Bull Environ Contam Toxicol (2014) 93:478–482
DOI 10.1007/s00128-014-1340-4

Profile of Natural Redox Mediators Production of Laccase-Producing Fungus *Pleurotus ostreatus*

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Received: 25 December 2013 / Accepted: 17 July 2014 / Published online: 10 August 2014

Abstract Polycyclic aromatic hydrocarbons (PAHs) are highly toxic organic pollutants which are abundant and environmentally widespread. Anthracene is a simple PAH that can be oxidized by laccases, copper-containing oxidase enzymes, produced by some plants, fungi, and bacteria. In this work, the extracellular culture fluid (CF) of laccase-producing fungus *Pleurotus ostreatus* was separated to crude laccase (CL) and aqueous ultrafiltrate (AU) fractions. The rate of anthracene oxidation by CF was 68.7 % while oxidation by CL was only 27.8 %. The addition of AU enhanced anthracene oxidation rate by CL to 60.4 %, indicating that the natural redox-mediators were present in the CF. The laccase-catalyzed anthracene oxidation rate increased with increased AU concentration, implying that oxidation rate is positively related to the concentration of natural mediators when laccase activity is constant. The AU from fungal culture containing bran or straw enhanced laccase-catalyzed anthracene oxidation; this enhancement increased further with prolonged fungus-cultivation, implying that both bran and straw induce the natural mediators. Our findings suggest increasing natural mediator levels may be an alternative strategy to improve the biodegradability of laccase-producing fungi.

Keywords Laccase · Natural redox mediators · Oxidation · Anthracene

Laccases are multicopper oxidases that catalyze the one-electron oxidation of phenolic substrates or aromatic amines. Laccases have been isolated from plants, bacteria, and insects, as well as fungi. Of these, the laccases found in basidiomycetous and ascomycetous fungi, including the white-rot basidiomycetes responsible for lignin degradation in nature, are the best characterized (Giardina et al. 2010). However, one limiting challenge with these enzymes is that frequently the substrates cannot be oxidized directly by the enzyme, either because they are too bulky to enter the enzyme's active site or because they have a particular high redox potential, and thus are only able to oxidize compounds with low ionization potentials (<7.45 eV; Riva 2006). This limitation can be addressed through the use of low-molecular-weight compounds that, once oxidized by the enzyme to stable radicals, can act as redox mediators, functioning as redox shuttles between the enzyme active site and the substrate to allow the oxidization of high-redox-potential substrates that would not be substrates of laccase (Riva 2006). To date, the most commonly used artificial redox mediators are 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 1-hydroxybenzotriazole (HBT), but industrial-scale application has been limited by the high cost of these chemicals (Johannes and Majcherczyk 2000).

Natural redox mediators are the compounds involved in the natural degradation of lignin by laccase-producing

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fungi, and are thought to derive from fungal metabolism (Johannes and Majcherczyk 2000). In nature, laccase-producing fungi strengthen their lignin biodegradation capabilities by secreting natural mediators (Riva 2006). Some fungal metabolites, such as phenol, aniline, 4-hydroxybenzoic acid and 4-hydroxybenzyl alcohol, have been shown to act as laccase mediators (Johannes and Majcherczyk 2000). Compared with artificial compounds, the utilization of natural mediators could be both eco-friendly and cost-effective (Camarero et al. 2005). However, few studies have been performed to investigate natural mediator production by fungi.

Collins et al. (1996) described a method to measure the natural redox mediators in culture fluid (CF) of strain *Pleurotus ostreatus*. In this method, the extracellular CF from laccase-producing fungus was separated to crude laccase (CL) and aqueous ultrafiltrate (AU) ($\leq$10 kDa in size) by ultrafiltration. Natural redox mediators were considered to be present only if AU enhanced laccase oxidation of PAHs (Collins et al. 1996). The work of Collins et al. (1996) did not identify the fungal natural mediators, but it established the method to use for such studies (Collins et al. 1996). Polycyclic aromatic hydrocarbons (PAHs) are a group of persistent organic pollutants that are widely distributed in the environment. In this work, we study the PAH anthracene as laccase substrate. Anthracene is acutely toxic and is recognized as a priority pollutant by the US Environmental Protection Agency (Liu et al. 2001). We separated the extracellular CF of laccase-producing fungus *Pleurotus ostreatus* into CL (containing laccase) and AU (containing possible natural mediators) fractions to determine the presence of natural redox mediators in the CF, according to the method of Collins et al. (Collins et al. 1996). We found a positive relationship between the natural redox mediator concentration and the stimulatory effect on anthracene oxidation. In addition, the profiles of natural mediator production for strain *Pleurotus ostreatus* were successfully outlined.

