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Full Length Research Paper

Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by white rot-fungus *Pseudotrametes gibbosa* isolated from the boreal forest in Northeast China

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This study compared laccase production and the degradation of polycyclic aromatic hydrocarbons (PAHs) by aboriginal white rot-fungus *Pseudotrametes gibbosa* (found in the northeast forest area of China) and *Pleurotus ostreatus* (which has been studied both domestically in China and overseas). The results showed that the laccase activity of *P. gibbosa* was 2841.3 U/l, which was 6 times more than that of *P. ostreatus* under the same culture conditions. The degradation of Anthracene and pyrene induced by *P. gibbosa* were 43.43 and 24.26%, while the removal efficiencies induced by *P. ostreatus* were only 30.12 and 18.76%. The results also showed a positive correlation between the PAHs degradation and laccase activity, and *Pseudotrametes gibbosa* had significant potential due to its higher laccase production and more potent degradation of PAHs. This study provides technical support for pollution amelioration using aboriginal white-rot fungus.

Key words: White-rot fungus, laccase, polycyclic aromatic hydrocarbons, degradation.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are an important class of persistent organic pollutants (POPs) in the environment which originate mainly from the incomplete combustion of fossil fuels, volcanic eruptions and forest fires, and are released into the environment in the form of exhaust and solid residues. In recent decades, with the continuing development of industry (particularly the annually increasing exploitation of petroleum and coal), the aromatic substances constantly emitted into the environment have increased, resulting in an ongoing increase in PAHs levels. This pattern of increasing environmental PAHs comprises a serious risk not only for humans, but for whole ecosystems. Gaspare et al. (2009) analyzed surface sediment and oyster samples from the inter-tidal areas of Dares Salaam for 23 PAHs including the 16 compounds prioritized by US-EPA using GC/MS, and as a result, the total concentration of PAHs in the sediment was found to range from 78 to 25,000 ng/g dry weight, while the oyster concentrations ranged from 170 to 650 ng/g dry weight. It is thus evident that PAHs pollution has become ubiquitous in the region, and that remediation of environmental PAHs requires our immediate attention.

White-rot fungus is a specie of mycelial fungus which is saprophytic on trees and leads to a decaying xylon white sponge-like mass. It can produce extracellular oxidase in the cell lumen. In addition to the decomposition of lignin, white-rot fungus, with its capacity for high efficiency, low consumption and broad-spectrum degradation of pollutants, is widely applied in wastewater treatments and bioremediation of polluted soil (and other urgent environmental applications), which include the degradation of dye, TNT,

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Abbreviations: PAHs, Polycyclic aromatic hydrocarbons; **POPs,** persistent organic pollutants; **GC/MS,** gas chromatography-mass spectrophotometer; **Lac,** laccase; **MnP,** manganese peroxidase; **LiP,** lignin peroxidase; **ABTS,** 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; **PCB,** polychlorinated biphenyl; **US-EPA,** United States Environmental Protection Agency.

PAHs and other toxic organic pollutants.

Laccase (Lac), manganese peroxidase (MnP) and lignin peroxidase (LiP) are the major lignin degradation enzymes of white-rot fungus (Wu et al., 2008). Laccase is a multicopper oxidase which can degrade a variety of complex structures of xenobiotics (Sealey and Ragauskas, 1998; Couto, 2007; Pozdnyakova et al., 2006). Knapp and Newby, (1999) selected the five most effective strains for the decoloration study of a chemical industry effluent. All five yielded 70 - 80% decoloration. Of these, the best were the strains of *Coriolus versicolor*. Vahabzadeh et al. (2004) studied the white-rot fungus *Phanerochaete chrysosporium* treatment of molasses wastewater from an ethanolic fermentation plant. Under the condition of diluting and adding a certain quantity of the spore to the waste water, the removal efficiency reached 75% on the fifth day. Gomaa et al. (2008) studied the ability of the white rot-fungus *P. chrysosporium* to decolorize Victoria Blue B (VB) in textile dyes. The inhibition of laccase production (by adding various inhibitors to the shaken cultures) exerts a potent influence on the decolorization of VB. When adding sodium azide and aminotriazole to inhibit the activities of endogenous catalase and cyto-chrome P-450 oxygenase, the decolorization efficiency decreased by 100 and 70%, respectively, while benzoate resulted in only a 50% decrease. Chupungars et al. (2008) studied PAH degradation by *Agrocybe* sp. CU-43. At 100 ppm, fluorene was 99% degraded within six days, while at the same concentration, 99 and 92% degradation of phenanthrene and anthracene, respectively, occurred within 21 days, and fluoranthene and pyrene were reduced by 80 and 75%, respectively, within 30 days. In a soil model, Agrocybe sp. CU-43 completely degraded 250 ppm fluorene at room temperature within four weeks.

