

Laccase Characterization and Discovering Metabolites of Laccase-Catalyzed Degradation of 17 α -Ethinylestradiol Using Fungi from Oak Mountain State Park

Samuel Beck¹ and Corey M. Johnson², Ph.D.

¹Louisiana State University, Baton Rouge, LA 70803; email: sbeck4@tigers.lsu.edu

²Department of Chemistry and Biochemistry, Samford University, Birmingham, AL 35229; email: cjohnso9@samford.edu

ABSTRACT

Endocrine disrupting chemicals (EDC's) are industrial and pharmaceutical pollutants, however some EDC's (estrogens) are naturally synthesized by humans and animals. EDCs can reach waterways through wastewater facility effluent since many treatment plants are not yet capable of removing these pollutants. Research has shown that white rot fungi have the ability to break down wastewater contaminants such as EDC's. These contaminants are broken down by lignolytic fungal enzymes, specifically lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase. Laccase has the advantage of catalyzing oxidation reactions with a concomitant reduction of oxygen to water. The use of oxygen as a second substrate (electron acceptor), rather than the peroxide required by MnP and LiP, makes laccase a more attractive enzyme for use in applications such as removal of wastewater contaminants. Even though removal of estrogenic activity in the presence of laccase has been demonstrated, little is known about the metabolites of laccase-catalyzed degradation of estrogens, and the mechanism of estrogen breakdown has not been determined. Likewise, there is not yet a consensus on what fungal source is the best producer of laccase. For this project, an optimal Oak Mountain State Park (OMSP) fungal source for laccase production was determined. Additionally, laccase from each source was purified and kinetically characterized with a natural substrate analog. We also began development of a new assay making use of a ruthenium-containing fluorescent probe to directly monitor the oxidation of estrogen. Finally, we identified the metabolites of laccase-catalyzed estrogen degradation. Turkey tail exhibited the best laccase activity. EE2 degradation resulted in the formation of dimers, trimers, tetramers, pentamers, and hexamers. We were unable to purify the cultured laccase.

INTRODUCTION

Estrogen, a steroid hormone, is an endocrine disrupting chemical (EDC) that can be found in wastewater effluents. Currently, wastewater treatment plants are designed to breakdown pathogens, waste particles, and nutrients, but do not effectively remove estrogen and other EDC's [1, 2]. Estrogen is metabolized by the body and excreted in an inactive, but water-soluble state [3, 4]. However, estrogens return to their active hormonal state in wastewater, possibly by exposure to microbial compounds [5-7]. Therefore, excess active estrogen is able to enter wastewater by means of mammalian excrement and improper disposal of pharmaceutical waste [8].

Exposure to external sources of estrogen and other endocrine disrupting chemicals through contaminated water can alter the natural processes of development, reproduction, and immunity in all types of animals, including humans [9, 10]. EDC's have lifelong effects during early developmental stages of offspring [11, 12]. When pregnant women are unknowingly dosed by low concentrations of EDC's, their children can experience severe developmental problems, including, but not limited to, brain sexual dimorphisms, prostate size, immune responses, male mammary gland development, thymus weight, and estrous cyclicity [13]. Concentrations as minute as 0.1 ng/L can be destructive to aquatic systems [14]. Studies in the U.K. [15] have shown estrogenic activity can continue several kilometers away from the sewage spillage site. Both male and female gudgeon (*Gobio gobio*) in close proximity to sewage sites had intersexual gonads and male fish acquired the female-specific yolk protein vitellogenin. [16]. Many other wildlife species have been disturbed or dislocated because of EDC's [17-21]. Therefore, EDC's have the potential to seriously alter human and wildlife physiology. Consequently, there is a great need to develop new methods for removal of estrogen and other EDC's from wastewater effluents.

White rot fungus naturally degrades lignin and is capable of degrading other stubborn chemicals and dyes by secreting the following three enzymes: manganese peroxidase (MnP), lignin peroxidase (LiP), and laccase [22]. Laccase is a multi-copper oxidase that was discovered in the lacquer tree in Japan [23], hence the enzyme's name. Since then, laccase has been found in bacteria [24], fungi [25, 26], and other plants [27]. It is capable of oxidizing polyphenols, methoxy-substituted phenols, diamines, and a number of other compounds. Laccase catalyzes oxidation reactions with a concomitant reduction of oxygen to water [28]. Interestingly, laccase is also known to eliminate the estrogenic activity of a variety of EDC's, including estrogen [29]. The use of oxygen as a second substrate (electron acceptor), rather than the peroxide required by MnP and LiP, makes laccase a more attractive enzyme for use in applications such as removal of wastewater contaminants.

This study was three pronged. First, an optimal Oak Mountain State Park (OMSP) fungal source for laccase production was determined. While previous studies in Dr. Denise Gregory's lab (Samford University) have shown the efficacy of laccase from white rot fungi in natural and synthetic estrogen degradation, the best fungal source has not been determined (Gregory lab; *unpublished*). OMSP is a rich source of fungal material. Turkey tail (*Trametes versicolor*) and portobello (*Agaricus bisporus*) from the park will be tested for laccase production in parallel to the laccase from shiitake (*Letinula edodes*). Shiitake is the leading producer of laccase activity identified to date (Gregory lab; *unpublished*). Identical cultures of the three white rot fungi were characterized by measuring mycelial growth, excreted protein concentration and laccase activity.

Second, the laccase from each source was purified and kinetically characterized with a natural substrate analog. While highly similar, the laccase from each source is a different enzyme with a different amino acid sequence, as shown by a multiple sequence alignment (Figure 1). Variation in sequence is often accompanied by variation in folded protein structure and function. Laccase isolated from an identical culture of each source was purified by a series of chromatographic steps. Laccase activity was measured and kinetic constants were determined.

In addition, the ability of each laccase to oxidize estrogen may differ substantially, and the mechanism of oxidation has not been fully elucidated. We began development of a new assay making use of a ruthenium-containing fluorescent probe to directly monitor the oxidation of estrogen. The fluorescence of this probe is quenched by oxygen, the other essential substrate of the laccase-catalyzed reaction.

Once optimized, the rate of oxygen depletion in the presence of estrogen will be measured directly for each fungal laccase. These kinetic data will provide information on the mechanism of estrogen oxidation for each laccase.

Third, we identified the metabolites of laccase-catalyzed estrogen degradation. While removal of estrogenic activity has been demonstrated (29), the metabolites of estrogen degradation have not been determined. It is essential that the product(s) of laccase treatment be identified to ensure that the degradation of estrogen does not result in an equally or more dangerous chemical. Incubations of highly concentrated, purified enzymes and estrogen were done under optimized conditions. The upscale production of the metabolites allowed identification by mass spectrophotometry analysis. Accomplishing the three aims of this study provides information applicable to EDC removal and essential to improving the methods of wastewater treatment facilities.

MATERIALS AND METHODS

Fungal Cultures

Samples of portobello, turkey tail, and shiitake collected from OMSP were cultured for 32 days on potato dextrose agar (PDA) at room temperature (~22°C). Mycelium for each fungal source was purchased from Out Grow (McConnell, IL) and cultured as positive controls. Three penny sized plugs were cut from each sample and placed into separate 100 mL liquid potato dextrose (PD) solutions with 2.6 mM L-asparagine and 55 µM ammonium tartrate [30]. A second batch of 100 mL liquid PD solution was made with 250 µL of copper sulfate added. The cultures remained at uncontrolled room temperature for 12 days. The samples were filtered and partially purified after the incubation period.

Filtration and Purification

The fruiting fungal bodies were separated from the solution by aseptically vacuum filtering through pre-weighed Fisherbrand® P5 filter paper [31]. The filter papers were dried in a Fisher Scientific Isotemp oven at 150° C after filtering and weighed to determine mycelial growth weight. The filtrate from each source was placed into Amicon® Ultra Centrifugal Filters with a molecular weight cut-off of 30 kDa. The centrifugal units were spun at 3000 x *g* in a Thermo REC MultiRF at 4° C for 15 minutes. Approximately one third of each cultured filtrate (CF) was set aside for kinetic assays. The remaining CF was further purified through ion-exchange and gel filtration at 4° C (32).