Materials and Methods

The laccase-producing strain *Pleurotus ostreatus* was obtained from the Henan Academy of Agricultural Sciences. Previous work found that this strain could not produce manganese peroxidase and lignin peroxidase. ABTS, ancetonitrile (Chromatographic Grade) and pure laccase (CAS: 80498-15-3, from *Trametes versicolor*) were purchased from Sigma–Aldrich (Shanghai, China). Anthracene (Chromatographic Grade) was obtained from Supelco (Bellefonte, USA). GY medium was prepared according to Elisashvili et al. (2008), which contained glucose (10 g/L), yeast extract (2.5 g/L), KH_2PO_4 (2 g/L), $MgSO_4{\cdot}7H_2O$ (0.5 g/L), and the pH value was adjusted to 5 with 20 mM acetic acid. All chemicals used in the media were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). GYB and GYS medium was prepared by addition of 20 g/L bran and straw to GY medium, respectively. The bran and straw were supplied by the Henan Academy of Agricultural Sciences.

The CF, AU and CL were prepared according to (Collins et al. 1996). Briefly, three potato dextrose agar (PDA) plugs (8 mm diameter) of *Pleurotus ostreatus* were introduced to 500 mL conical flasks containing 150 mL of autoclaved GYB medium and incubated for 10 days (120 r/min, 28°C). The culture media from incubation flasks were adjusted to pH 5 and centrifuged for 10 min (5000 g, 4°C); the supernatant was used as CF. Then, the CF was filtrated by ultrafiltration tubes (10 kDa, Millipore, USA). Ultrafiltrate was autoclaved at 121°C for 30 min to be used as the AU. The extracellular proteins on the ultrafiltration membrane were collected and washed three times with distilled water and were used as the CL.

To determine the effect of AU on anthracene oxidation by laccase, the experiments were performed in 5 mL anthracene-oxidation systems in 15 mL serum bottles (Field et al. 1993), which included 4.5 mL laccase-containing fluid, 0.5 mL methanol and anthracene. The final laccase activity was 2 U/mL and anthracene concentration was 1 mg/L, respectively. Laccase-containing fluid was added in the form of 4.5 mL CF or CL solution. CL solution was prepared by dissolving CL in sodium acetate buffer (20 mM, pH5), AU, or GYB medium. The influence of the ABTS on anthracene oxidation was determined by adding it to CL-buffer solution reaction mixtures to a final concentration of 1.0 mM. The treatment with inactivated enzyme addition (boiled for 30 min) was the negative control, and each treatment was performed in triplicate.

To evaluate the effects of various AU concentrations on laccase-catalyzed anthracene oxidation, the AU, diluted by sodium acetate buffer (20 mM, pH5) to various concentrations (100 %, 50 %, 20 %, 10 % of original concentration), was added to bottles containing anthracene oxidation system. The anthracene oxidation system contained 4.5 mL AU, 10 U pure laccase, 0.5 mL methanol and 1 mg/L anthracene. The treatment with inactivated enzyme addition (boiled for 30 min) served as the negative control. Each treatment was performed in triplicate.

The relative fungal production of the natural redox mediators was evaluated by determining the stimulatory effect of the AUs on the anthracene oxidation by laccase. Briefly, the AU, prepared from three fungal culture media (GY, GYB and GYS) were cultivated for different lengths of time (0, 2, 4, 6, 8, 10, 12, 14 and 16 days), were added to bottles containing the anthracene oxidation system. The anthracene-oxidation system contained 4.5 mL AU, 10 U

pure laccase, 0.5 mL methanol, and 1 mg/L anthracene. Another treatment was prepared by replacing AU by an equal volume of acetate buffer (20 mM, pH5). The treatment with the inactivated enzyme (boiled for 30 min) served as the negative control. Each treatment, including the control, was done in triplicate.