This investigation focused on the PAH degradation effect of aboriginal white rot-fungus *Pseudotrametes gibbosa* (found in the northeast forested area of China) and *Pleurotus ostreatus* (which has been studied both domestically in China and overseas) with the goals of identifying a potent degradative aboriginal fungus and providing technical support for bioremediation utilizing aboriginal white-rot fungus.

MATERIALS AND METHODS

Strains

P. gibbosa was isolated from the Northeast Forest located at Changbai Mountain and maintained in the forest pest pathology laboratory of Northeast Forestry University. *P. ostreatus* was purchased from the Institute of Applied Microbiology, Heilongjiang Province.

Culture conditions

Solid medium: 200 g of peeled potatoes were cut into small and moderate pieces and completely immersed in distilled water, and cooked for 20 ~ 30 min to filter out a leaching solution of potato with gauze, 20 g glucose, 20 g agar, 3.0 g KH_2PO_4, 1.5 g $MgSO_4$, 0.01 g VB_1 and distilled water were added to bring the constant volume to 1 L.

Liquid medium I (used in the culture of *P. gibbosa*): 10 g/l corn meal (lixivium boiled filtered), 0.44 g/l NH_4Cl, 0.2 g/l KH_2PO_4, 0.05 g/l $MgSO_4$, 0.01 g/l $CaCl_2$, 1.0 g/l tween 80, 1ml/l inorganic solution, 0.5ml/l vitamin solution, pH 7.0. The inorganic solution consists of the following: 3.0 g/l $MgSO_4 \cdot 7H_2O$, 0.5 g/l $MnSO_4$, 1.0 g/l NaCl, 0.1g/l $FeSO_4 \cdot 7H_2O$, 0.1 g/l $CoSO_4$, 0.082 g/l $CaCl_2$, 0.1 g/l $ZnSO_4$, 0.01 g/l $CuSO_4 \cdot 5H_2O$, 0.01 g/l $KAl(SO_4)_2$, 0.01 g/l H_3BO_3, 0.01 g/l $NaMoO_4$; vitamin solution, 0.002 g/l biotin, 0.002 g/l folic acid, 0.005 g/l VB_1, 0.005 g/l VB_2, 0.01 g/l VB_6, and 0.005 g/l nicotinic acid).

Liquid medium II (used in the culture of *P. ostreatus*): 200 g of peeled potatoes cut into small and moderate pieces and completely immersed in distilled water and cooked for 20 ~ 30 min to filter out a leaching solution of potato with gauze, with 20 g glucose, 3.0 g KH_2PO_4, 1.5 g $MgSO_4$, 0.01 g VB_1 and distilled water to a constant volume 1 L.

Crude enzyme liquid preparation

The test strain was inoculated into liquid medium culture for 20 days, and samples were taken from the first day to the 20th day. 1 ml medium was removed in a centrifuge tube using a pipette gun, centrifuged at 9000 r/min for 10 min, then the supernatant was used as the crude enzyme liquid.

Assay for laccase activity

One unit (U) of laccase activity was defined as the amount of enzyme oxidizing 1 µmol ABTS/min. The assay mixture contained 0.2 mol/l acetic acid-sodium acetate buffer solution (pH 5.0), 5 mmol/l ABTS and a certain amount of enzyme solution. The oxidation of ABTS was followed by an absorbance increase of 0 – 3 min at 420 nm with spectrophotometer (Gao et al., 2008).

Degradation experiments

Acetone solution containing anthracene and pyrene (Sigma products, purity > 99%) was placed in a 150 ml flask overnight, after the acetone had become completely volatile, the addition of 50 ml sterile liquid medium and preparation of the solution containing anthracene and a pyrene, in which the solution concentration of anthracene and pyrene was 5 mg/l, respectively. Then 3 agar tablets with the diameter of 10 mm were inoculated in the solution with anthracene and a pyrene, and there were three parallel samples as well as a blank sample. The culture conditions were 25℃, 130 r/min, and a degradation period of 21 days, with the degradation efficiency determined every 7 days.