The DEAE-Sepharose® (Sigma Aldrich) resin used for ion exchange was rinsed using 100 mL of 50 mM sodium acetate buffer (pH 5). The high salt buffer consisted of 500 mM sodium chloride in 50 mM sodium acetate (pH 5). The low salt buffer consisted of 50 mM sodium acetate (pH 5). The purified filtrate was collected in approximately 5 mL fragments. Established activity assays (protocol below) were used to determine which fragments contained laccase. Sephadex® G-100 (Sigma Aldrich) was used for gel filtration. The filtrate was collected in approximately 3 mL fragments. The same activity assays were performed to find the fragments with laccase. The fragments after gel filtration with laccase were again concentrated using Amicon® Ultra Centrifugal Filters with the same molecular weight cut-off. The total protein content of each CF and purified filtrate was determined according to Bio-Rad Protein Assay,

derived from the Bradford assay [34]. A SDS page was run to determine the purity of laccase in the final turkey tail and shiitake purified filtrate.

Laccase Assays

Turkey tail and portobello laccase from Sigma Aldrich were purchased and used as positive controls for the laccase activity assay. The more active turkey tail was used to optimize assay conditions. We used a Cary 50 Bio UV/Vis Spectrophotometer at 470 nm and 24.9° C to detect the oxidation of 2,6 dimethoxyphenol (DMP). The reaction mixture was composed of five different concentrations of DMP (0.020-1 mM), 50 mM sodium acetate (pH 5), 1.563 and 3.125 nM laccase, and distilled water [35]. The varying concentrations of the substrate DMP were assayed and the formation of the oxidized diquinone product, 3,3',5,5'-tetramethoxy-biphenyl-4,4'-diol, was monitored at 470 nm ($\epsilon_{470} = 22 \text{ mM}^{-1} \text{ cm}^{-1}$) over time in triplicate. Kinetic constants were determined. Two negative controls were used to ensure that activity was only the result of the laccases' oxidation of DMP. One negative control lacked laccase, while the other lacked DMP. The purchased laccase was stored at pH 5 in sodium acetate buffer and at pH 7 in phosphate. Storage conditions were optimized for solubility and activity.

CF (500 μL) from each fungal source was assayed using the same method with the exception of the purchased laccase. The activity of each laccase was determined after concentrating each by centrifugation (partial purification) and after the chromatographic steps (full purification).

Estrogen Degradation Assay

We attempted to create a new assay to detect the degradation of estrogen by sensing the luminescence of tris(2,2'-bipyridyl)ruthenium(II) ($\text{Ru}(\text{bpy})_3^{2+}$) purchased from Sigma Aldrich. Oxygen is an excellent quencher of the emission of $\text{Ru}(\text{bpy})_3^{2+}$. When laccase oxidizes DMP or estrogen, the other substrate, O_2 , is reduced to $2\text{H}_2\text{O}$. Therefore, by measuring the extent to which $\text{Ru}(\text{bpy})_3^{2+}$ is quenched at a given point in the oxidation reaction, the oxygen concentration at that point is determined [35, 36].

Optimal instrumental conditions for detecting the fluorescence of $\text{Ru}(\text{bpy})_3^{2+}$ were first found by measuring the fluorescence at its strongest point, when oxygen is absent. A sample of 10 μM $\text{Ru}(\text{bpy})_3^{2+}$, 40 mM sodium acetate buffer (pH 5), and distilled water were placed into a QS 10 mm quartz cuvette and sparged with nitrogen for 5 minutes. A cap was used to slow the reabsorption of oxygen by the environment. The cuvette was inserted into a Cary Eclipse Fluorescence Spectrophotometer to run a prescan. The excitation and emission wavelength was 285.93 nm and 610.86 nm, respectively.

To test the method in the presence of laccase, we first used DMP as the substrate. The reaction mixture was composed of 10 μM $\text{Ru}(\text{bpy})_3^{2+}$, 1 mM DMP, 40 mM sodium acetate buffer (pH 5), and distilled water. The mixture was first sparged with oxygen for five minutes and then run in the spectrophotometer to establish a baseline. After two minutes, 1.5625 nM turkey tail laccase from Sigma Aldrich was added to the reaction mixture. Reactions ran for 15 minutes.

An estrogen, 17 α -Ethinylestradiol (EE2; Sigma Aldrich) was also tested. Due to limited solubility in water, EE2 (0.3 mM) was resuspended in pure ethanol. Laccase activity assays and controls were

optimized for ethanol content. Assays were performed with increasing amounts of ethanol to ensure the enzyme would remain active. The reaction mixtures were composed of 0-40% ethanol, 50 mM sodium acetate, 1.5625 nM turkey tail laccase (Sigma), 1 mM DMP, and distilled water.

Determining the Metabolites of Laccase-catalyzed EE2 Oxidation

The reaction mixture was composed of 50 mM ammonium acetate (pH 5), 0.3 mM EE2, 50 nM Turkey Tail laccase (Sigma), and distilled water. The solution contained 30% ethanol. A control reaction was made that lacked laccase. The samples sat at room temperature for approximately 24 hours. The sample was vacuum filtered through Whatman™ #1 filter paper. The filter paper was allowed to dry at room temperature. After approximately 24 hours, samples were centrifuged in 1.5 mL Eppendorf Flex-Tubes® using an Eppendorf Centrifuge 5418 at 12,000 rpm for 1 minute. These samples were then placed into Amicon® Ultra Centrifugal Filters with a molecular weight cut-off of 30 kDa. The centrifugal units were spun at 3000 x *g* in a Thermo REC MultiRF at 4° C for 25 minutes to remove laccase. The filtrate was run through a mass spectrophotometer (MS). Precipitated product from the reaction was re-dissolved in 1 mL pure ethanol. This was also subjected to MS analysis. The control reaction solution (EE2 with no enzyme) was run through the MS as well.

RESULTS

Positive Control Laccase

The turkey tail laccase (Sigma) was used as a positive control. We were able to graphically estimate kinetic constants (Table 1) using a Lineweaver-Burk plot (Figure 2a-2b). Finding the k_{cat} and K_m is useful because it has been found that the $\log(k_{cat}/K_m)$ is proportional to the redox potential of the enzyme (28). The redox potential of laccase is directly correlated to how well it can oxidize a substrate. As expected, no activity occurred during the two negative controls. Figures 3a and 3b show the difference in activity between laccase stored at pH 7 in phosphate buffer and laccase stored at pH 5 in sodium acetate. Five activity assays were run at each pH, all displayed higher activity when stored at pH 7. While laccase is optimally active at pH 5, increased activity was observed when the enzyme was stored at pH 7.

Culture Filtrate

The mycelial growth weight was measured for each 100 mL culture. The difference in weight of the filter papers from before and after filtration were averaged from three samples each. The portobello culture produced the most mycelium of the three fungi (Figure 4). The total protein content of each CF was found using the standard Bio-Rad Protein assay. Portobello had the highest protein content of all the fungi (Figure 5).

The culture filtrate from each source was assayed for activity. We graphically estimated the kinetic constants (Table 2) using a Lineweaver-Burk plot (Figures 6a-6b). Only V_{max} and K_m were estimated for the partially purified culture filtrates. Turkey tail CF produced the most laccase activity.

Purification

Upon vacuum filtration through filter paper, the final volume of filtrate was approximately half of the original volume. After the cultures were spun in the centrifugal filters, approximately 24-28 mL of turkey tail and shiitake CF remained, a 6-fold reduction in volume. The portobello filtrate did not filter as well. Only 45 mL of CF were recovered from portobello filtrate. Two chromatographic steps concentrated the enzymes. After flowing through the ion-exchange resin, approximately 76% of the original volume of turkey tail CF remained. Likewise, 20% of the portobello CF and 62.5% of the initial volume of shiitake CF remained. Approximately 38-45% of turkey tail and shiitake remained after gel filtration. The portobello laccase was lost completely in this step. No visible enzyme activity was observed upon screening of the fractions. Following the second centrifugation, final volumes of 200 μ L of turkey tail and 500 μ L of shiitake purified filtrate remained and was stored at 4°C.