All the bottles mentioned above were flushed with 100 % oxygen for 5 min and incubated statically in darkness at 28°C. After 24 h, another 5 mL of methanol was added to terminate the incubation. After vortexing for 1 min, 50 μL samples were centrifuged (13,000×*g*, 4°C) for 10 min and 20 μL of the supernatant were analyzed by HPLC system.

Laccase activities were determined by methods according to Ryu et al. (2000).

Anthracene concentration was analyzed with an Agilent 1100 system. A reversed phase column C_{18} (4.6 mm × 250 mm, partical size 2.2 μm, Agilent-880975-914), 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 anthracene.

The oxidation product of anthracene by laccase was analyzed by GC–MS (Agilent 6890N/5975C system, USA), equipped with a GC column (HP-5MS, 30 m × 0.25 mm), the oven temperature was programmed from 80°C with a 2 min hold and 10°C per minute increment to 250°C with 1 min hold.

SPSS for Windows software was used for statistical analysis and one-Way ANOVA to determine difference between treatments at a significant level of 0.01 or 0.05.

Results and Discussion

The important role of redox mediators in the improvement of laccase catalysis has motivated considerable interest in searching and screening low-cost, high-efficiency mediators (Morozova et al. 2007). Much attention focused on synthetic compounds while natural mediators were overlooked (Johannes and Majcherczyk 2000; Canas et al. 2007; Larson et al. 2013). The effect of AU on anthracene oxidation rate by various fractions of fungal CF is shown in Fig. 1. Anthracene oxidation rate by CF was 68.7 % while the value by CL-buffer treatment was only 27.8 %, indicating that some compounds present in the CF, enhanced laccase catalysis. The CL–AU treatment transformed 60.4 % of anthracene, higher than the CL-buffer treatment, further demonstrating that the component, present in AU and ≤10 kDa in size, enhanced the anthracene oxidation. The anthracene oxidation rate by CL-medium treatment was 27.0 %, the same level as CL-buffer treatment, implying that the active component originated from fungal metabolism and not from the media. The artificial mediator

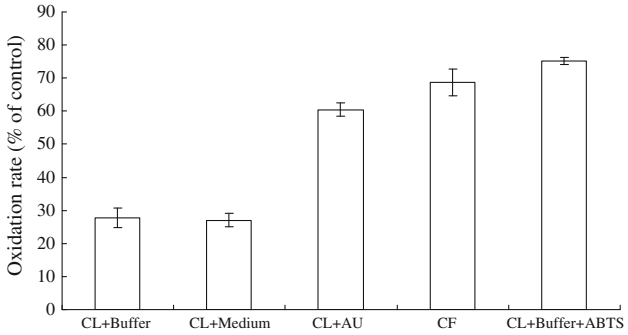

Fig. 1 Oxidation of anthracene by various fractions of CF of strain *Pleurotus ostreatus* (28°C, 24 h). Values were means of triplicate, and *error bars* stood for standard deviations. The anthracene oxidation system included 4.5 mL laccse-containing fluid (10 U), 0.5 mL methanol and 1 mg/L PAH. Laccse-containing fluid was added in the form of CL dissolved in buffer (CL + Buffer), CL dissolved in medium without inoculation (CL + Medium), CL dissolved in AU (CL + AU), CL dissolved in buffer with ABTS (CL + Buffer +ABTS), and culture fluid (CF), respectively