Extraction of PAHs

The extraction of PAHs utilized the full flask including liquid and agar tablets. For the liquid sample in the flask, n-hexane was utilized as the extracting solvent, and was added in equal volume. After two extractions, the n-hexane containing PAHs was collected into a matrass flask. In order to fully extract polycyclic aromatic hydrocarbons of samples in the flask, the agar tablets of the flask were also extracted at the same time, adding 20 ml 1:1 of the mixed n-hexane and acetone solution into the flask, after which the ultrasonic extraction lasted for 20 min, and after repeating twice, the organic phase containing PAHs was collected into the above matrass flask. The extract was concentrated to 1 ml using a rotary evaporator (Shanghai Shensheng Biotech Ltd. Corp., R205) at

Table 1. Recovery efficiency of PAHs.

Compound	Recovery efficiency (%)
Anthracene	66.43
Pyrene	69.31

45°C and then transferred to a gas chromatography vial for analysis.

PAH analysis

The quantitative determination of PAHs used gas chromatography and a hydrogen flame detector (Aglient 6890N GC, Aglient Technologies). The chromatographic conditions were as follows: a HP-5MS capillary column (30 m × 0.25 mm × 0.25 mm) was used with a temperature raising procedure. The gasification chamber temperature was 260°C, the detector was 280°C and the flow rate was 1.0 ml min^{-1}. The split injection split ratio was 50:1 and the injection volume was 1 µl (Guo et al., 2007).

RESULTS AND DISCUSSION

The recovery efficiency of PAHs

The isolation of PAHs by liquid-liquid extraction utilizes different solubility components in the mutually exclusive phases to achieve separation and enrichment. Although the liquid-liquid extraction device is relatively simple, choosing a suitable extractant is critical, and requires solvents with low water miscibility and high selectivity to PAHs, and the boiling point of solvent itself must be sufficiently low and volatile (Lin, 2007).

The extraction of the residual PAHs on agar tablets utilizes an ultrasonic extraction procedure. As an ultrasonic wave may undermine the original structure of an unstable compound while not affecting the structural stability of PAHs, this particular method was deemed better. The ultrasonic extraction method used was simple, fast, relatively small in the amount of solvent consumed and had a high extraction efficiency. Our previous research used this ultrasonic procedure to extract PCBs from the soil, and the efficiency of purification and extraction reached 70 to 80% (Gao et al., 2007).

The recovery efficiency of the PAHs is shown in Table 1. The low recovery efficiency of anthracene showed that low-ring polycyclic aromatic hydrocarbons had a low water-solubility, and with an increase in the number of benzene rings, the solubility and vapor pressure was lower, the octanol/water partition coefficient was high and the 4-ring pyrene was soluble in organic solvents (Bercaru et al., 2006).

The degradation effect on PAHs by *P. ostreatus*

Under the conditions of 25°C and 130 r/min, the degradation efficiency of anthracene and pyrene by *P. ostreatus* in liquid medium was measured. Figures 1 and 2 are, respectively, the laccase production and the degradation effects on anthracene and pyrene by *P. ostreatus.*

As shown in Figures 1 and 2, the degradation effect on anthracene and pyrene by *P. ostreatus* was more obvious with prolonged degradation time. With the strains undergoing growth, the secretion of laccase increased and the catalytic oxidation volume of anthracene and pyrene also increased. 7 days before the testing, laccase activity increased rapidly and began a gradual degradation of anthracene and pyrene. At 7 - 14 days, when the strains exhibited peak activity, the degradation of anthracene and pyrene was increased. By 14 days, the cumulative degradation efficiencies were 23.34 and 15.62%, respectively.

