterial-fungal co-cultures for PAH degradation (Balcom and Crowley, 2010). It was found in previous studies that benzo[a]pyrene biodegradation is significantly more enhanced by defined fungal-bacterial co-cultures than by single cultures (Machín-Ramírez et al., 2010). On the contrary, when bacteria and fungi were combined as co-cultures, the ability of pyrene degradation in saline-alkaline soil decreased as observed by Zhong et al. (2011), who found that the interactions between *Mycobacterium* strain and A1-PYR and *Sphingomonas* strain PheB4 in the mixed cultures could lead to both positive effects with an increase in degradation and negative effects with a decrease in the degradation of PAH. Those contradictory effects might depend on the type of PAHs and the species of the PAH-degrading bacteria. Six different combinations of three microorganisms were tested for their ability to remove PAHs in soil originating from PAH-contaminated sites. Our results indicate that the combination of different bacterial and fungal species show different abilities to degrade PAHs, and are consistent with the work of Kim and Lee (2007) showing that *Penicillium* sp. co-cultured with *Rhodococcus* sp. was more efficient in the removal of four PAHs.

3.4 Pyrene degradation in saline-alkaline soil of CK

In the present study, the pyrene degradation of CK1, namely the autoclaved soil, was very low until day 20, and reached 14.8% at the end of 30-day incubation (Fig. 1), which is due to the fact that the indigenous microflora in soil would be repopulated in 20–50 days after autoclaving the soil, for soil microorganisms could not be completely destroyed (Li et al., 2009). It was also reported that some spores are able to survive under the autoclaving conditions and these specific microorganisms are able to colonize the soil again (Berns et al., 2008). Furthermore, the pyrene degradation rates in CK3 and CK4 were much higher than those in CK1 and CK2 (Fig. 1), indicating that the immobilized carrier materials can highly promote the activity of indigenous microorganisms. Similar results were found in the study of Xu and Lu (2010) in which peanut hull powder was used as a biocarrier for the immobilization of indigenous hydrocarbon-degrading bacteria to enhance the degradation of crude oil under laboratory conditions.

4 Conclusions

The PAH removal ability of free and immobilized microorganisms was investigated in the present work. It was suggested that *Mycobacterium* sp. B2 and *Pantoea* sp. B1 have a high pyrene biodegradation capability

in saline-alkaline soil, which could be used for PAHs bioremediation of saline-alkaline soil. Among all the microorganisms, bacteria generally have a better pyrene degradation ability than fungi do in saline-alkaline soil. Due to the positive and negative interactions between fungus and bacteria, the combination of different bacterial and fungal species shows a different ability to degrade PAHs and might have a lower degradation than pure microbial cultures. The use of immobilized microorganisms can promote PAH biodegradation in saline-alkaline soil contaminated to different extents, which has the potential for good removal efficiency and the tolerance to environmental factor stress. Furthermore, the immobilization enhances the pyrene degradation capability of fungi much more than that of bacteria in saline-alkaline soil. In conclusion, the use of immobilized microorganisms with farm byproducts and nutrients as carrier materials and bulking agents for the remediation of PAH-contaminated saline-alkaline soil *in situ* is a promising technology.

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