Estrogen Degradation Assay

In order to measure the rate of laccase oxidation of EE2, conditions were optimized for EE2 solubility and enzyme activity. EE2 solubility was increased with ethanol content. Laccase activity tests with pure ethanol ranging from 0 - 40% in solution were performed (Figures 7a-7d). Based on the results of these tests, final ethanol concentration in the reactions with EE2 was 30%. Reaction solutions contained 1 mM DMP, 50 mM sodium acetate (pH 5), 1.5625 nM turkey tail laccase (Sigma), ethanol, and distilled water. Compared to controls, no detectable change in fluorescent signal (oxygen concentration) was observed compared to controls. UV/vis assays verified active enzyme and DMP oxidation. This assay is still being optimized.

Metabolite Identification

The control reaction that was run through the MS had a high peak at 296 m/z, the molecular weight of EE2. The reaction solution that ran through the centrifugal filters showed evidence of a dimer at 590 m/z and a high peak at 312 m/z (Figure 8). The precipitate clearly showed dimers at 590 m/z and trimers at 885 m/z (Figure 9). Lower peaks were also found, most likely tetramers at 1178 m/z, pentamers at 1497 m/z, and hexamers at 1769 m/z (Figure 9).

DISCUSSION

The activity assays run with the positive control turkey tail laccase (Sigma) provided results of high accuracy. Rogalski and Höfer reported the same K_m value when turkey tail oxidizes DMP [37, 38]. As expected, the V_{max} was cut in half when the concentration of laccase was halved. Also, the $\log(k_{cat}/K_m)$ was the same for both concentrations. This was an internal control and an anticipated result. The redox potential of the enzyme should not change with the concentration. Overall, the positive control presented reliable data that gives verification to the assay method used. It was also found that it is

better to store the turkey tail laccase (Sigma) in phosphate buffer (pH 7) instead of in sodium acetate (pH 5). The laccase is better able to retain its activity.

Turkey tail laccase was the most active of all the CF laccases tested versus DMP. This is contradictory to our hypothesis and previous results (Gregory lab; *unpublished*). Whether its high activity toward DMP is reflective of its ability to oxidize estrogen is currently under investigation. Turkey tail had the highest maximum velocity and the lowest K_m value. An unpurified laccase would be a cheap method for use in wastewater facilities. It would save cost and time. Turkey tail may be a good source of laccase to use in such a situation. Its inherently low K_m value allows it to oxidize substrates easily in low concentrations. Portobello CF did not indicate laccase activity with DMP as the substrate in ten minute reactions. However, when the reaction mixture was allowed to sit for 24 hours, the solution would change from clear to yellow, indicating laccase activity. The amount of mycelial growth weight and total protein content did not correspond to laccase activity.

We believe the thick mycelial growth of the portobello solution inhibited its ability to be filtered in the centrifugal units, even after vacuum filtering through filter paper. Further studies should centrifuge the portobello filtrate to form a pellet, then carefully transfer the liquid to the centrifugal units. It is unclear why the portobello laccase was lost after gel filtration. No bands were formed when the laccase from the purified turkey tail and shiitake was run on a SDS page. Without knowing the laccase concentration, k_{cat} could not be determined, and therefore the redox potential of each enzyme could not be estimated. Continuation of this research should include more attempts to purify laccase from upscaled cultures.

The estrogen degradation assay remained unsuccessful, but largely because of time constraints. Further studies should not disregard the importance of the assay, and should take more time in developing the assay. For further testing, enzyme concentration and reaction time should be increased. Also, varying concentrations of the ruthenium complex in reaction need to be tested. Identification of kinetic constants using estrogenic compounds as substrates will quickly determine which laccase has the highest efficacy for degradation and usefulness for treatment facilities. Although laccase continued to display decent activity in 30% ethanol reaction solution, activity was decreased. Further studies should test different methods for dissolving estrogen into solution that is less disadvantageous towards laccase activity.

It is imperative to discover the metabolite of EE2 after it is degraded by laccase. EE2 could be degraded into a substance that is just as harmful. We hypothesized that a precipitate would form after EE2 is degraded due to the formation of a dimer. One of the mechanisms of laccase is radical coupling. DMP undergoes the same type of degradation when it forms a diquinone and precipitates out of solution. The EE2 and laccase incubations exhibited a white precipitate after sitting overnight. The control reaction did not have any precipitate. When we first tried to use vacuum filtration to separate the precipitate, we were unable to scrape the precipitate off the filter paper. We then tried to centrifuge the samples to form a pellet. However, the precipitate went back into solution after centrifugation. We had to put the reaction solution into centrifugal filters to separate the laccase. The solution that went through the filter and the control reaction was run through the MS. The control reactions peak at 296s m/z, the molecular weight of EE2, ensured we had the correct settings for the solutions. The filtrate solution displayed a high peak at 312 m/z. This could be the soluble, hydroxylated derivative of EE2. Lloret et al. reported the same molecule upon EE2 degradation with laccase [39]. A

small peak was also formed at 592 m/z, proof of dimer formation. However, novel metabolites were identified from the re-dissolved precipitate product. The white precipitate that forms upon EE2 degradation has a high tendency to stick to surfaces. The inside of the pipette tip used for transferring the reaction solution had a white film on the inside. We cleaned it out with ethanol and ran it through the MS. High peaks formed at 592 m/z and 884 m/z, are consistent with the formation of dimers and trimers. Smaller peaks were also found consistent with the formation of tetramers, pentamers, and hexamers. To our knowledge, this is the first time anyone has observed polymers of this size for laccase-catalyzed oxidation of estrogen. The mechanistic details of this novel finding are currently under investigation.

CONCLUSIONS

The kinetic assays used to characterize each laccase proved to be reliable from the kinetic constants that were estimated using the control turkey tail laccase (Sigma). We were able to estimate kinetic constants from the CF, but we failed in purifying the laccase from the cultures. Turkey tail CF was the most active and may be most beneficial for wastewater treatment facilities. Further studies should make sure to purify each laccase in order to find the $\log(k_{cat}/K_m)$. Knowing this number would give insight to the redox potential of each laccase, and better help in the determination of the best laccase to use in degradation methods. The amount of mycelial growth and total protein content did not relate to a higher production of laccase. Assaying estrogen degradation using the fluorescence of a ruthenium complex was unsuccessful. However, more time and consideration should be put into the conception of the assay. Estrogen assays would be a significant step towards improving biodegradation techniques of EDC's. The metabolites of EE2 after degradation by laccase were found. EE2 degradation resulted in the formation of dimers, trimers, tetramers, pentamers, and hexamers. Continued studies should test the metabolites for toxicity.

REFERENCES

- [1] Tabak HH, Bloomhuff RN, Bunch RL. 1981. Steroid hormones as water pollutants II. Studies on the persistence and stability of natural urinary and synthetic ovulation-inhibiting hormones in untreated and treated wastewaters. *Dev Ind Microbiol* **22**: 497–519.
- [2] Snyder SA, Keith TL, Verbrugge DA, Snyder EM, Gross TS, Kannan K, Giesy JP. 1999. Analytical methods for detection of selected estrogenic compounds in aqueous mixtures. *Environ Sci Technol* **33**: 2814–2820.
- [3] Andreolini F, Borra C, Caccamo F, Di Corcia A, Samperi R. 1987. Estrogen conjugates in late-pregnancy fluids-Extraction and group separation by a graphitized carbon-black cartridge and quantification by high-performance liquid-chromatography. *Anal Chem* **59**: 1720–1725.

