Efficient remediation of 17 α -ethinylestradiol by *Lentinula edodes* (shiitake) laccase

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ABSTRACT

Removal of the endocrine disrupting chemicals (EDCs) which commonly pollute wastewater has been observed upon incubation with white-rot fungi. To date, there is no consensus on what is the optimal fungal source for the enzymes that catalyze remediation of EDCs. Herein, two white-rot fungi cultures (*Lentinula edodes* and *Phanerochaete chrysosporium*) were tested for an ability to remove the pollutant 17 α -ethinylestradiol (EE2). Mass spectrometry indicates a moderate removal of EE2 for simple potato dextrose cultures of both fungi within one week (50% EE2 remaining in *L. edodes*; 57% remaining in *P. chrysosporium*). Due to an absence of measured laccase activity from *P. chrysosporium*, it was hypothesized that the improved EE2 remediation in *L. edodes* was due to laccase. Supplementation of *L. edodes* cultures that improve production of laccase exhibit a corresponding increased ability to remove EE2 (20% EE2 remaining). *P. chrysosporium* cultures did not respond to supplementation accordingly (48% remaining). Consistent with laccase-specific remediation by *L. edodes*, incubations of EE2 in the presence of a purified laccase produced identical mass spectra to those observed from *L. edodes* cultures. The dominant soluble product observed for each has a mass consistent with hydroxylated EE2 (m/z 313). To our knowledge, this is the first demonstration of EE2 remediation by *L. edodes* culture. In addition, data indicate that the removal of EE2 is improved by increased production of the high-activity laccase secreted by the fungus.

Key words

Bioremediation – Shiitake – Laccase – Wastewater treatment – Pharmaceuticals

1. Introduction

Currently, wastewater treatment plants are not adequately designed to remove endocrine-disrupting chemical (EDC) pollutants, such as 17 α -ethinylestradiol (EE2) (Guttendorf and Westendorf, 2001). Some white rot fungi secrete enzymes capable of modifying these pollutants and removing the associated estrogenicity (Auriol *et al.*, 2008; Auriol *et al.*, 2007; Lloret *et al.*, 2010). While pure enzymatic degradation may be effective, use of fungal culture for these remediation applications is projected to be less expensive (Tsujiyama *et al.*, 2013). Therefore, it is of significant interest to identify fungal sources that produce enzymes capable of EDC removal (Roy-Arcand, *et al.*, 1991; Ullah *et al.*, 2000; Lloret *et al.*, 2013).

White rot fungi secrete three enzymes of interest: manganese peroxidase (MnP), lignin peroxidase (LiP), and laccase (Cabana, 2007). Laccase is preferable due to its use of dissolved oxygen as a second substrate, rather than hydrogen peroxide. Another advantage of laccase is its demonstrated broad substrate specificity (Xu *et al.*, 1996). Often, it is the redox potential of the active site type 1 (T1) copper ion, rather than geometric or electrostatic complementarity, that determines if laccase can catalyze the oxidation of a given substrate (Xu *et al.*, 1996). Fungal laccases exhibit a range of redox potentials (430 - 800 mV) (Baldrian P, 2006). Therefore, the optimal fungal source will produce a “high-activity” laccase with a high redox potential, capable of modifying (oxidizing) estrogenic pollutants.

Lentinula edodes (shiitake) is the most popular cultivated edible mushroom, yet its ability to specifically remove EE2 has not been reported. In addition, *L. edodes* is a known producer of laccase (Tsujiyama, *et al.*, 2013), yet whether *L. edodes* laccase is a high-activity laccase capable of EE2 modification has not been determined. Herein, liquid chromatography-mass spectrometry (LC-MS) is used to quantitate EE2 removal from cultures of *L. edodes* and *Phanerochaete chrysosporium*. The most efficient removal of EE2 from cultures has been observed in *L. edodes*, which (unlike *P. chrysosporium*) produces a high-activity laccase. Consistent with a laccase-specific EE2 removal, the incubation products extracted from culture display almost identical mass spectra to that obtained from incubations with

purified laccase. To our knowledge, this bioremediation application has not been demonstrated previously for *L. edodes* culture. Correspondingly, the data suggests that the removal of EE2 is improved by increased production of the high-activity laccase in *L. edodes*.

