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研究报告

Important role of natural mediators in oxidation of anthracene and pyrene by laccase producing fungi

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Abstract: [Objective] The role of natural mediators in oxidation of anthracene and pyrene by laccase producing fungi were evaluated. **[Methods]** In this work, enzyme activities assay and nondenaturing PAGE were employed to analyze laccase activity. **[Results]** The results showed that the fungus *Pycnoporus sanguineus* Z-1 and *Fomes fomentarius* Z-5 produced laccase, with maximal production of 11.90 and 4.83 U/mL, respectively, but not lignin peroxidase and manganese peroxidase. However, the culture fluid of *Fomes fomentarius* Z-5, with lower laccase activity, oxidized 74.3% of anthracene and 12.4% of pyrene, higher than that of *Pycnoporus sanguineus* Z-1, which suggested that the natural mediators might exist in the fungal culture and influenced the anthracene and pyrene oxidation. A further experiment demonstrated that all the treatments with addition of ultrafiltrate, boiled ultrafiltrate or boiled culture fluid improved the antracene and pyrene oxidation. The enhancement levels of ultrafiltrate, boiled ultrafiltrate and boiled culture fluid from *Fomes fomentarius* Z-5 were higher than those of *Pycnoporus sanguineus* Z-1, implying that the natural mediators in *Fomes fomentarius* Z-5 culture was more efficient in improving PAHs oxidation than in *Pycnoporus sanguineus* Z-1 culture. **[Conclusion]** The findings indicated that natural mediators played a important role in oxidation of substrates by laccase producing fungi and these might account for the phenomenon that *Fomes fomentarius* Z-5 culture, with lower laccase activity, oxidized more anthracene and pyrene than *Pycnoporus sanguineus* Z-1 culture.

Keywords: Laccase, Polycyclic aromatic hydrocarbons, White rot fungi, Oxidation

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天然介体在产漆酶真菌氧化蒽和芘中的重要作用

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摘 要:【目的】研究氧化还原介体在产漆酶真菌氧化蒽和芘的作用。【方法】通过非变性电泳和酶活力分析。【结果】发现血红密孔菌 Z-1 和木蹄层孔菌 Z-5 只产漆酶，其最大酶产量分别为 11.90 U/mL 和 4.83 U/mL，不产木质素过氧化酶和锰过氧化物酶。木蹄层孔菌 Z-5 的胞外液尽管具有较低的漆酶活性，但是氧化了 74.3%的蒽和 12.4%的芘，高于血红密孔菌 Z-1 对蒽和芘的氧化率，提示天然介体可能存在于真菌胞外液中并且影响了漆酶对多环芳烃的氧化。实验进一步表明，木蹄层孔菌 Z-5 灭活和不灭活的超滤液以及灭活的胞外液对纯漆酶氧化多环芳烃的促进作用均大于血红密孔菌 Z-1，说明木蹄层孔菌 Z-5 的天然介体比血红密孔菌 Z-1 能够更为有效地促进多环芳烃氧化。【结论】氧化还原结体在产漆酶真菌降解底物过程中发挥了重要作用，这也解释了木蹄层孔菌 Z-5 胞外液尽管漆酶活性不高，但是具有较大多环芳烃氧化率的原因。

关键词: 漆酶，多环芳烃，白腐真菌，氧化

1 Introduction

White-rot fungi are a kind of fungi that can cause white rot of wood, and they are unique in their abilities to degrade a wide variety of organic pollutants, mainly due to the secretion of a low-specificity enzyme system[1]. The enzyme system included lignin peroxidase (LiP) and manganese peroxidase (MnP) and laccase[2]. To date, all three extracellular enzymes have been reported with ability of oxidizing polycyclic aromatic hydrocarbons (PAHs)[2]. Unlike LiP and MnP, laccase are blue multicopper oxidases that can catalyze the one-electron oxidation of phenolic substrates or aromatic amines to their corresponding products[3]. It has been widely found in plants, bacteria, and insects; however, only laccases from white rot fungi were extensively studied due to their promising application prospects[4]. Laccases only can oxidize low redox potential compounds, which limits their application[3]. Some low molecule compounds, so-called 'mediators', can act as redox shuttles between the enzyme active site and targeted substrate to oxidize persistent pollutants with high redox-potential[3]. Up to now, the most commonly used artificial redox mediator is 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS), but the high price or possible toxicity limited its application on industrial scale[5].