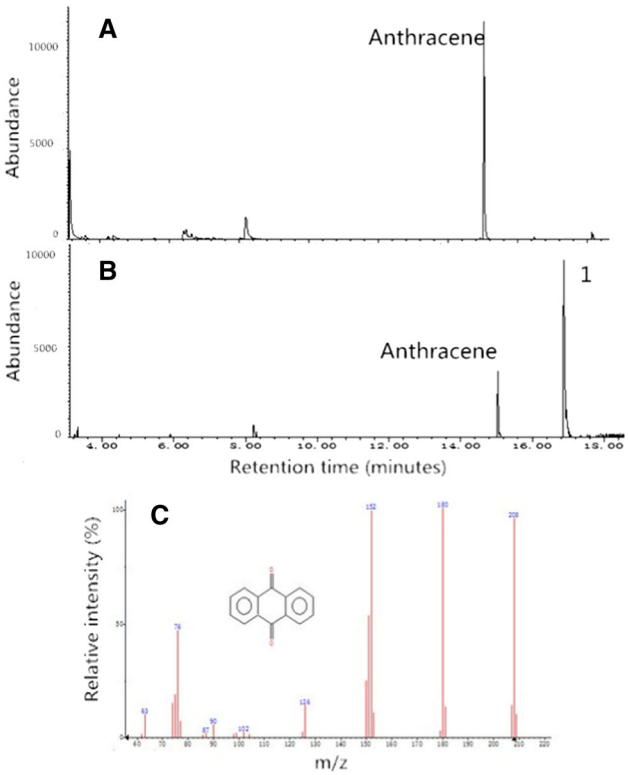

Fig. 2 Identification of main products of anthracene by CL with ABTS. **a** GC of control reaction, **b** GC of the products after 24 h incubation and **c** mass spectrum of peak 1 in **b**

ABTS also enhanced the anthracene oxidation rate, to 75.2 %, consistent with previous findings (Collins et al. 1996; Canas et al. 2007; Camarero et al. 2008). We determined the oxidation product of anthracene by GC–MS and found that a new peak with a molecular ion of m/z 208

appeared at retention time of 17.0 min (Fig. 2a, b). The new product was further identified as 9,10-anthraquinone based on the fragments profile in mass spectrum (Fig. 2c). These results indicated that some compounds in the CF, with size ≤10 kDa, could act as ABTS in the oxidation of anthracene. That is to say, natural mediators were present in the CF of strain *Pleurotus ostreatus*.

We predicted that the concentration of mediators would alter their stimulatory effects on laccase catalysis. Previous work reported that anthracene oxidation rate by laccase increased with increased concentration of mediator phenol (from 0 to 1 mM), aniline (from 0 to 0.1 mM), and ABTS (from 0.1 to 2 mM) (Johannes et al. 1996; Johannes and Majcherczyk 2000). To see if this was true in this assay, we measured the effect of AUs with different concentrations (100 %, 50 %, 20 %, 10 % of original concentration) on laccase-catalyzed anthracene oxidation (Fig. 3). Pure laccase without AU only oxidized 25.6 % of anthracene; addition of AUs increased oxidation in a concentration-dependent manner. Addition of AU without dilution (100 % of original concentration) improved the anthracene oxidation significantly and the oxidation rate reached 70.9 %. These results implied that laccase-catalyzed anthracene oxidation rate was positively related to natural mediator concentration when laccase activity was constant. This finding allowed us to compare the concentration of natural mediators in different fungal culture fluid by determining the stimulatory effect of AU on laccase-catalyzed anthracene oxidation. To do this, we used three media (GY, GYB and GYS medium) to cultivate strain *Pleurotus ostreatus* and prepare AU. We found (Fig. 4) that compared to the control (without AU), addition of AUs from GY medium under any fungus cultivation conditions did not enhance laccase-catalyzed anthracene oxidation. In contrast, addition of AUs from GYB medium after 6 day and GYS medium after 10 day fungus-cultivation, enhanced the anthracene oxidation rate significantly. The oxidation rate increased with the prolongation of strain *Pleurotus ostreatus* cultivation and finally reached 79.8 % and 45.3 %, for GYB and GYS, respectively (Fig. 4). These results suggested that both

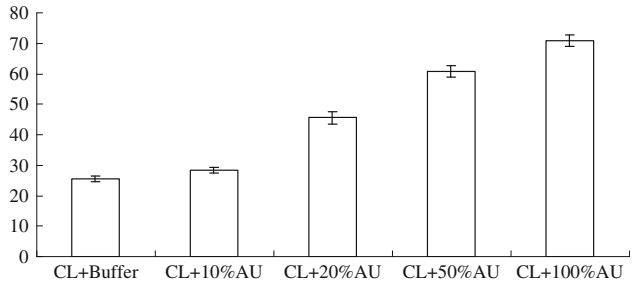

Fig. 3 Oxidation of anthracene by pure laccase by addion of AUs with different concentrations (28°C, 24 h). Values were means of triplicate, and *error bars* stood for standard deviations. The anthracene oxidation system included 4.5 mL laccse-containing fluid (10 U), 0.5 mL methanol and 1 mg/L anthracene. Laccase-containing fluid was added in the form of pure laccase dissolved in 4.5 mL AU of different concentration (100 %, 50 %, 20 %, 10 % of original concentration) or 4.5 mL acetate buffer (20 mM, pH5)