2. Materials and methods

2.1 Strain and Culture medium

Sterile liquid cultures of *L. edodes* (ATCC 28760) were commercially purchased from Out-Grow.com (McConnell, IL). *P. chrysosporium* (BKM-F-1767) was donated by the Center for Forest Mycology Research, USDA (Madison, WI). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

2.2 Incubation and Extraction

Sterile, stationary liquid (20 mL) cultures were prepared in petri dishes containing potato dextrose media (PD). Supplemented potato dextrose (PD+) contained 55 $\mu\text{mol L}^{-1}$ ammonium tartrate, 2.6 mmol L^{-1} L-asparagine and 180 $\mu\text{mol L}^{-1}$ copper sulfate (Cavallazzi *et al.*, 2005). Each trial contained three dime-sized plugs of the fungus of interest (cultured and cut from potato dextrose agar plates). After ten days of incubation in liquid culture at 25 °C, 10 mg mL^{-1} EE2 was added to each petri dish. Negative controls lacking fungus or EE2 were performed in parallel. Triplicate samples of each dish were taken one, three and seven days after the addition of EE2. Reaction solutions were extracted according to the protocol of Lee (Lee *et al.*, 2005). Briefly, solutions were extracted twice with a 1:1 ethyl acetate and hexane mixture. The aqueous layer was concentrated and re-suspended in the mobile phase (1:1 acetonitrile in 5 mmol L^{-1} ammonium acetate). Terfenadine (10 ng mL^{-1}) was added as an internal standard for mass spectral analysis.

Incubations (3 mL) of EE2 in the absence and in the presence of purified laccase from *Trametes versicolor* (Sigma) were composed of 50 mmol L^{-1} ammonium acetate buffer (pH 5), 1.75 units of *Trametes versicolor* laccase (estimated by dry weight from 0.5 U mg^{-1} bottle), 1 mmol L^{-1} EE2, and

deionized water. Sodium ions interfere with mass spectral analysis; therefore, ammonium acetate buffer was preferable for these incubations. These reactions were run over night at 25 °C in the dark but exposed to the air. Due to limited solubility in water, stock solutions of EE2 were suspended in pure ethanol.

2.3 Liquid Chromatography-Mass Spectrometry (LC-MS)

LC-MS analysis was performed at the Pharmaceutical Sciences Research Institute, McWhorter School of Pharmacy, Samford University. LC-MS-MS detection was performed using an Applied Biosystems 4000 QTRAP (Applied Biosystems, Foster City, CA) triple quadrupole mass spectrometer operated in the positive ion mode. Additional details of the protocol are provided in the supplementary material. EE2 in PD (or PD+) media from *L. edodes* and *P. chrysosporium* cultures was quantified by comparing the peak area ratio of the EE2 to the internal standard terfenadine (10 ng mL⁻¹) for each day of analysis. EE2 was expressed as the percent remaining each day as compared to the Day 1 value. Each sample was run in triplicate (n = 3).

2.4 Enzyme assay

Laccase activity was determined by monitoring the oxidation of 2, 6-dimethoxyphenol (DMP) to 2,2',6,6'-tetramethoxydibenzo-1,1'-diquinone at 470 nm ($\epsilon_{470\text{ nm}} = 49,600\text{ mol L}^{-1}\text{cm}^{-1}$) (Wariishi et al., 1992; Suzuki *et al.*, 2003). Reaction mixtures (1 mL) included 1 mmol L⁻¹ DMP, 50 mmol L⁻¹ sodium acetate buffer (pH 5) and 550 µL of laccase-containing culture. One unit of enzyme activity is defined as the amount of laccase needed to produce 1 µmol L⁻¹ quinone dimer product per minute in 1 mL of enzyme solution at 25 °C. Laccase activity assays were done in the presence of 0-40% v/v ethanol to determine the limits of activity with ethanol present. In order to maximize estrogen concentration and retain most of the enzyme activity, 30% v/v ethanol was used.