- [4] Guengerich FP. 1990. Minireview: Metabolism of 17 α -ethynylestradiol in humans. *Life Sci* **47**: 1981–1988.
- [5] Belfroid AC, Van der Horst A, Vethaak AD, Schäfer AJ, Rijs GBJ, Wegener J, Cofino WP. 1999. Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in The Netherlands. *Sci Total Environ* **225**: 101–108.
- [6] Ternes TA, Kreckel P, Müller J. 1999. Behaviour and occurrence of estrogens in municipal sewage treatment plants-II. Aerobic batch experiments with activated sludge. *Sci Total Environ* **225**: 91–99.
- [7] Panter GH, Thompson RS, Beresford N, Sumpter JP. 1999. Transformation of a non-oestrogenic steroid metabolite to an oestrogenically active substance by minimal bacterial activity. *Chemosphere* **38**: 3579–3596.
- [8] Bound JP, Voulvoulis N. 2005. Household disposal of pharmaceuticals as a pathway for aquatic contamination in the United Kingdom. *The National Institute of Environmental Health Sciences* **113**: 1705-1711.
- [9] Gray LE, Ostby J, Ferrell J, Rehnberg G, Linder R, Cooper R, Goldman J, Slott V, Laskey J. 1989. A dose-response analysis of methoxychlor-induced alterations of reproductive development and function in the rat. *Fundam Appl Toxicol* **12**: 92-108.
- [10] Guzelian PS. 1982. Comparative toxicology of chlordecone (kepone) in humans and experimental animals. *Annu Rev Pharmacol Toxicol* **22**: 89-113.
- [11] Gray LE. Chemical-induced alterations of sexual differentiation: a review of effects in humans and rodents. 1992. In: Chemically induced alterations in sexual and functional development: the wildlife/human connection (Colborn T, Clement C, eds). Princeton, NJ: *Princeton Scientific Publishing*, 203-230.
- [12] Petersen RE, Theobald HM, Kimmel GL. 1993. Developmental and reproductive toxicity of dioxins and related compounds: cross species comparisons. *Crit Rev Toxicol* **23**: 283-335.
- [13] Melnick R, Lucier G, Wolfe M, Hall R, Stancel G, Prins G, Gallo M, Reuhl K, Ho S M, Brown T, Moore J, Leakey J, Haseman J, Kohn M. 2002. Summary of the National Toxicology Program's report of the endocrine disruptors low-dose peer review. *Environ Health Perspect* **110**: 427–431.
- [14] Auriol M, Filali-Meknassi Y, Tyagi, RD, Adams CD, Surampalli R Y. 2006. Endocrine disrupting compounds removal from the wastewater treatment plant, a new challenge. *Process Biochem* **41**: 525–539.
- [15] Rodgers-Gray T, Jobling S, Morris S, Kelly C, Kirby S, Janbakhsh A, Harries J, Waldock M, Sumpter J, Tyler C. 2000. Long-term temporal changes in the estrogenic composition of treated sewage effluent and its biological effects on fish. *Environ Sci*

Technol **34**: 1521-1528.

[16] Jones PD, De Coen WM, Tremblay L, Glesy JP. 2000. Vitellogenin as biomarker for environmental estrogens. *Water Science and Technology* **42**: 1-14.

[17] Moccia R, Fox G, Britton AJ. 1986. A quantitative assessment of thyroid histopathology of herring gulls (*Larus argentatus*) from the Great Lakes and a hypothesis on the causal role of environmental contaminants. *J Wild Dis* **22**: 60-70.

[18] Shugart G. 1980. Frequency and distribution of polygony in Great Lakes herring gulls in 1978. *Condor* **82**: 426-429.

[19] Gibbs PE, Pascoe PL, Burt GR. 1988. Sex change in the female dog-whelk, *Nucella lapillus*, induced by tributyltin from antifouling paints. *J Mar Biol Assoc UK* **68**:715-731.

[20] Reijnders PJH. 1986. Reproductive failure in common seals feeding on fish from polluted coastal waters. *Nature* **324**: 456-457.

[21] Bishop CA, Brooks RJ, Carey JH, Ng P, Norstrom RJ, Lean DRSJ. 1991. The case for a cause-effect linkage between environmental contamination and development in eggs of the common snapping turtle(*Chelydra s. serpentina*) from Ontario, Canada. *J Toxicol Environ Health* **33**: 521-548.

[22] Wesenberg D, Kyriakides I, Agathos S. 2003. White-rot fungi and their enzymes for the treatment of industrial dye effluents. *Biotechnology Advances*. **22**: 161-187.

[23] Yoshida, H. 1883. Chemistry of Lacquer. *Journal of the Chemical Society (Tokyo)* **43**, 472-486.

[24] Givaudan A, Effosse A, Faure D, Potier P, Bouillant , Bally R. (1993). Polyphenol oxidase from *Apospirillam lipoferum* isolated from the rhizosphere: evidence for a laccase in non-motile strains of *ATospirillzlm lipoferum*. *FEMS Microbiol Lett* **108**: 205-210.

[25] Eggert C, Temp U, Eriksson KE. 1996. The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: purification and characterization of the laccase. *Appl. Environ. Microbiol* **62**: 1151-1158.

[26] Solomon EI, Sundaram UM, Machonkin TE. 1996. Multicopper oxidases and oxygenases. *Chem Rev* **96**: 2563–2605.

[27] Harvey BM, Walker JRK. 1999. Studies with plant laccases: I. Comparison of plant and fungal laccases. *Biochem Mol Biol Biophys* **3**: 45–51.

