*Pedosphere* **24**(3): 359–366, 2014
ISSN 1002-0160/CN 32-1315/P

Published by Elsevier B.V. and Science Press

PEDOSPHERE

www.elsevier.com/locate/pedosphere

# Influencing Factors and Product Toxicity of Anthracene Oxidation by Fungal Laccase*[1]

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(Received August 18, 2013; revised March 24, 2014)

## ABSTRACT

The transformation profiles of polycyclic aromatic hydrocarbons (PAHs) by pure laccases from *Trametes versicolor* and *Pycnoporus sanguineus*, and the optimal reaction conditions (acetonitrile concentration, pH, temperature and incubation time) were determined. Anthracene was the most transformable PAH by both laccases, followed by benzo[*a*]pyrene, and benzo[*a*]anthracene. Laccase-mediator system (LMS) could not only improve the PAH oxidation but also extend the substrate types compared to laccase alone. 5% or 10% (v/v) of acetonitrile concentration, pH 4, temperature of 40 °C, and incubation time of 24 h were most favorable for anthracene oxidation by laccase from *T. versicolor* or *P. sanguineus*. The gas chromatography-mass spectrometry analysis indicated that 9,10-anthraquinone was the main product of anthracene transformed by laccase from *T. versicolor*. Microtox test results showed that both anthracene and its laccase-transformation products were not acute toxic compounds, suggesting that laccase-treatment of anthracene would not increase the acute toxicity of contaminated site.

*Key Words*: laccase-mediator system, polycyclic aromatic hydrocarbons, *Pycnoporus sanguineus*, reaction condition, *Trametes versicolor*

**Citation:** Li, X. Z., Cheng, Q., Wu, Y. C., Feng, Y. Z., Liu, W. W. and Lin, X. G. 2014. Influencing factors and product toxicity of anthracene oxidation by fungal laccase. *Pedosphere*. **24**(3): 359–366.

## INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are fused-ring aromatic compounds mainly formed during the incomplete combustion of organic compounds and has been widely distributed in the environment (Juhasz and Naidu, 2000; Bamforth and Singleton, 2005). Sixteen PAHs are listed by the US Environmental Protection Agency (EPA) as the priority pollutants (Keith and Telliard, 1979). Presently, bioremediation is a common method in removal of PAHs in the environment (Haritash and Kaushik, 2009; Hadibarata *et al.*, 2011; Ye *et al.*, 2011; Hadibarata and Kristanti, 2012). Unfortunately, some factors such as the suboptimal growth conditions (McErlean *et al.*, 2006), high toxicity (Wu *et al.*, 2008) or interactions with the indigenous microbes (Andersson *et al.*, 2000) have often caused the failures of conventional bioremediation. Recently, enzyme-based bioremediation has increasingly been received attention for its less limiting factors in remediation (Durán and Esposito *et al.*, 2000; Torres *et al.*, 2003), and laccase is one of the most promising remedial enzymes (Riva, 2006).

Laccase belongs to the copper-containing polyphenol oxidases, and presently, the enzyme from white rot fungi was most widely applied (Collins *et al.*, 1996; Johannes *et al.*, 1996; Majcherczyk *et al.*, 1998; Dodor *et al.*, 2004). With the assistance of a cluster of four copper atoms, which form the catalytic core of the enzyme, laccase is able to oxidize a number of substrate molecules to the corresponding reactive radicals (Riva, 2006). In the presence of some chemicals so-called "mediators", the substrates of laccase extend to compounds with high ionization potential (IP) (Keum and Li, 2004; Riva, 2006). In the processes of laccase catalysis, $O_2$ from ambient air is the only requirement and $H_2O$ is the unique byproduct (Riva, 2006; Morozova *et al.*, 2007), suggesting that the reaction process

*[1]Supported by the National High-Tech R&D Program of China (No. 2007AA061101), the Key Laboratory of Soil Environment and Pollution Remediation, Institute of Soil Science, Chinese Academy of Sciences, and the Zhejiang Provincial Natural Science Foundation of China (No. Y5110147).
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is eco-friendly. Laccase could oxidize a widely range of xenobiotics, including PAHs (Sei *et al.*, 2008; Zhang *et al.*, 2008). However, the profile as well as the optimal remediation conditions of PAH oxidation by laccase was still not clear, which disadvantages the application of the enzyme.