3. Results and discussion

3.1 *L. edodes* and *P. chrysosporium* cultures reduce EE2 levels

L. edodes fungal cultures were tested for the ability to remove the estrogen EE2. This was done in parallel with a white rot fungus (*P. chrysosporium*) known to possess this capability (Zhou *et al.*, 2015). EE2 was extracted from culture samples on day 1, 3 and 7, then quantitated using LC-MS relative to an internal standard. Cultures lacking fungus were used as controls for non-enzymatic degradation of EE2. The controls displayed no significant difference in EE2 levels within the first 7 days (Figure S1). Cultures containing fungi all exhibited the ability to remove EE2. In one week, EE2 levels were reduced to an average of 57% of its initial level (day 1) in *P. chrysosporium*, whereas *L. edodes* cultures had an average of 51% EE2 remaining (Figure 1). *L. edodes* is known to secrete significantly larger amounts of laccase than *P. chrysosporium* (Podgornik *et al.*, 2001). The slightly improved removal of EE2 observed for *L. edodes* cultures suggests that the *L. edodes* laccase was participating in remediation. Therefore, in order to test this hypothesis, both cultures were grown in media supplemented to upregulate laccase production (Cavallazzi *et al.*, 2005). To measure specific laccase activity for each culture on days in which EE2 levels were sampled, we employed an *in vitro* enzyme activity assay.

3.2 Determination of laccase activity in cultures

Aliquots of each culture were tested for laccase activity throughout the week. Rates were measured using the lignin analog 2, 6-dimethoxyphenol (DMP) as a substrate in an *in vitro* spectrophotometric enzyme activity assay (Wariishi *et al.*, 1992; Suzuki *et al.*, 2003). DMP modification by manganese peroxidase or lignin peroxidase would require stoichiometric amounts of MnSO_4 and peroxide to be present; therefore, this is an assay specific to laccase. *L. edodes* cultures exhibited an average of 0.079 units of laccase on day one, which corresponds to a rate of $2.6 \mu\text{M hr}^{-1}$ of quinone dimer product (Figure 2A). An average of 0.168 units of laccase was observed on day seven, which corresponds to a rate of $5.5 \mu\text{M hr}^{-1}$ of quinone dimer product (Figure 2B). The increase in units over the first week is attributed to the continual growth of the *L. edodes* culture. This corresponds to the improved removal of EE2 observed on day 7 in the *L. edodes* cultures (Figures 1, 3). *P. chrysosporium* produced approximately

5% of the laccase activity seen in *L. edodes*, which is within error of zero activity (Figure 2A, B). These data verify a strong presence laccase in the *L. edodes* cultures, when compared to the *P. chrysosporium*.

3.3 Improved EE2 removal upon increased units of laccase in *L. edodes* cultures

Previously, some inducers of laccase production were identified for *L. edodes* cultures (Cavallazzi *et al.*, 2005). Since improved degradation of other known environmentally hazardous compounds have been observed under these conditions (Tsujiyama *et al.*, 2013), we tested the effect of media supplementation (PD+) on EE2 removal for both *P. chrysosporium* and *L. edodes* (Figure 3A). The laccase activity assay was used to measure units of laccase activity in the cultures (Figure 3B).

Both fungal cultures displayed improved EE2 remediation in the supplemented media (PD+) (Figure 1 and Figure 3A). However, PD+ cultures for *P. chrysosporium* had no significant effect on EE2 removal from day 3 to day 7 ($n = 3$) (Figure 3A). Interestingly, the ability of *L. edodes* to remove EE2 was greatly enhanced in PD+ media. *L. edodes* reduced EE2 levels to an average of 20% its initial amount on day 1 ($n=3$) (Figure 3A). This is an increased reduction of EE2 by 30%, when compared to the remediation achieved in PD media (Figure 1). Correspondingly, use of PD+ media significantly improved the production of active laccase units to over twice that in simple PD cultures (Figure 3B). Together, these data are consistent with the hypothesis that the decrease in EE2 is a direct result of an increased presence of active laccase in *L. edodes* culture.

It is worth mentioning that the units of laccase produced from *L. edodes* culture (< 0.3 units per mL) are significantly less than the amount reported for some other fungal sources, such as *Trametes versicolor* (Tran *et al.*, 2010). Further optimization of culture conditions must be achieved before *L. edodes* is the optimal choice for the production of high-activity laccase. However, an efficient remediation of EE2 by *L. edodes* laccase is evidenced by the data herein. This capability is only attributable to high activity laccases (laccases with a high redox potential T1 copper ion). In this sense, the *L. edodes* is comparable to the better-studied *T. versicolor*.