- [28] 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.
- [29] Auriol M, Youssef FM, Adams C, Rajeshwar T, Noguero TN, Pina B. 2007. 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.
- [30] Cavallazzi J, Kasuya C, Soares M. 2005. Screening of Inducers for Laccase Production by *Lentinula edodes* in Liquid Medium. *Brazilian Journal of Microbiology* **36**: 383-387.
- [31] Praveen K, Viswanath B, Usha KY, Pallavi H, Reddy GVS, Naveen M, Reddy BR. 2011. Lignolytic Enzymes of a Mushroom *Stereum ostrea* Isolated from Wood Logs. *Enzyme Research* **2011**. 1-6.
- [32] Rittstieg K, Suurnakki A, Suortti T, Kruus K, Guebitz G, Buchert J. 2002. Investigations on the laccase-catalyzed polymerization of lignin model compounds using size-exclusion HPLC. *Enzyme and Microbial Technology* **31**: 403-410.
- [33] Wariishi H, Valli K, Gold MH. 1992. Manganese (II) oxidation by manganese peroxidase form basidiomycete *Phanerochaete chrysosporium*, kinetic mechanism and role of chelators. *Journal of Biological Chemistry* **267**: 88-95.
- [34] Cuber M, Demas JN, Bare WD, Pham CV. 2007. An improved method for studying the enzyme-catalyzed oxidation of glucose using luminescent probes. *J. Chem. Educ.* **84**: 1511.
- [35] Bateman RC Jr., Evans JA. 1995. Using the glucose oxidase/peroxidase system in enzyme kinetics. *J. Chem. Educ.* **72**: A240.
- [36] Bradford M. 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Biochimica et Biophysica Acta* **533**: 525.
- [37] Rogalski J, Dawidowicz AL, Leonowicz A. 1990. Purification and immobilization of the inducible form of extracellular laccase of the fungus *Trametes versicolor*. *Acta Biotechnol* **10**: 261-269.
- [38] Höfer C, Schlosser D. 1999. Novel enzymatic oxidation of Mn^{2+} to Mn^{3+} catalyzed by a fungal laccase. *FEBS Lett* **451**: 186-190.
- [39] Lloret L, Eibes G, Moreira MT, Feijoo G, Lema JM. 2013. Removal of estrogenic compounds from filtered secondary wastewater effluent in a continuous enzymatic membrane reactor. Identification of biotransformation products. *Environ. Sci. & Technol.* **47**: 4536-4543.

```

gi|289099|gb|AAC18877.1|      --MRLSNALVLAACISSVAKTRTFDFDLVNRRLAPDGFERDVTVINGEFPPTLIQVNK 58
gi|357594898|gb|AET86512.1|  --MFYFSSFLLLGPIGGALAAIGPVTDLHIVNSFIQPDGFNRSGLAEGIFPGPLITGNK 58
gi|390980641|gb|AFM31222.1|  MGLQRFSFFVTLALVARSLAAIGPAASLVVANAPVSEDFGLRDAIVVNGVFPSPLIITGNK 60
      : . : : . : : * . : : : * : : * * * * * . : : * * * * * * *
      : . : : . : : * . : : : * : : * * * * * . : : * * * * * * *

gi|289099|gb|AAC18877.1|      GDSVRIPLHNKLTSPTRRSVSIHWHGFFQARTSGQDGPFSFVNQCPQPPNITFTYEFSSVA 118
gi|357594898|gb|AET86512.1|  GDSFQINVIDELTNGTMLLSTSIHWHGLFQKTTNWADGPAFVNQCPIAANDSFLYNFNVP 118
gi|390980641|gb|AFM31222.1|  GDRFQLNVVDTLTNHTMLKSTSIHWHGFFQAGTNWADGPAFVNQCPIASGHSFLYDFHVP 120
      * * . : : : * * * * * . : : * * * * * * * . : : * * * * * * *

gi|289099|gb|AAC18877.1|      EQSGTFWYHSHLSTQYCDGLRGAFIVYDPRDPLRHLYDVDEESTVITLAEWYHILAPDAT 178
gi|357594898|gb|AET86512.1|  DQAGTFWYHSHLSTQYCDGLRGPLVYDQDPYVDLYDVDDSTVITLADWYHVPAPQAG 178
gi|390980641|gb|AFM31222.1|  DQAGTFWYHSHLSTQYCDGLRGPVVYDPKDPHASRYDVDNESTVITLTDWYHTAARLGP 180
      : * : * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

gi|289099|gb|AAC18877.1|      NEFFSSGIIIPVQDGLINGKGRFNGGPL-TPFAVVNVRGKRYRLRVIAISCRPFPTFSV 237
gi|357594898|gb|AET86512.1|  AVP-----TSDATLINGLGRSVNGPADAPFAVVNVRGSRYRFRLVISCDPNLFSI 231
gi|390980641|gb|AFM31222.1|  KFP-----LGADATLINGLGRSASTPT-AALAVINVQHGKRYRFRLVISCDPNYTFSI 233
      * : * * * * * * * * * * * * * * * * * * * * * * * * * * *

gi|289099|gb|AAC18877.1|      DNHSLVMEADGVEHDFVEVQNVDIYAAQRVSVILHANQPIDNYWIRAPMTGGNPDNRPN 297
gi|357594898|gb|AET86512.1|  DGHTFTVIEADGVNHEPIVADSIIQIFAAQRYSFILTANQTADNYWIRANPNNGHTG---- 287
gi|390980641|gb|AFM31222.1|  DGHNLTVIEVDGINSQPLIVDSIIQIFAAQRYSFVLNANQTVGNVWVRANPNFGTVG---- 289
      * * . : : * * * * * : : * : : * * * * * * * * * * * * * * * *

gi|289099|gb|AAC18877.1|      LNIISLTAILRYHGARGHVEPTTVNVPGHKLL-DQEMHPIRQE-GPGKLGDPDPKHITLN 355
gi|357594898|gb|AET86512.1|  FAGGINSAILRYSGAPVADEPTTQTSAN-LLQETSILVPRENPGAPGNATANGVNDLNLV 346
gi|390980641|gb|AFM31222.1|  FAGGINSAILRYQGAPIAEPPTTQTPSVIPLIETNLHPLARMPVPGSPTPGGVDKALNLA 349
      : . : . * * * * * * * . : * * * * * * * . : : * * * * * * *

gi|289099|gb|AAC18877.1|      IAQPNAPFFDINGISYISPTVPVLLQILSGAKRPEDVLPSEQIFFVFPKNSLIEVNIPG-- 413
gi|357594898|gb|AET86512.1|  LS-FVGGRFEEINGVSVFPPVTVVLLQILSGATTAAELLPSSVYTLPLNSVIQLSFTNVA 405
gi|390980641|gb|AFM31222.1|  FN-FNGTINFFINNATFTPTTVVLLQILSGAQIAQDLLPAGSVYPLPAHSSIEITLPATA 408
      : . * * * * : * * * * * * * * * * * * * * * * * * * * * *

gi|289099|gb|AAC18877.1|      ---EGAHPFHLHGHNFDVVLASNDDTFNFKNPPRRDVYP---INGGNITFRFFTDNPG 465
gi|357594898|gb|AET86512.1|  VAAVGGPHPFHLHGHTFDVVRSAGSTYNYINPPRRDVVSTGA--AIDNVTIRFTTDNAG 463
gi|390980641|gb|AFM31222.1|  -LAPGAPHPFHLHGHAFAVVRSAGSTITYNYNDPIFRDVVSTGTAAAGDNVTIRFQTDNPG 467
      . * * * * * * * * * * : : * : * * * * * * * * * * * * *

gi|289099|gb|AAC18877.1|      AWFLHCHIDWHLEAGLAIVFAEAPEDNVSGPQSQITPQDWLDCPEYNAIEPEFQ--- 520
gi|357594898|gb|AET86512.1|  PWFLHCHIDWHLEAGFAIVFAEDAPDVA---AVNPVVDANQLCPTYDALTPAQLGNN 518
gi|390980641|gb|AFM31222.1|  PWFLHCHIDFHLDAAGFAIVFAEDVADVK---AANPVPKAWSDLCPIYDGLSEANQ--- 519
      * * * * * * * * * * * * * * * * * * * * * * * * * * * *

```

Figure 1. Multiple sequence alignment of laccase from portobello (*Agaricus bisporus*; row 1), shiitake (*Letinula edodes*; row 2) and turkey tail (*Trametes versicolor*; row 3). Conserved residues are indicated by *. Hypervariable residues have no mark. Sequences were obtained from ncbi.nlm.nih.gov and the alignment was generated using clustal2.1 (EBI, 2014).

Table 1: Kinetic constants derived from a Lineweaver-Burke plot. The laccase was turkey tail purchased from Sigma.

Laccase Concentration (<i>nM</i>)	V_{\max} (<i>min</i> ⁻¹)	K_m (<i>mM</i>)	k_{cat} (<i>s</i> ⁻¹)	$\log(k_{\text{cat}}/K_m)$ (<i>s</i> ⁻¹ <i>mM</i> ⁻¹)
3.125	0.6708	0.013	16.266	3.09
1.563	0.3237	0.015	15.698	3.02

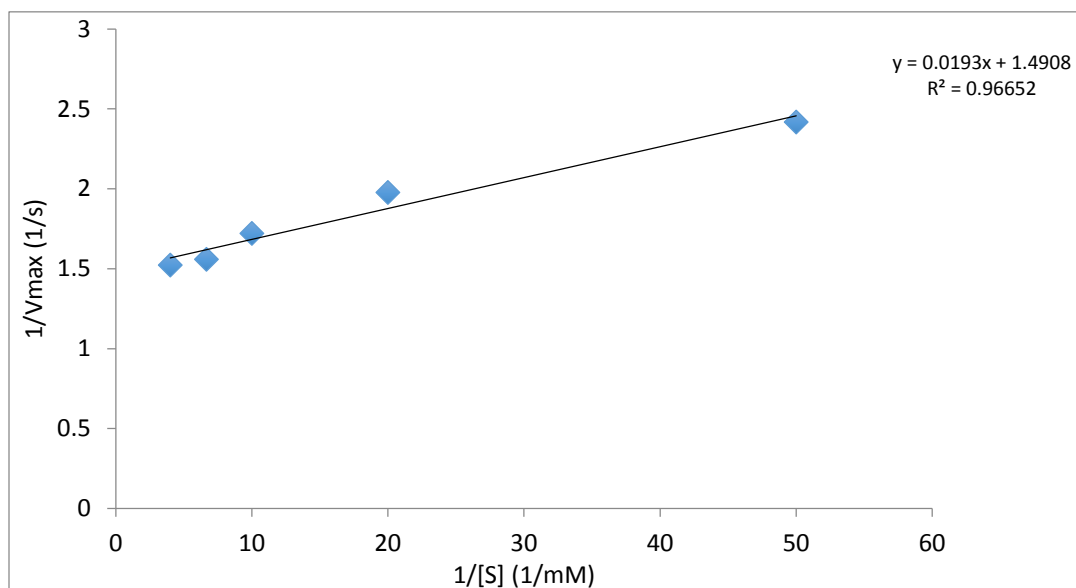


Figure 2A. Lineweaver-Burk plot for turkey tail laccase (Sigma). Laccase concentration was 3.125 nM. Concentrations of DMP used were 0.02, 0.05, 0.1, 0.15, and 0.25 mM. Each of the assays were done in triplicate and averaged. The linear trendline generates an equation that was used to derive the kinetic constants.

