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# Mineralization of pyrene induced by interaction between *Ochrobactrum* sp. PW and ryegrass in spiked soil

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ABSTRACT

This study was conducted to investigate the capability of pyrene-degrading bacterium *Ochrobactrum* sp. PW and ryegrass (Lolium multiflorum) grown alone and in combination on the degradation of pyrene in soil. After 60 days of ryegrass growth, plant biomass, pyrene-degrading microbial mass, soil enzyme activity (catalase activity and polyphenol oxidase activity) and residual concentration of pyrene in soils were determined. Higher dissipation rates were observed in PW inoculation treatments: ryegrass+PW rhizosphere soil (RP-r) and ryegrass+PW non-rhizosphere soil (RP-nr), than planting of ryegrass alone, rhizosphere (R-r) or non-rhizosphere (R-nr). The inoculation with PW significantly ($p < 0.05$) increased the dry weight of ryegrass root and shoot, nearly 2.8 and 3.3 times higher than ryegrass treatment. The pyrene-degrading microbial mass indicated that a much larger mass of bacteria, actinobacteria were present in RP treatment. The catalase activity in all different treatments were significantly ($p < 0.05$) higher than in with treatment R-nr, and the polyphenol oxidase activity was also significantly ($p < 0.05$) increased by inoculation with PW, leading to enhanced mineralization of pyrene from soil. Our results suggest that adding of PAHs-degrading bacteria to soil can enhance remediation of PAHs contaminated soil, while improving plant growth.



## 1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are one class of are a group of persistent organic pollutants (POPs) that have accumulated ubiquitously in the environment (Zhang et al., 2015). They may present in high concentrations in soil because of naturally occurring deposits, incomplete combustion of fossil fuels and biomasses, development of traffic and transportation industry and so on (Samanta et al., 2002; Wilcke, 2007). These pollutants are toxic, possess carcinogenic, mutagenic and teratogenic properties and have potential to accumulation in organisms, PAHs pose a significant risk to environmental and human health (Beg et al., 2003; Moscoso et al., 2012; Samanta et al., 2002). The contamination of soil environment by PAHs is paid widely attention in the whole world (Oleszczuk, 2009). Thus, the deleterious properties of PAHs have made their remediation a critical need.

Currently, three major technologies are available to deal with the PAHs contaminated soil: chemical, physical and bioremediation. Because of the lower cost, thorough mineralization, less secondary pollution, bioremediation has been suggested to be an efficient and economical technology to clean up contaminated soils by PAHs (Margesin and Schinner, 1997; Sayara et al., 2011). As an environment friendly approach, bioremediation includes phytoremediation, microbial remediation and microbe-plants combined remediation. The growth of plants may be restrained when the concentration of contaminants was at a very high level in soil, so the activities of microbe in the rhizosphere also were reduced inducing less PAHs mineralization. On the other hand, the efficient PAHs degrading bacteria can endure the high concentration of PAHs due to the production of PAHs degrading enzymes and surfactant (Husain, 2008), but it should grow in a strict condition to achieve the mineralization of PAHs. So when added to the soil the high-efficient PAHs degrading bacteria could not compete with the indigenous flora limiting the growth of the strains. Thus, the bioavailability of PAHs in soil, plant uptake and transformation capacity and the biological diversity in soil would greatly influence the efficiency of PAHs remediation.

There are two principal factors in PAHs degradation Plant-microorganism combined bioremediation: The reactions of PAHs degradation by microorganisms are usually ascribed to their

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oxidative enzymes (Baldrian and Wiesche, 2000), which can have effects on K-region and bay-region of the PAHs and ultimately render them non-toxic (Kim et al., 2005). On the other hand, degradation of PAHs by microorganisms has been shown stimulated by root exudates and fine root turnover through providing readily available organic matters that would elevate microbial population (Miya and Firestone, 2001) and cometabolites that induce the cometabolism of PAHs (Ruuhola, 2000). Ryegrass is widely used in the phytoremediation of PAH-contaminated sites owing to its fibrous root system and large specific root surface area (Kang et al., 2010).

In the present research, the degradation mechanisms in soil and interactions among pyrene-degrading bacteria and plant as well as their effects on PAH dissipation are still poorly understood. The objectives of this experiment were therefore to investigate: (1) the effect of pyrene-degrading bacterium and ryegrass, and their interaction on PAHs mineralization and (2) the principal factor in PAHs dissipation by pyrene-degrading bacteria and ryegrass. Some possible mechanisms related to enhanced bioremediation including number of bacteria, actinobacteria and fungi as well as soil oxidoreductase activity and its relationships with plant biomass and pyrene dissipation.

## 2. Materials and methods

### 2.1. Chemicals and media

Pyrene with a purity of 99.9% was purchased from Sigma-Aldrich Co. Ltd. All the other solvents and chemicals used in this study were of reagent grade or better. Mineral salt medium (MSM) contained the following chemicals (per liter): $K_2HPO_4$, 4 g; $Na_2HPO_4$, 4 g; $(NH_4)SO_4$, 2 g; $MgSO_4$, 0.2 g; $CaCl_2$, 1 mg; $FeSO_4 \cdot 7H_2O$, 1 mg; pH 7.0–7.2. Peptone beef medium contained: beef extract, 1.5 g; peptone, 2.5 g; agar, 2.5 g; distilled water, 500 ml.