3.4 Laccase-specific EE2 removal

To verify laccase-specific removal, EE2 was tested as a substrate for purified laccase from *Trametes versicolor* (TvLcc) (Sigma-Aldrich). A sequence alignment of TvLcc and *L. edodes* laccase show 66.5% identity and 87% similarity (Figure S2). The amino acid sequences for *L. edodes* and *T. versicolor* laccase were obtained from the National Center for Biotechnology Information (NCBI) database. TvLcc is a well-characterized, high redox potential laccase, capable of oxidizing estrogenic pollutants (Auriol et al., 2008; Blaquez, 2008). Incubations of EE2 in the presence of purified laccase produced mass spectra matching that produced from EE2 in the presence of *L. edodes* cultures. The primary product observed in the soluble fraction for each reaction mixture was identical (m/z 313), whereas controls lacking enzyme or fungi retained the EE2 peak (m/z 297) (Figure 4A). This product peak (m/z 313) has been reported previously and is proposed to be a hydroxylated form of EE2 (Lloret, 2013). In addition, collision-induced dissociation of the m/z 313 peak produced almost identical spectra for both the *L. edodes* cultures and incubations with purified laccase (Figure 4B, C). The most intense fragment for both conditions was m/z 123 (Figure 4B, C insert), whereas the predominant fragment for EE2 control was m/z 107 (Figure 4A insert). Again, a difference of m/z 16 was observed, which suggests a hydroxylated daughter ion. The exact location of hydroxylation was not determined by this work. The structure shown is one possibility, due to the stability afforded the radical at the ortho position once the phenoxy radical is generated. The mass spectral results show almost identical oxidation products for incubations of EE2 from *L. edodes* culture or with purified laccase, suggesting a laccase-specific contribution to the bioremediation of EE2 in *L. edodes* culture.

TvLcc is well-characterized as a “high-potential” laccase (800 mV) (Jönsson et al., 1995). The high level of homology for the *L. edodes* laccase with this enzyme is consistent with its demonstrated EE2 remediation. While *P. chrysosporium* is capable of EE2 removal, it does not produce laccase, the preferred ligninolytic enzyme. In addition, residual estrogenic activity from EE2 has been reported from *P. chrysosporium* cultures (Cajthaml et al., 2009). This finding illustrates the importance of product identification and characterization. The laccase-catalyzed oxidation products for a variety of pollutants are currently under investigation in our laboratory.

4. Conclusion

In conclusion, the goal of this work was to illustrate for the first time that *L. edodes* (shiitake mushroom) culture can be used for the bioremediation of EE2. Enzyme activity assays suggest that an increase in units of *L. edodes* laccase is primarily responsible for the improved bioremediation. According to the data, *L. edodes* laccase is a high-activity laccase capable of EE2 remediation. This application has not been reported previously for the *L. edodes* laccase.

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Declaration of interest

No conflict of interest declared.

References

- Auriol, M., Filali-Meknassi, Y., Adams, C.D., Tyagi, R.D., Noguerol, T.N., and Pina, B. (2008) Removal of estrogenic activity of natural and synthetic hormones from a municipal wastewater: Efficiency of horseradish peroxidase and laccase from *Trametes versicolor*. *Chemosphere*. 70, 445-452.
- Auriol, M., Filali-Meknassi, Y., Tyagi, R.D., and Adams, C.D. (2007) Laccase-catalyzed conversion of natural and synthetic hormones from a municipal wastewater. *Water Research*. 41(15), 3281-3288.
- Baldrian, P. (2006) Fungal laccases: occurrence and properties. *FEMS Microbiology Review*. 30, 215–242.
- Blanquez, P., and Guieysse, B. (2008) Continuous biodegradation of 17 β -estradiol and 17 α -ethynylestradiol by *Trametes versicolor*. *Journal of Hazardous Materials*. 150, 459-462.
- Cabana, H., Jones, J.P., and Agathos, S.N. (2007) Elimination of endocrine disrupting chemicals using white rot fungi and their lignin modifying enzymes: a review. *Engineering in Life Sciences*. 7, 429–456.
- Cajthaml, T., Kresinova, Z., Svobodova, K., and Moder, M. (2009) Biodegradation of endocrine-disrupting compounds and suppression of estrogenic activity by lignolytic fungi. *Chemosphere*. 75, 745-750.
- Cavallazzi, J., Kasuya, C.M., and Soares, M.A. (2005) Screening of inducers for laccase production by *Lentinula edodes* in liquid medium. *Brazilian Journal of Microbiology*. 36, 383-387.
- Gutendorf, B., and Westendorf, J. (2001) Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens. *Toxicology*. 166, 79–89.
- Jönsson, L., Sjöström, K., Haggström, I., and Nyman, P.O. (1995) Characterization of a laccase gene from white-rot fungus *Trametes versicolor* and structural features of basidiomycete laccases. *Biochimica et Biophysica Acta*. 1251, 210-215.