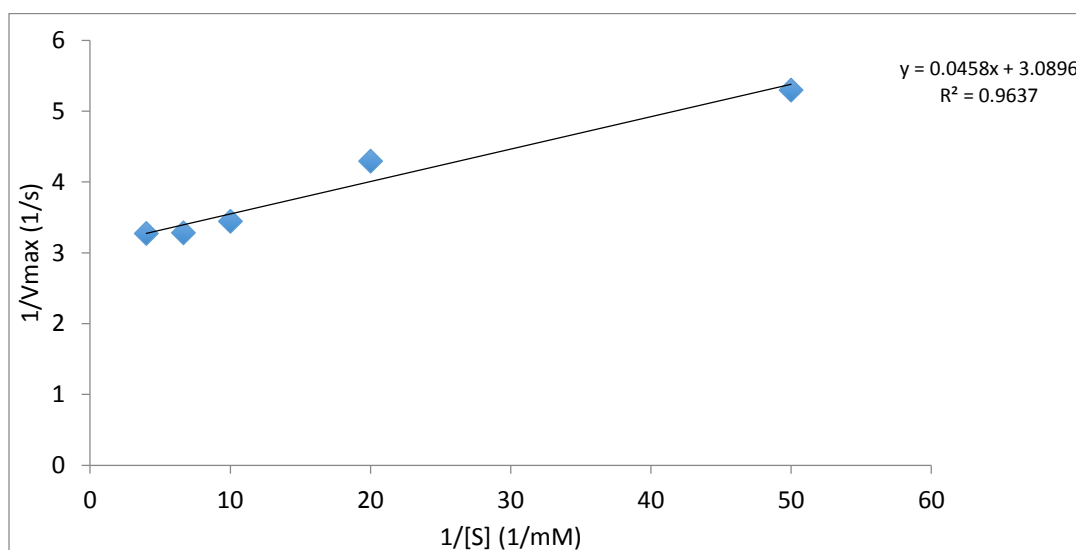


Figure 2B. Lineweaver-Burk plot for turkey tail laccase (Sigma). Laccase concentration was 1.5625 nM. Concentrations of DMP used were 0.02, 0.05, 0.1, 0.15, and 0.25 mM. Each of the assays were done in

triplicate and averaged. The linear trendline generates an equation that was used to derive the kinetic constants.

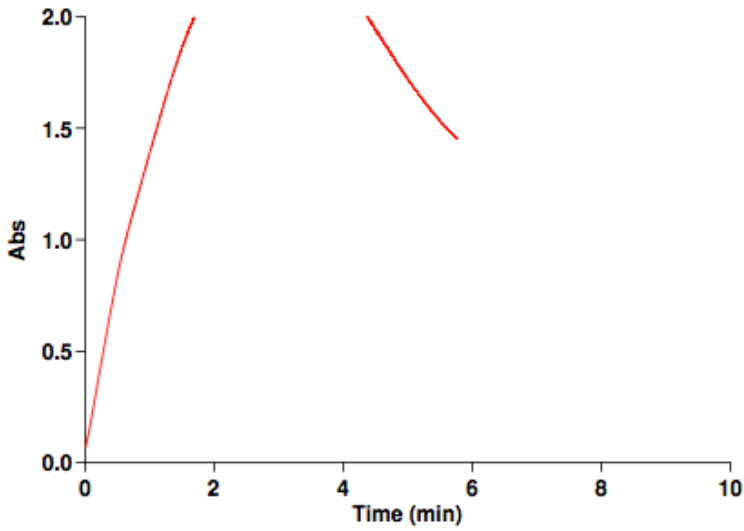


Figure 3A. Kinetic trace of turkey tail laccase activity assay after storage in pH 7 phosphate buffer. Absorbance higher than 2 is beyond the detection limits of the spectrophotometer.

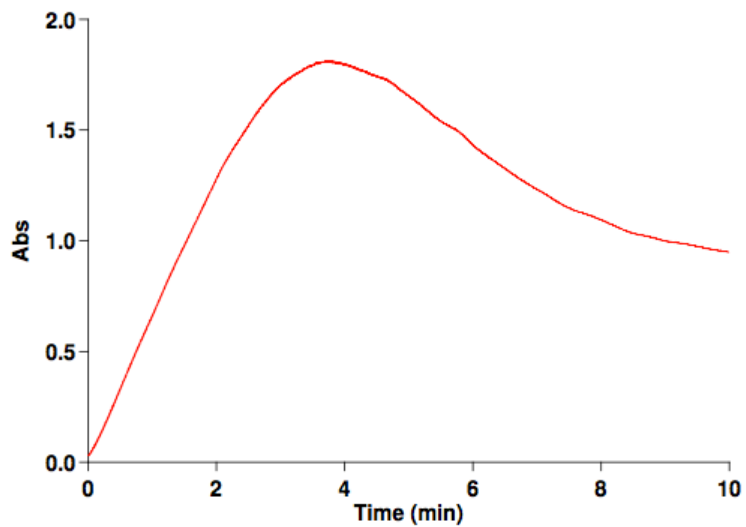


Figure 3B. Kinetic trace of turkey tail laccase activity assay after storage in pH 5 sodium acetate buffer.

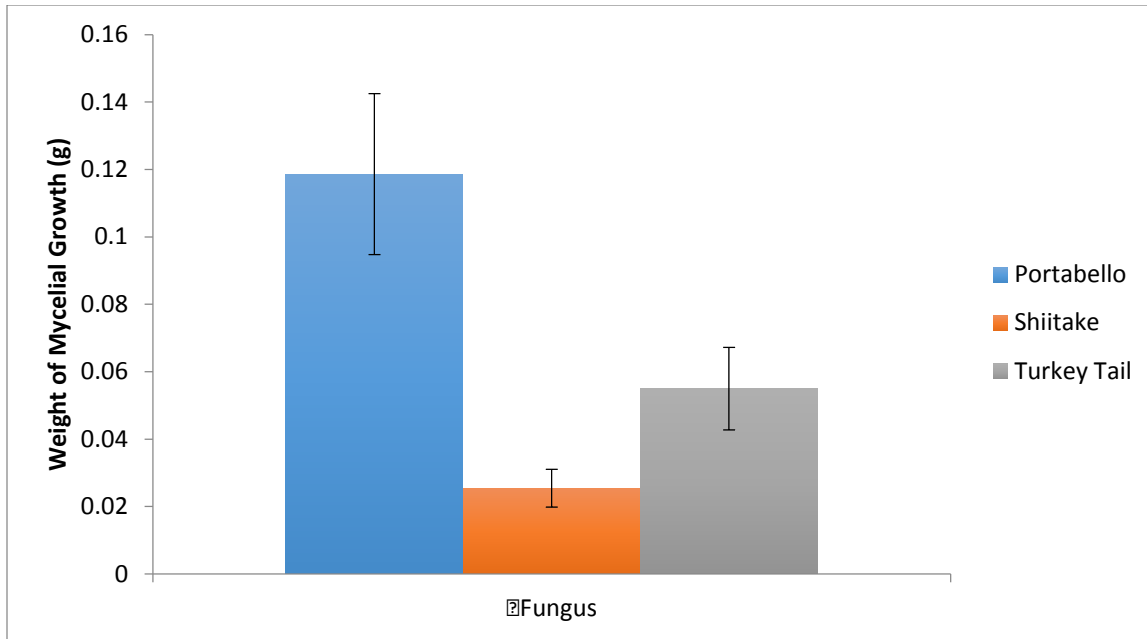


Figure 4. Mycelial growth weight of each culture. Total volume for each culture was 300 mL. Cultures were vacuum filtered using pre-weighed Fisherbrand® P5 filter paper. The paper was dried in an oven after filtration and weighed again. Weight of the paper was averaged +/- standard error.