Anthracene is one of the EPA priority pollutants (Keith and Telliard, 1979). Though no considerable toxicity, carcinogenesis, or mutagenesis is found (Bu-Abbas *et al.*, 1994), anthracene pollution is still of great concern due to its similar structure with carcinogenic PAHs such as benzo[*a*]pyrene and benzo[*a*]anthracene (Bonnet *et al.*, 2005; Mohammadi and Nasernejad, 2009) and the relative high level in the environment (Hu *et al.*, 2009). Although anthracene may undergo adsorption, volatilization, photolysis, and chemical degradation in the environment, microbial degradation is the major degradation process (Haritash and Kaushik, 2009). Once anthracene enters the body, it appears to endanger the skin, stomach, intestines and the lymphatic system, being a probable inducer of tumors (Hadibarata *et al.*, 2012). Because of its low molecular weight compared to most of other PAHs, anthracene has a higher solubility and can be found at more significant levels in water (Stucki and Alexander, 1987). So, anthracene is usually studied as a model PAH (Bonnet *et al.*, 2005).

The aim of this study was to determine the profile of PAH oxidation by laccase, as well as the optimal reaction conditions, and the products and their toxicity of laccase-transformation of PAHs, by using anthracene as a model aromatic compound.

## MATERIALS AND METHODS

### *Enzymes and chemicals*

Pure laccases from *Trametes versicolor* and *Pycnoporus sanguineus* were obtained from Sigma-Aldrich Corporation, Shanghai, China (CAS 80498-15-3) and the Institute of Microbiology, Chinese Academy of Sciences, respectively. Fifteen PAHs were purchased from Supelco Inc., Bellefonte, USA. 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonicacid) (ABTS) was purchased from Sigma-Aldrich Corporation, Shanghai, China.

### *Laccase activity assay*

Laccase activity was determined by measuring the oxidation of ABTS at 30 °C. Briefly, a 2 mL reaction mixture was prepared including 1.8 mL B&R buffer (0.1 mol $L^{-1}$ boracic acid, 0.1 mol $L^{-1}$ phosphoric acid and 0.1 mol $L^{-1}$ acetic acid; pH adjusted to 5.0 with NaOH), 0.1 mL ABTS (20 mmol $L^{-1}$), and 0.1 mL enzyme solution. The increase in absorbance at 420 nm was monitored with a spectrophotometer (model 752, CANY, Shanghai, China) to determine laccase activity ($\varepsilon_{420}$ = 36 000 mol $L^{-1}$ $cm^{-1}$) by the formula of $\Delta A \times 20 \times 10^6/36\,000$, where $\Delta A$ is the increment of absorbance per min when it is stable. One unit of laccase activity is defined as the amount of enzyme able to oxidize 1 μmoL ABTS $min^{-1}$.

### *Oxidation of 15 PAHs*

The experiment was performed in 15 mL glass tubes with 5.0 mL phosphate buffer solution (pH 5.0, 50 mmol $L^{-1}$) containing 10% (v/v) of acetonitrile and 10 U of total laccases from *T. versicolor* and *P. Sanguineus*. Fifteen PAHs were added to the mixture and their initial concentrations are present in Table I. Mediator ABTS was added to some treatments at an initial concentration of 1 mmol $L^{-1}$ to evaluate its effect on the PAH oxidation. Reaction tubes were closed tightly with screw caps, shaken violently by hand, and incubated in darkness for 48 h at 25 °C. After incubation another 5.0 mL acetonitrile was added to terminate the reaction. The screw caps were closed tightly and the tubes were shaken again. After 1-h incubation, reaction mixtures were centrifuged at 11 000 × *g* for 10 min and 10 μL supernatant was collected to determine the PAH concentration by an ultra fast liquid chromatograph (UFLC) system. Instead of fresh laccase, inactivated laccase (boiled for 30 min) was added to serve as the corresponding control and each treatment was performed in triplicate.

### *Reaction conditions of anthracene oxidation*

A series of single factor experiments were performed in 15 mL glass tubes with 5.0 mL acetonitrile-buffer solution containing laccase and anthracene to assess the impacts of reaction conditions (acetonitrile concentration, pH, temperature and reaction time) on the laccase (from *T. versicolor* and *P. sanguineus*) catalysis. Different acetonitrile concentrations were set with 1%, 2%, 5%, 10% and 20% (v/v) acetonitrile-phosphate buffer (pH 5.0, 50 mmol $L^{-1}$). This experiment was performed at 25 °C in the reaction mixture of pH 5.0 containing 1 mg $L^{-1}$ anthracene and 30 U laccase. The pH values of the medium varied from 3 to 7 were set by using citrate buffer (pH 3.0 and 4.0, 50 mmol $L^{-1}$) and phosphate buffer (pH 5.0, 6.0 and 7.0, 50 mmol $L^{-1}$) and the experiment was carried out at 25 °C in the mixture containing 10% acetonitrile-buffer solution, 1 mg $L^{-1}$ anthracene, and 30 U laccase. The effects of temperature on laccase catalysis were deter-