Natural redox mediators referred to the compounds involved in the natural degradation of lignin by laccase-producing fungi, and they were supposed to be originated from fungal metabolism[5]. In nature, the presence of such compounds improved the biodegradation of laccase-producing fungi greatly[6]. Compared with artificial compounds, the utilization of natural mediators would be both eco-friendly and cost-effective[6], thus attracting the considerable interesting. Collins et al (1996) described a method to investigate the natural mediators, in which the extracellular culture fluid of laccase producing fungus was separated to crude laccase and ultrafiltrate ($\leqslant$ 10 kD in size) by ultrafiltration. Natural redox mediators were considered to be existed only if ultrafiltrate enhanced the PAHs oxidation by laccase[7].

PAHs are a group of persistent organic pollutants and widely distributed in environment and some of them are substrates of laccase. In our present

work, two PAHs, anthracene and pyrene were employed. We separated ultrafiltrate (containing possible natural mediators with elimination of enzyme) from extracellular culture fluid of two white rot fungi *Pycnoporus sanguineus* Z-1 and *Fomes fomentarius* Z-5, to determine their effect on the PAHs oxidation by laccase, according to the method of Collins[7]. The main objects of the work were to determine the role of natural redox mediators in biodegradation of laccase-producing fungi.

2 Materials and Methods

2.1 Chemicals, microorganisms and media

Anthracene and pyrene were purchased from Supelco (Bellefonte, USA). ABTS and pure laccase (CAS: 80498-15-3, from *Trametes versicolor*) were purchased from Sigma-Aldrich (Shanghai, China). The organisms used for laccase producing were *Pycnoporus sanguineus* Z-1 and *Fomes fomentarius* Z-5, collected from Tianmu mountain, Zhejiang province.

Liquid medium contained (g/L): glucose 10.0, yeast extract 2.5, KH_2PO_4 2.0, $MgSO_4 \cdot 7H_2O$ 0.5 and bran 2.0. The value of pH was adjusted to 5 with acetic acid[8].

2.2 Preparation of culture fluid and aqueous ultrafiltrate of fungus

Culture fluid (CF) and aqueous ultrafiltrate (AU) were prepared by the method of Collins[7]. Briefly, three agar plugs (8 mm diameter) cut out from the margin of a 7 day mycelium grown on PDA solid medium were inoculated in 250 mL conical flasks containing 100 mL liquid medium and incubated in a rotary shaker (120 r/min, 28 °C). After 9 d incubation, the culture medium was centrifuged for 5 min (11 000×g, 4 °C) and the supernatant was used as CF[9]. The CF, adjusted pH to 5 and boiled for 30 min, was used to BC. BC was filtrated by ultrafiltration membrane (10 kD), and the ultrafiltrate was used as AU. AU was further boiled for 30 min to use as boiled ultrafiltrate (BU). All compounds in AU and BU were≤10 kD.

2.3 Oxidation of PAHs by CF

To evaluate the oxidation efficiency of PAHs by CF, the experiments were performed in 15 mL brown tubes. The 5 mL reaction mixture consisted of 4.5 mL CF and 0.5 mL acetonitrile containing anthracene or pyrene. Deactivated CF (boiled for 30 min) was added to some treatments to serve as control. The final concentration of single PAH was 10 mg/L. Reaction tubes were closed tightly with screw cups and shaked violently by hand, and then incubated in the dark for 24 h (30 °C, 150 r/min). After another 5 mL acetonirile was added to terminate the reaction, the screw caps were closed tightly and the tubes were shaked again. After 1 h incubation, reaction mixtures were centrifuged at 13 000×g for 10 min and 20 μL supernatant were analyzed by HPLC system. All treatments, including controls, were triplicate. The oxidation rate was calculated by the formula:

$$\frac{\text{PAH concentration}_{\text{Control}} - \text{PAH concentration}_{\text{Treatment}}}{\text{PAH concentration}_{\text{Control}}}$$

2.4 Effects of the aqueous ultrafiltrate and boiled ultrafiltrate on enzymatic oxidation of PAHs

An experiment to evaluate the possible natural mediators on the enzymatic oxidation of PAHs was performed by the modified method according to method of Collins[7]. Briefly, the experiment was carried out in 5 mL reaction mixture included 4 mL either AU or BU, 0.5 mL phosphate buffer (pH 5.0, 50 mol/L) containing 25 U pure laccase, and 0.5 mL acetonitrile containing 100 mg/L anthracene or pyrene. The initial laccase activity and single PAH concentration was 5 U/mL and 10 mg/L. One treatment was added 4 mL medium (without organism inoculated) instead of AU or BU to evaluate the oxidation ability of pure laccase. Another treatment with addition of deactivated laccase (boiled for 30 min) served as control. All treatments, including controls, were triplicate. The incubation conditions and analysis methods were performed according to mentioned above.