- Lee, S., Koo, B., Choi, J., Choi, D., An, B., Jeung, E., and Choi, I. (2005) Degradation of bisphenol A by white rot fungi, *Stereum hirsutum* and *Heterobasidium insulare*, and reduction of its estrogenic activity. *Biological and Pharmaceutical Bulletin*. 28(2), 201-207.
- Lloret, L., Eibes, G., Lu-Chau, T.A., Moreira, M.F., Feijoo, G., and Lema, J.M. (2010) Laccase-catalyzed degradation of anti-inflammatories and estrogens. *Biochemical Engineering Journal*. 51, 124-131.
- Lloret, L., Eibes, G., Moreira, M.T., Feijoo, G., and Lema, J.M. (2013) Removal of estrogenic compounds from filtered secondary wastewater effluent in a continuous enzymatic membrane reactor. Identification of biotransformation products. *Environmental Science & Technology*. 47, 4536-4543.
- Podgornik, H., Stegu, M., Zibert, E., and Perdih, A. (2001) Laccase production by *Phanerochaete chrysosporium* an artefact caused by Mn(III)? *Letters in Applied Microbiology*. 32: 407-411.
- Roy-Arcand, L., and Archibald, F.S. (1991) Direct dechlorination of chlorophenolic compounds by laccase from *Trametes (Coriolus) versicolor*. *Enzyme Microbiology and Technology*. 13, 194-203.
- Suzuki, K., Hirai, H., Murata, H. and Nishida, T. (2003) Removal of estrogenic activities of 17 β -estradiol and ethinylestradiol by ligninolytic enzymes from white-rot fungi. *Water Research*. 37: 1972-1975.
- Tran, N.H., Uruse, T., and Kasakabe, O. (2010) Biodegradation Characteristics of Pharmaceutical Substances by Whole Fungal Culture *Trametes versicolor* and its Laccase. *Journal of Water and Environmental Technology*, 8(2): 125-140.
- Tsujiyama, S., Muraoka, T., and Takada, N. (2013) Biodegradation of 2,4-dichlorophenol by shiitake mushroom (*Lentinula edodes*) using vanillin as an activator. *Biotechnology Letters*. 35, 1079-1083.
- Ullah, M.A., Bedford, C.T. and Evans, C.S. (2000) Reaction of pentachlorophenol with laccase from *Coriolus versicolor*. *Applied Microbiology and Biotechnology*. 53, 230-234.

- Wariishi, H., Valli, K., and Gold, M.H. (1992) Manganese (II) oxidation by manganese peroxidase from basidiomycete *Phanerochaete chrysosporium*, kinetic mechanism and role of chelators. *Journal of Biological Chemistry* 267, 23688-23695.
- Xu, F. (1996) Oxidation of Phenols, Anilines, and Benzenethiols by Fungal Laccases: Correlation between Activity and Redox Potentials as Well as Halide Inhibition. *Biochemistry* 35, 7608-7614.
- Xu, F., Shin, W., Brown, S.H., Wahleithner, J.A., Sundaram, U.M., and Solomon, E.I. (1996) A study of a series of recombinant fungal laccases and bilirubin oxidase that exhibit significant differences in redox potential, substrate specificity, and stability. *Biochimica et Biophysica Acta*. 1292, 303-311.
- Zhou, L., Luo, Q., Lu, J., and Huang, Q. (2015) Transformation of 17 β -estradiol by *Phanerochaete chrysosporium* in different culture media. *Bulletin of Environmental Contamination and Toxicology*. 95(2), 265-271.
- National Center for Biotechnology Information (NCBI) database.
<https://www.ncbi.nlm.nih.gov/protein/AET86512.1>