### 2.2. Isolation of pyrene-degrading bacteria and phylogenetic analysis

Enrichment was carried out by adding 5 g PAHs contaminated soil of coking plant (Beijing, China) into 100 ml MSM medium, and incubated with shaking at 30 °C, 150 r/min for 5 days in the dark. Then 10 ml of supernatant was transferred into a new MSM medium with pyrene as sole carbon source, after incubation for 5 days, the same amount of supernatant was transferred several times with an increasing concentration of pyrene until it was 5 mmol $l^{-1}$. At the end of enrichment, pyrene-degrading bacterium was separated by plate streaking in peptone beef medium repeatedly.

16S rDNA was amplified from genomic DNA of isolated bacteria using the primer pair 8F: 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R: 5′-GGTTACCTTGTTACGACTT-3′. The PCR conditions were 35 cycles of 94 °C for 4 min , 63 °C for 1 min, 72 °C for 1 min, followed by one cycle of 72 °C for 7 min. The PCR products were purified and sent to be sequenced. The sequences were submitted to the GenBank database to align with published sequences using NCBI BLASTN. The software MEGA (version 4.0) was used to construct phylogenetic trees.

### 2.3. Soil and plant

The soil (0–25 cm) samples used in the experiment were obtained from Tiantongyuan of Beijing, China. This soil had been classified as a typical brown soil, and had no background concentration of exposure to PAHs and other contaminants. The soil was air-dried and sieved to 2 mm mesh. The soil pH was 8.4, organic matter content 3.5% and the cation exchange capacity (CEC) 15.9 cmol $kg^{-1}$.

The Perennial ryegrass seeds were disinfected in 10% $H_2O_2$ (w/w) solution for 10 min, followed by thorough washing with distilled water. The seeds were then germinated in dark at 25 °C. Seedlings with uniform appearance were selected for the experiment use 3 days later.

### 2.4. Experimental design

Soil was spiked with a high concentration of pyrene in acetone (10% of the total quantity of soil to be used was spiked first). When acetone evaporated off overnight, the resulting PAH-spiked soil sample was mixed with the residual PAH-free soil and passed through a 2 mm sieve to achieve homogeneity. The soil was then packed into plastic bags, and placed in the dark at room temperature for 2 weeks. After aging, partial of spiked soil was inoculated with PW (name of the pyrene-degrading bacteria obtained in 2.2) with a population of $3.5 \times 10^9$ CFU $g^{-1}$ (CFU: Colony-Forming Units) soil, then packed the soils into different root boxes as required (12 cm in length, width and height, Fig. 1).

Each root box contained 1 kg of spiked soil, and distilled water was added to adjust the moisture of soil to 60% of field water capacity and maintained for 3 days. The experiment had two treatment groups: (1) planting of ryegrass (R); (2) planting of ryegrass plus PW inoculation (RP). Four replicates of each group were prepared. Under each treatment the root box was used to separate the soil into two parts: the rhizosphere soil (r) and non-rhizosphere soil (nr). The ryegrass seedlings were transplanted in the middle of the root box (12 seedlings each box). The seedlings transplantation was considered as the start of the experiment. The soil moisture content was adjusted once a day to 60% of field water capacity with distilled water. All the root boxes were arranged randomly in an incubator, with temperature control (20–25 °C), and with illumination (with a light intensity of 250 μmol $m^{-2}$ $s^{-1}$, under a 14/10 h – light/dark cycle). After 60 days growing, the shoot of ryegrass were collected firstly, then the root boxes were disconnected, the rhizosphere soils, non-rhizosphere soils and the root of ryegrass were collected separately into plastic bags, and waited for the following analysis.

### 2.5. Analytical methods

#### 2.5.1. Plant biomass

The harvested plant shoots and roots were fully washed in tap water followed by distilled water to remove residual soil particle on the surfaces, then freeze-dried for 48 h and weighed.

#### 2.5.2. Number of pyrene-degrading microbe

To enumerate the viable pyrene-degrading microbial population, aqueous extracts of 10 g soil samples were added to 90 ml sterilized water, then serially diluted and spread on different nutrient agars for bacteria, actinobacteria and fungi, which contained 100 mg $l^{-1}$ pyrene. Three replicates were prepared of each dilution. Plates were incubated for 3–5 days at 28 °C. The number was expressed as colony-forming units (CFU) $g^{-1}$ soil.

#### 2.5.3. Soil enzyme activity analysis

Catalase (E.C. 1.11.1.6) activity in soils was determined by the method of Temple and Johnson (1964) and Lee et al. (2007) and with some modifications, it was measured by adding hydrogen peroxide and determining the disappearance rate of $H_2O_2$ over time. Four grams of fresh soil was mixed with 40 ml distilled water and 5 ml 0.3% $H_2O_2$, shaken for 20 min at 150 r/min. After incubation, 5 ml of 1.5 M $H_2SO_4$ was introduced to terminate the

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