The degradation effect on PAHs by *Pseudotrametes gibbosa*

At 25°C and 130 r/min, the degradation effect of anthracene and pyrene by *P. gibbosa* in liquid medium I was determined. Figures 3 and 4, respectively, show the laccase production and the efficiency of degradation of anthracene and pyrene by *P. gibbosa.*

The production of laccase and the degradation of anthracene and pyrene by *P. gibbosa* were significantly higher than *P. ostreatus*. From 1 - 7 days, the secretion of laccase was low, the activity was low, the PAH degradation efficiency was low and the degradation efficiencies of anthracene and pyrene were 15.34 and 8.83%, respectively. The reason was that the addition of PAHs inhibited the growth of *P. gibbosa*, which requires a short-term adaptation phase. From 7 -14 days, the strains were at peak activity. The high availability of laccase in the liquid medium was conducive to the degradation of PAHs, enhancing the degradation effect, and producing degradation efficiencies of anthracene and pyrene at 32.54 and 18.51% and an increase of 17.2 and 9.68%, respectively. From 14 - 21 days, the capacity to secrete laccase declined, and the laccase activity and degradation efficiency of anthracene and pyrene decreased gradually. The degradation efficiencies were 43.43 and 24.26%.

It can be seen that these two fungi had a similar pattern of enzyme production and degradation process. The first step was an adaptation phase to PAHs, with enzyme production gradually increasing. Then, when a certain amount of PAH degradation was achieved, after peak activity and maintenance periods, the relative degradation of PAHs reached its highest level in the period of greatest production. Finally, the activity gradually decreased to zero, but this stage still maintained a certain amount of PAH degradation until the degradation efficiency ultimately became zero.