FIGURE LEGENDS

Figure 1. *L. edodes* and *P. chrysosporium* fungal cultures increase removal of EE2. EE2 in PD media cultures of *L. edodes* and *P. chrysosporium* was quantified by comparing the peak area ratio of the EE2 to the internal standard terfenadine (10 ng mL^{-1}) for each day of analysis. The average values of three cultures for each sample ($n = 3$) are expressed as the percent EE2 remaining from the amount measured on day 1. PD cultures that lacked fungus were monitored in parallel with the fungal cultures. The EE2 levels from these controls were used to normalize the data from fungal culture and control for non-enzymatic degradation. Error bars represent standard deviation.

Figure 2. *L. edodes* cultures secrete increased levels of active laccase compared to *P. chrysosporium*. Each 1 mL reaction mixture was composed of 1 mmol L^{-1} 2, 6-dimethoxyphenol (DMP), 50 mmol L^{-1} sodium acetate buffer (pH 5) and $550 \text{ }\mu\text{L}$ of media from the fungal culture. Oxidation of DMP to 2,2',6,6'-tetramethoxydibenzo-1-1'-diquinone was measured at 468 nm ($\epsilon_{468 \text{ nm}} = 49,600 \text{ M}^{-1}\text{cm}^{-1}$) over time at $25 \text{ }^{\circ}\text{C}$. The filled circles are *L. edodes*. The filled triangles are *P. chrysosporium*. (A) Day 1 of fungal cultures. (B) Day 7 of fungal cultures.

Figure 3. *L. edodes* cultures in supplemented media (PD+) exhibit increased laccase production and improved EE2 removal. A) EE2 in PD+ media cultures of *L. edodes* and *P. chrysosporium* was quantified by comparing the peak area ratio of the EE2 to the internal standard terfenadine (10 ng mL^{-1}) for each day of analysis. The average values of three cultures for each sample ($n = 3$) are expressed as the percent EE2 remaining from the amount measured on day 1. PD+ cultures that lacked fungus were monitored in parallel with the fungal cultures. The EE2 levels from these controls were used to normalize the data from fungal culture and control for non-enzymatic degradation. Error bars represent standard deviation. According to student's t test for day 7, $p = 0.10$. B) Average units of laccase activity

per mL of *L. edodes* culture in PD or PD+ media as measured using DMP as a substrate ($n = 4$).

According to student's t test for day 7, $p = 0.01$.

Figure 4. *L. edodes* cultures and purified laccase generate the same EE2 remediation products according to mass spectral analysis. The MS scans were done in positive ion mode, and protonated masses are reported. Product ion scans of A) EE2 incubated in the absence of *L. edodes* or purified laccase (control). The fragmentation pattern is shown here for an EE2 control sample (m/z 297). Insert – The structure of EE2 (peak observed m/z 297). The dashed lines indicate the proposed site of fragmentation due to collision-induced dissociation (CID) (produces ion product at m/z 107). B) EE2 after incubation with *L. edodes* culture. The fragmentation pattern is shown here for the most prominent soluble oxidation product (m/z 313). Insert – The structure of hydroxylated EE2 (peak observed m/z 313). The dashed lines indicate the proposed site of fragmentation due to CID (produces ion product at m/z 123). The site of oxidation (hydroxylation) has not been determined. The structure shown is one possibility. C) EE2 after incubation with purified laccase. The fragmentation pattern is shown here for the most prominent soluble oxidation product (m/z 313). The dashed lines indicate the proposed site of fragmentation due to CID (produces ion product at m/z 123). The site of oxidation (hydroxylation) has not been determined. The structure shown is one possibility.

Figure 1.

Figure 2.

Figure 3.

Figure 4

ESTROGEN

REMEDIATION

Shiitake
Laccase

O_2

H_2O