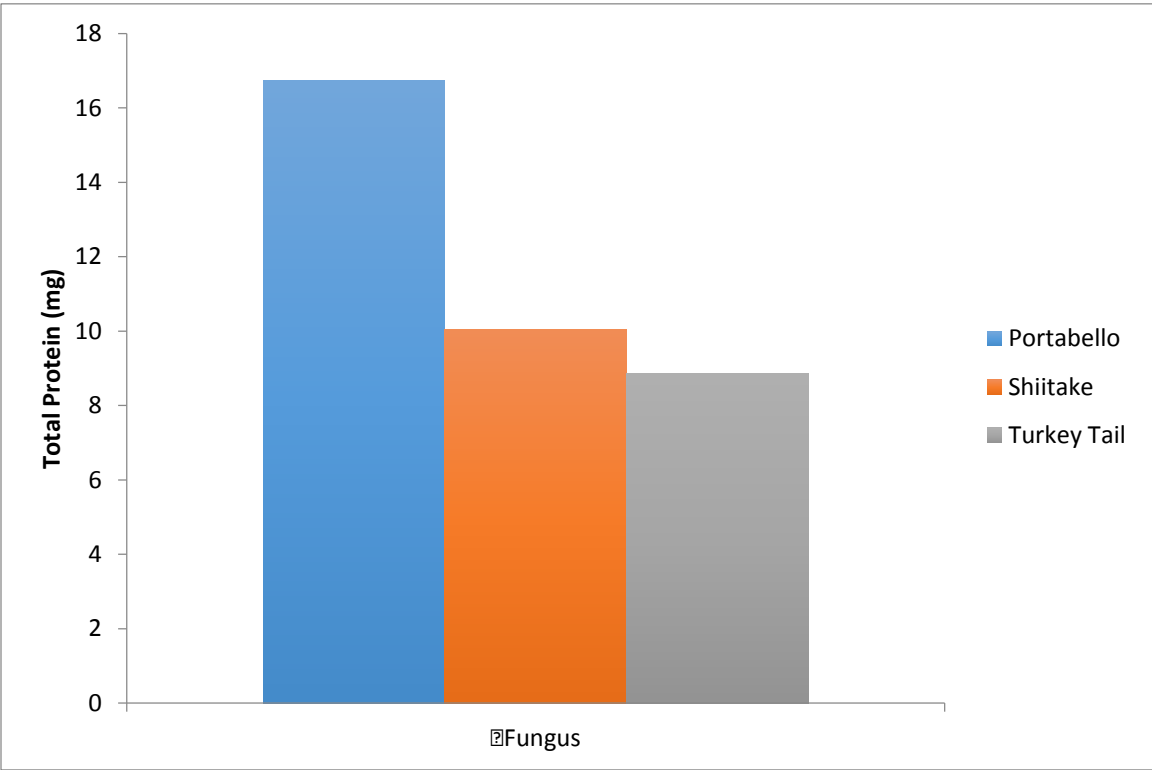


Figure 5. Total protein content in the CF from each source. The total protein content was determined using the Bio-Rad Protein assay, derived from Bradford.

Table 2: Kinetic constants were derived from a Lineweaver-Burke plot. We used 500 μL from each source in the reaction mixtures. Portobello did not display activity. Laccase concentration was unknown.

Laccase Source	V_{\max} (1/min)	K_m (mM)
Shiitake	0.01369	0.058
Turkey Tail	0.0772	0.0188

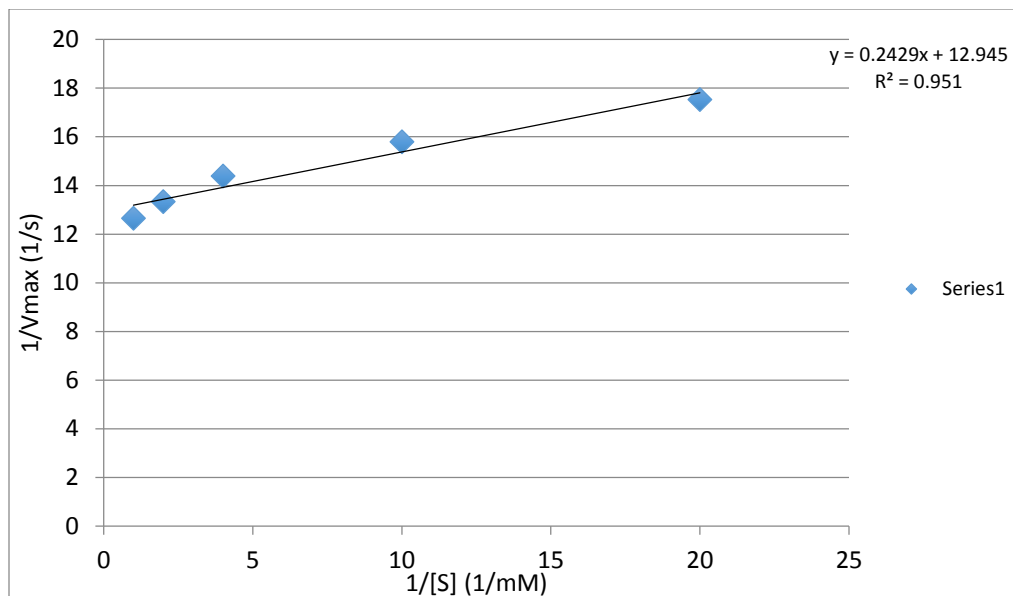


Figure 6a. Lineweaver-Burke plot for turkey tail CF. We added 500 μ L CF to the reaction solution, laccase concentration was unknown. Concentrations of DMP were 1, 0.5, 0.25, 0.1, and 0.05 mM. All assays were done in triplicate and averaged. The linear trendline generates an equation that was used to derive the kinetic constants.

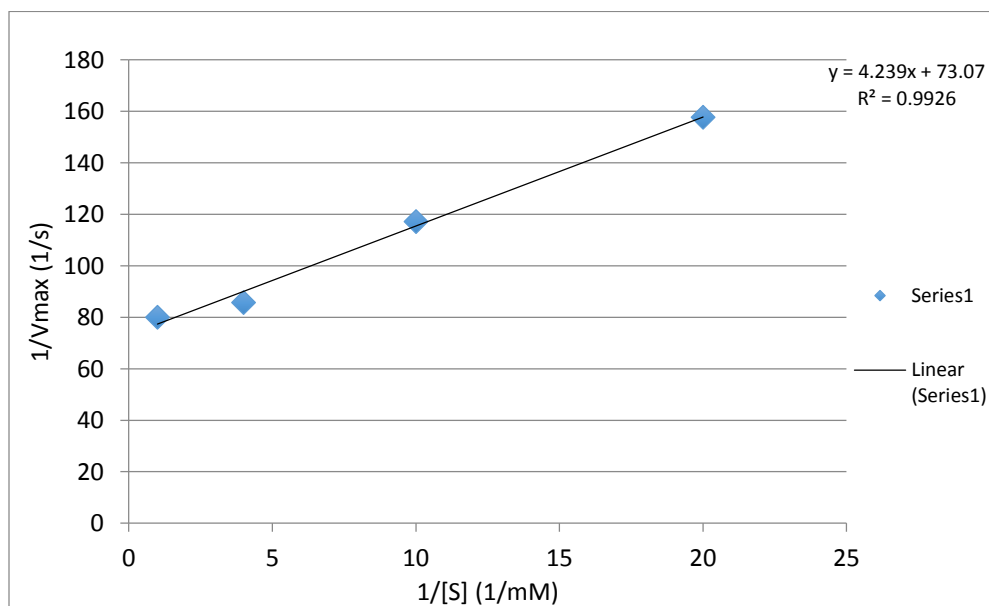


Figure 6b. Lineweaver-Burke plot for shiitake CF. We added 500 μ L CF to the reaction solution, laccase concentration was unknown. Concentrations of DMP were 1, 0.25, 0.1, and 0.05 mM. All assays were done in triplicate and averaged. The linear trendline generates an equation that was used to derive the kinetic constants.

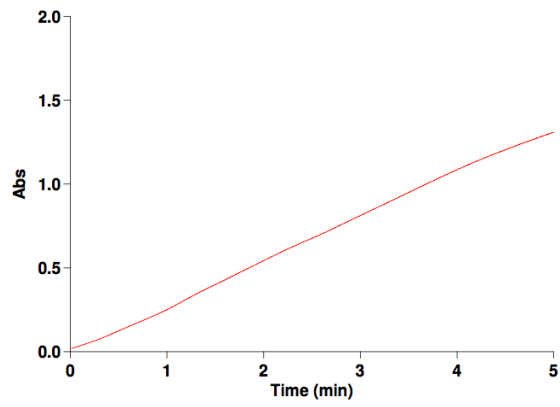


Figure 7a: Laccase activity assay with no ethanol.

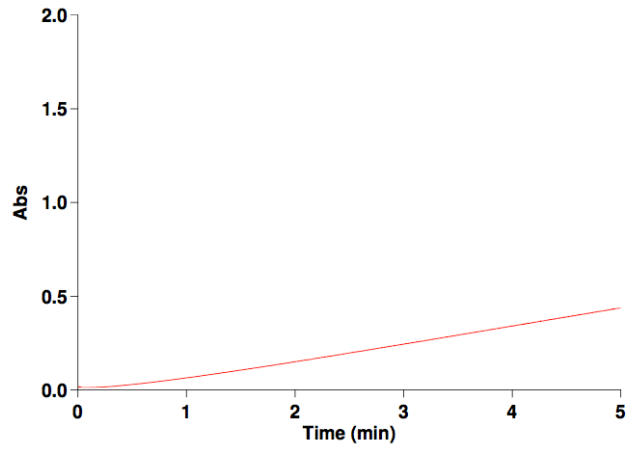


Figure 7b: Laccase activity assay with 20% ethanol.