Comparison of the degradation effect of anthracene and pyrene by the two strains

Through the earlier studies, it can be seen that the yield

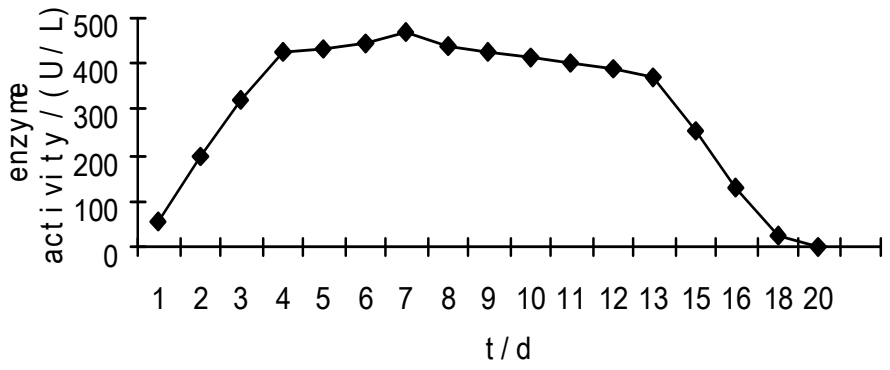

Figure 1. Laccase activity of *P. ostreatus.*

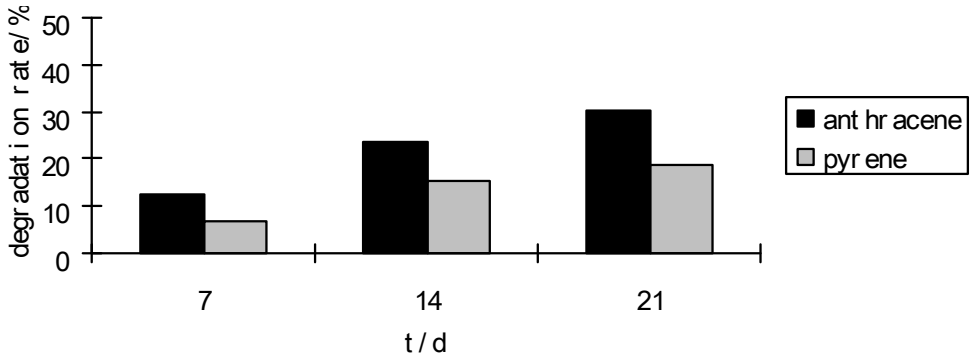

Figure 2. Anthracene and pyrene degradation by *P. ostreatus.*

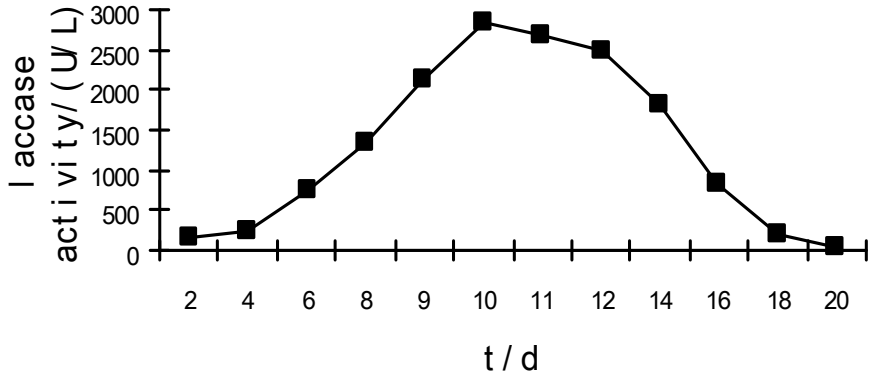

Figure 3. Laccase activity of *P. gibbosa.*

of enzyme in the liquid medium was higher than that of *P. ostreatus* in liquid medium II, while the laccase production also had a close relation with the degradation of PAHs. The degradation efficiencies of anthracene and pyrene by *P. ostreatus* were 18.76 and 30.12%, respectively (Figure 2), while the degradation efficiencies of anthracene and pyrene by *P. gibbosa* were 43.43 and 24.26%, respectively (Figure 4), so the degradation effect of *P. gibbosa* was clearly better than *P. ostreatus*. The main reasons are as follows:

(1) The different liquid medium: *P. ostreatus* was used in liquid medium II, in which the nutrient composition was relatively simple and able to support strain growth, it did not promote secretions of a large amount of laccase, so the degradation efficiency of anthracene and pyrene was

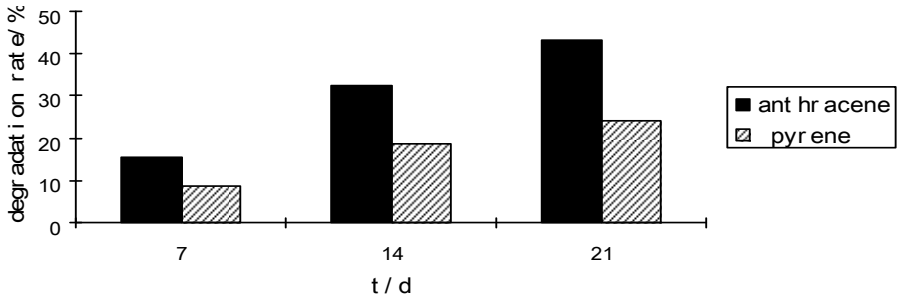

Figure 4. Anthracene and pyrene degradation of *P. gibbosa*.

lower. *P. gibbosa* was used in liquid medium I, which not only supported the growth of the strains, but also can promote the secretion of laccase.

(2) The laccase activity in the liquid medium: The laccase activity of *P. gibbosa* in the liquid media I was as high as 2841.36 U/l, which was more than 6 times that of *P. ostreatus*, the activity of which was only 471.15 U/l. It can be seen that the laccase activity bore a relation to the degradation of PAHs. That is to say, the higher the laccase activity, the more potent the degradation effect.

During the laccase catalysis oxidation process, adding certain low-molecular-weight compounds as mediators, such as ABTS, which plays the role of electronic transmission during the redox process, may have some effect. In the enzyme role, a mediator will form an intermediate with high activity that is reasonably stable, which can transfer the electrons from the oxygen molecules to the substrate, so that the substrate is degraded more effectively (Dodor et al., 2004).

(3) The effect of the surfactant: Tween 80 was added into the liquid medium I used by *P. gibbosa*. Surfactants, a class of substances with both hydrophobic and hydrophilic groups, have the roles of dispersion, emulsion and reduces the interface tension. The distribution role of the PAHs in the surfactant monomer-water, micelles, is reportedly potent. It reduced the capillary tension of PAHs in the environment, improved its solubility in water and promoted bio-availability of PAHs (Wang et al., 2006). At the same time, surfactants effectively protect the laccase activity. A surfactant can improve the permeability of cell membranes, thereby enhancing the degradation of PAHs.

Conclusion

The degradation of anthracene and pyrene by aboriginal white rot-fungus *P. gibbosa* in northern China was better than by *P. ostreatus* which has been studied both in China and overseas, and the degradation of anthracene and pyrene were 43.43 and 24.26%, respectively.

The laccase activity secreted by *P. gibbosa* was as high as 2841.36 U/l, which was more than 6 times than that of *P. ostreatus*. By comparing the activity and different degradation effects of anthracene and pyrene, it can be seen that laccase activity positively correlates with the degradation of PAHs.

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