2.5 Ligninolytic enzymes assay

The ligninolytic enzymes activities in fungal culture were assayed by analysis of CF at 4, 6, 8, 10 and 12 fungus-incubation days.

Laccase activities were determined by oxidation of ABTS at 30 °C. The 2 mL reaction mixture included 1.8 mL B&R buffer (0.1 mol/L boracic acid, 0.1 mol/L phosphoric acid and 0.1 mol/L acetic acid, pH adjusted to 5.0 with NaOH), 0.1 mL ABTS (20 mol/L) and 0.1 mL CF. The increase in absorbance at 420 nm was monitored with aspectrophotometer

(model752, CANY, China) to calculate laccase activity. The laccase activities were calculated by formula of $\Delta A \times 20 \times 10^6/36\ 000$. ΔA was increment of absorbance per min when it was stable. One unit of laccase activity was defined as the amount of enzyme able to oxidize 1 μmol ABTS/min.

LiP and MnP activities were determined by methods according to method of Ryu[10].

2.6 Nondenaturing PAGE

The CF of fungus was used as sample to perform nondenaturing PAGE according to the method of Pozdnyakova[11]. Ten percent of polyacrylamide gels were used without sodium dodecyl sulfate and β-mercaptoethanol. Native samples were dialyzed against (10 kD) 20 mol/L Tris-HCl (pH 6.8) and were applied to electrophoretic gels. After separation, the gels were visualized by 0.2% guaiacol for laccase activity and o-dianizidine in the presence of 100 μmol/L H_2O_2, with or without 100 μmol/L $MnSO_4$, for MnP and LiP activity.

2.7 HPLC analysis

PAHs samples were analysis by Agilent 1100 system. A reversed phase column C_{18} (4.6 mm×250 mm, partical size 2.2 μm), using a mobile phase with 100% ancetonitrile (elution 7 min, at a constant flow rate 1.0 mL/min, 30 °C), was used to separate PAHs.

2.8 Statistical analysis

SPSS for Windows software was used for statistical analysis.

3 Results and Discussion

3.1 Ligninolytic enzymes of fungi

The ligninolytic enzymes production of fungi *P. sanguineus* Z-1 and *F. fomentarius* Z-5 were investigated. Only laccase activities were detected in both fungal cultures whereas MnP and LiP were not found (Figure 1). At ninth incubation day, laccase production of *P. sanguineus* Z-1 reached the maximum of 11.90 U/mL, which was two times more than that of *F. fomentarius* Z-5 (4.83 U/mL at sixth incubation day), indicating that *P. sanguineus* Z-1 was more efficient in laccase producing.

The nondenaturing PAGE was also performed to detect the ligninolytic enzymes activities (Figure 2). When ABTS was used as the staining reagent, one band appeared in each gel of *P. sanguineus* Z-1 and *F. fomentarius* Z-5. ABTS can be oxidized to green color by laccase, thus can be used as the staining reagent. Pozdnyakova et al (2010) have used ABTS to detect laccase bands of *Pleurotus ostreatus* D1 by nondenaturing PAGE[11]. The band appeared in our work indicated that both *P. sanguineus* Z-1 and *F. fomentarius* Z-5 could produce laccase. We also used o-dianizidine in the presence of 100 μmol/L H_2O_2,

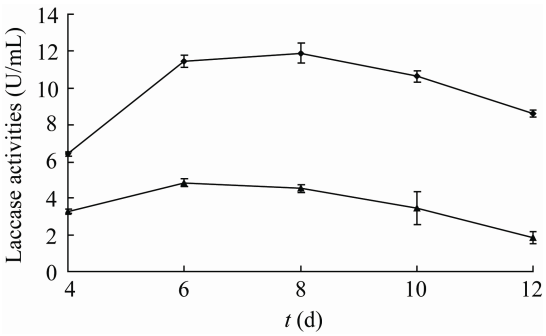

Figure 1 The laccase production of *P. sanguineus* Z-1 and *F. fomentarius* Z-5 in liquid medium