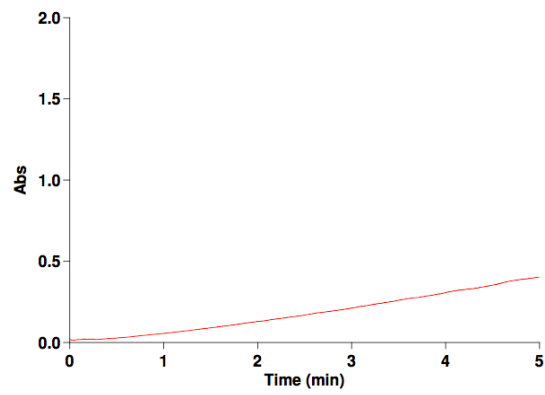


Figure 7c: Laccase activity assay with 30% ethanol.

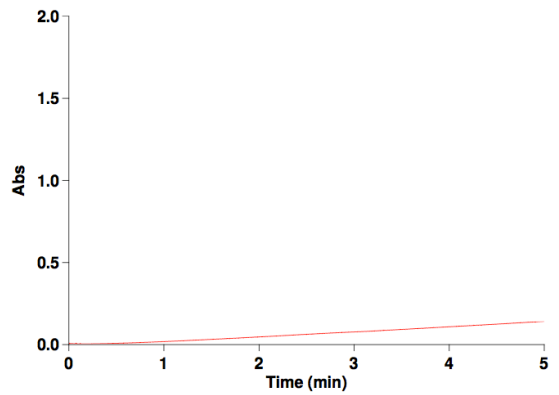


Figure 7d: Laccase activity assay with 40% ethanol.

C:\Johnson-14-7-21-EE2 with Laccase #100 RT: 1.39 AV: 1 NL: 1.79E5
T: - p ESI Full ms [70.00-700.00]

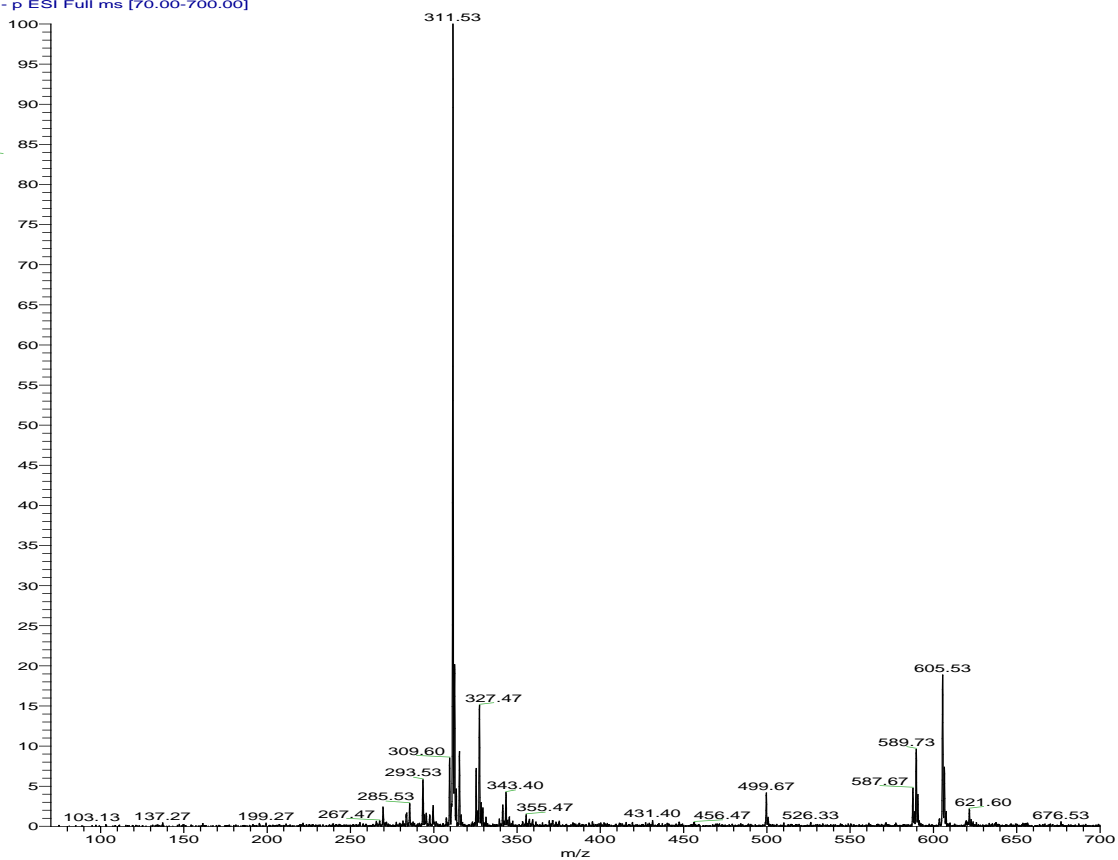


Figure 8. Soluble solution that was first run through centrifugal filters. The high peak at 311 has not been identified. Small peak at 587 was the first indication of a dimer.

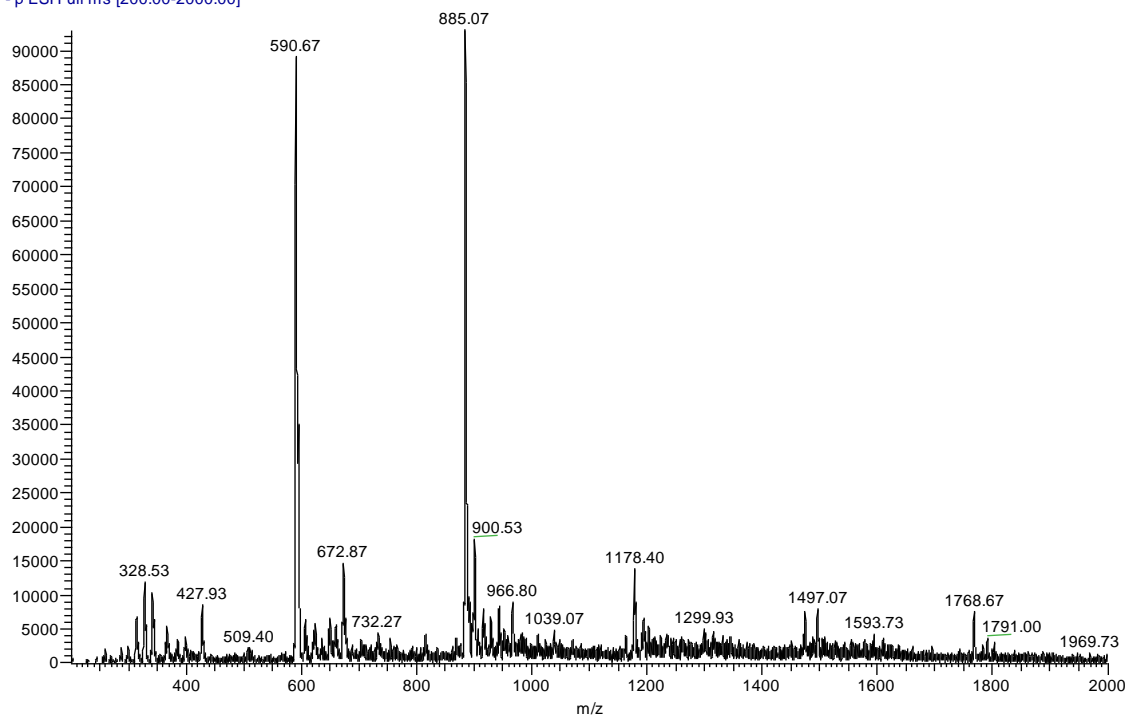


Figure 9. Solid precipitate that was dissolved in pure ethanol. High peaks at 590 and 885 confirm dimers and trimers. The other three smaller peaks at 1178, 1497, and 1768 are most likely tetramers, pentamers, and hexamers, respectively.