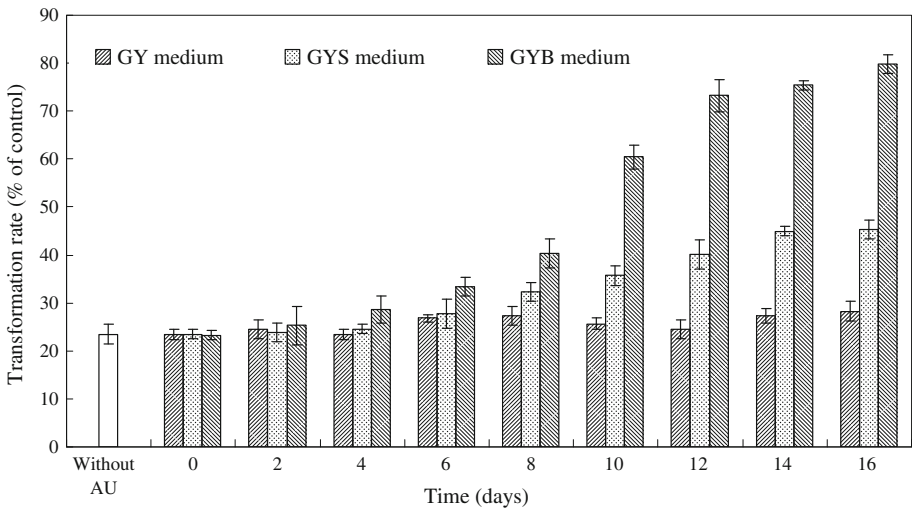

Fig. 4 The effects of AU of different fungus-cultivation durations on anthracene oxidation by laccase (28°C, 24 h). Values were means of triplicate, and *error bars* stood for standard deviations. The anthracene oxidation system included 4.5 mL laccse-containing fluid (10 U), 0.5 mL methanol and 1 mg/L anthracene. Laccse-containing fluid was added in the form of pure laccase dissolved in 4.5 mL of AU from three culture media (GY, GYS and GYB) of different fungus-cultivation durations (0, 2, 4, 6, 8, 10, 12, 14 and 16 day). The treatment of "Without AU" presented that 4.5 mL acetate buffer (20 mM, pH5) was added in stead of AU

bran and straw induced natural mediators and the concentration of these mediators increased with the length of fungus-cultivation, based on the previous observation that natural mediator concentration was positively related to their stimulatory effect on laccase-catalyzed anthracene oxidation (Fig. 3). The enhancement of anthracene oxidation rate by AU from GYB medium was higher than that from GYS medium (Fig. 4), implying that natural mediator concentration was higher in GYB medium than in GYS medium after fungus-cultivation. Therefore, we inferred that bran was more efficient in induction of mediators than straw. To our knowledge, this is the first report on the production of natural mediators by strain *Pleurotus ostriatus*.

Conclusions

In this work, we found that natural mediators were present in the extracellular culture fluid of strain *Pleurotus ostreatus* by determining the stimulatory effect of AU on the laccase-catalyzed anthracene oxidation. The stimulatory effect increased with increased natural mediator concentration. Both bran and straw could induce natural mediators and the yield of natural mediators increased with duration of fungal cultivation. Our findings suggested that this might be an alternative strategy to improve the biodegradability of laccase-producing fungi through enhancement of their natural mediator production. Natural mediators are preferred over artificial mediators due to lower costs and potentially fewer harmful environmental effects. However, further work is needed to identify these natural mediators and to determine the best methods to enhance their production for large-scale use.

Acknowledgments The work was financial supported by Foundation by Key Laboratory of Soil Environment and Pollution Remediation, Institute of Soil Science, Chinese Academy of Sciences and Zhejiang Provincial Natural Science Foundation of China (Y5110147).

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