图 1 *P. sanguineus* Z-1 和 *F. fomentarius* Z-5 在液体培养基中的漆酶产量

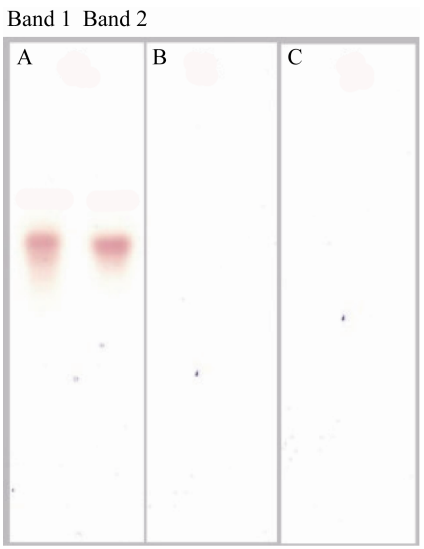

Figure 2 Nondenaturing PAGE of culture fluid from *P. sanguineus* Z-1 and *F. fomentarius* Z-5

图 2 *P. sanguineus* Z-1 和 *F. fomentarius* Z-5 的胞外液的聚丙烯酰胺凝胶电泳

Note: The gels were visualized by 0.2% guaiacol (A) and o-dianizidine in the presence of 100 μmol/L H_2O_2, with (B) or without 100 μmol/L $MnSO_4$ (C). The band "1" and "2" presented culture fluid of *P. sanguineus* Z-1 and *F. fomentarius* Z-5 were used as the sample, respectively.

注：字母 A、B 和 C 分别代表用愈创木酚、邻联茴香胺/H_2O_2/$MnSO_4$ 和邻联茴香胺/ H_2O_2 染色，Band 1 和 2 分别代表用 *P. sanguineus* Z-1 和 *F. fomentarius* Z-5 的半纯化漆酶作为样品.

with or without 100 μmol/L $MnSO_4$ as staining reagent, according to method of Pozdnyakova, but no band appeared in any gel, indicating that neither MnP nor LiP was produced by both fungi.

3.2 Anthracene and pyrene oxidation by CF

Anthracene and pyrene oxidation by CF from *P. sanguineus* Z-1 and *F. fomentarius* Z-5 were investigated (Figure 3). The results showed that the CF of both fungi could oxidize anthracene and pyrene. The oxidation rate of anthracene by CF from *P. sanguineus* Z-1 was 49.8%, lower than that of *F. fomentarius* Z-5 (74.3%). Similarly, the pyrene oxidation rate by CF from *P. sanguineus* Z-1 (5.4%) was also lower than that of *F. fomentarius* Z-5 (12.4%). These results indicated that the CF of *F. fomentarius* Z-5 was more efficient in PAHs oxidation than *P. sanguineus* Z-1. In light of the laccase activity of CF from *P. sanguineus* Z-1 was much higher than that of *F. fomentarius* Z-5 (Figure 1), it was not difficult to conclude that there was no obvious link between the laccase activity and the PAH oxidizing ability[9]. Therefore, we supposed that natural mediators might exist in the CF and they effect the enzymatic PAHs oxidation[7].

3.3 Effects of the AU, BU and BC on anthracene and pyrene oxidation by laccase

To determine the existence of possible natural mediators in CF, we added the AU, BU and BC to laccase-catalyzed PAHs oxidation system to evaluate their stimulatory effects (Figure 4). The experiment was carried out in 5 mL reaction mixtures and the commercial pure laccase from *Trametes versicolor* was used as oxidase. The results showed that the pure laccase oxidized only 15.9% of anthracene and 2.1% of pyrene, respectively, and all treatments that addition of BU, AU or BC improved the PAHs oxidation. For example, addition of AU from *P. sanguineus* Z-1 and *F. fomentarius* Z-5 increased anthracene oxidation rate to 38.7% and 59.2%, and addition of BU from *P. sanguineus* Z-1 and *F. fomentarius* Z-5 increased the value to 26.9% and 46.8%, respectively. Anthracene oxidation rate by addition of BC was on the same level with BU-treatment. In view of the enzymes were eliminated in both AU, BU and BC, it could be deduced that the chemical compounds, namely natural mediators, contributed to the enhancement of PAHs oxidation. That was to say, both *P. sanguineus* Z-1 and *F. fomentarius* Z-5 could produce natural mediators. The similar results were observed in Collins's work[7], in which ultrafiltration (≤10 kD) from CF of *Trametes versicolor* enhanced the anthracene oxidation by pure laccase. In addition, a new finding in our work was that the enhancement level of either BU, AU or BC form *F. fomentarius* Z-5 was higher than that from *P. sanguineus* Z-1, suggesting that the natural mediators in *F. fomentarius* Z-5 CF was more efficient in improving laccase catalysis. These might account for phenomenon that CF from *F. fomentarius* Z-5, with lower laccase activity, oxidized more anthracene or pyrene than *P. sanguineus* Z-1 (Figure 2). Our results also indicated that besides laccase, natural mediators also played an important role in PAHs oxidation by laccase-producing fungus.

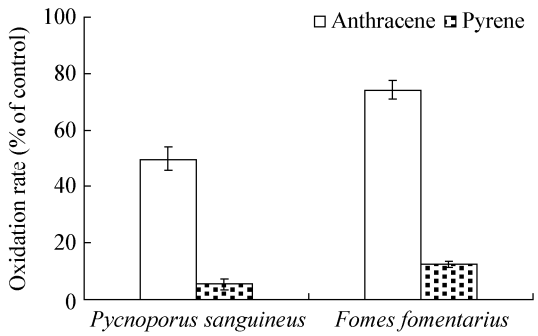

Figure 3 Oxidation rate of anthracene and pyrene by CF from *P. sanguineus* Z-1 and *F. fomentarius* Z-5

图 3 *P. sanguineus* Z-1 和 *F. fomentarius* Z-5 的胞外液对蒽和芘的氧化

Note: Values were means of triplicates, and error bars stood for standard deviations

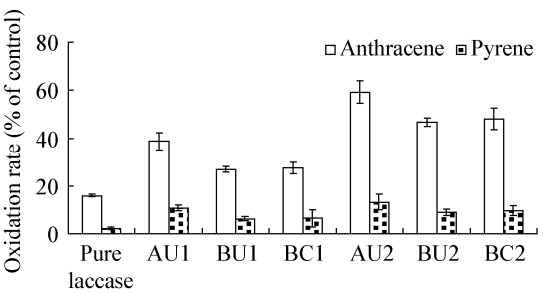

Figure 4 The effects of BU and AU from *P. sanguineus* Z-1 and *F. fomentarius* Z-5 on oxidation rate of anthracene and pyrene by pure laccase

图 4 *P. sanguineus* Z-1 和 *F. fomentarius* Z-5 的胞外超滤液和煮沸灭活的胞外液对纯漆酶氧化蒽和芘的影响

Note: 1: *P. sanguineus* Z-1; 2: *F. fomentarius* Z-5.

We further observed the improvement of AU on PAHs oxidation was more efficient than corresponding BU or BC, either from *P. sanguineus* Z-1 or *F. fomentarius* Z-5 (Figure 4), suggesting that the natural mediators might be thermo-labile, and they were probably diminished by the boil procedure.

3.4 The effects of boiled terms on the stimulatory effects of BU on oxidation rate of anthracene by pure laccase

To evaluate the thermal stability of the natural mediators, we determined the effluence of BU with different boiled terms (0, 30, 60 and 90 min) on the anthracene oxidation by laccase. The results showed that the improvement of enzymatic anthracene oxidation by BU without any boiled treatments was largest among all the treatments, which reached 42.3% (*P. sanguineus* Z-1) and 55.3% (*F. fomentarius* Z-5), respectively (Figure 5). When boiled terms of BU prolonged, the improvement of enzymatic anthracene oxidation decreased significantly, and the oxidation rate was on the same level by addition of BU boiled for 30 to 90 min. These results further indicated that natural mediators in the AU were thermo-labile and some of them could be removed by boiled treatment.

To improve biodegradability of laccase-producing fungi, much attention was paid on the enhancement of laccase production and nature mediators were usually neglected[12]. Our results indicated that laccase was indispensable while natural mediators were important in fungal remediation of pollutants. Though the artificial mediators such as ABTS was supplied commercially, the price was too high to apply in industrial scale[13]. Therefore, how to enhance the natural mediators yield of fungi might be a novel alternative to improve the biodegradation of laccase-producing fungi, and our further work would focus on this topic.

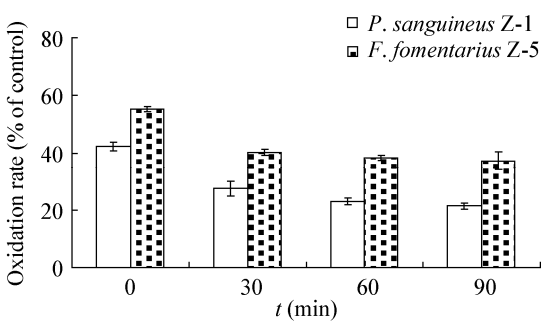

Figure 5 Effluence of BU with different boiled terms on the anthracene oxidation by laccase

图 5 不同煮沸时间的灭活胞外液对纯漆酶氧化蒽的影响

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