Laccase Mediated Transformation of 17β-Estradiol in Soil

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ABSTRACT

It is known that 17β-estradiol (E2) can be transformed by reactions mediated by some oxidoreductases such as laccase in water. Whether or how such reactions can happen in soil is however unknown although they may significantly impact the environmental fate of E2 that are introduced to soil by land application of animal wastes. We herein studied the reaction of E2 in a model soil mediated by laccase, and found that the reaction behaviors differ significantly from those in water partly because of the dramatic difference in laccase stability. We also examined E2 transformation in soil using ^{14}C-labeling in combination with soil organic matter extraction and size exclusion chromatography, and indicated that applied ^{14}C radioactivity was preferably bound to humic acids. The study provides useful information for understanding the environmental fate of E2 and for developing a novel soil remediation strategy via enzyme-enhanced humification reactions.

Capsule abstract

E2 was effectively transformed in soil by preferably binding to the humic acids through reactions mediated by laccase.

Keywords: Laccase; Oxidative Coupling; Humic Acid; Humification

INTRODUCTION

An increasing body of evidence demonstrates the presence of sex steroidal hormones routinely released to the environment by humans and animals[1-3]. Steroidal hormones present in the environment can elicit undesirable responses by altering the synthesis and function of endogenous hormones in living organisms[4], thus interfering with normal biological cycles such as reproduction, growth and development. The female sex hormones, estrogens, are of particular concern because estrogens are most frequently detected and their endocrine-disrupting effects on aquatic wildlife are widely documented[5-7]. In particular, 17β-estradiol (E2), the major and most potent form of natural estrogens, is capable of triggering responses in aquatic organisms at extremely low concentrations[8,9].

Land application of estrogen-containing solid wastes, such as poultry manure and municipal sludge, are considered common ways for hormones to enter soil and eventually water bodies via surface runoff and/or leaching[1,10-12]. Although some studies have shown that E2 has limited mobility and is degradable[13], others have consistently reported detection of E2 in surface[14], and groundwater[15-17]. It is thus critical to understand the transport and transformation of hormones in soil. This understanding is needed for a scientific assessment of the environmental risks associated with land application of hormone-containing biosolids and manures, and will help with the development of management and remediation methods to mitigate hormone release from soil to water.

Hormones may be transformed in soil by participating in the natural processes that affect the transformation and turnover of natural organic matter in soil systems. Two primary classes of processes, degradation and humification, are involved[18]. While degradation mechanisms operate to eventually mineralize contaminants, humification renders them bound covalently to soil

organic matrices resulting in their immobilization and detoxification[18]. Estrogens may undergo both types of transformations in soils. Studies with radio-labeled hormones in soil microcosms have shown limited mineralization and a major portion forming non-extractable bound residues[19-21]. Mineralization of estrogens was found to be low in broiler and breeder litters as well[22], which suggests the importance of bound-residue formation pathways. It has been documented previously that E2 in soil binds to the humic substances of organic matter, thus forming bound residue and restricting its movement to aqueous bodies[20].

Previous studies suggest that oxidative coupling reactions, a class of reactions that is critically involved in natural humification processes, may be one important mechanism by which xenobiotics form covalent bound residues in soil organic matter[23-25]. Oxidative coupling reactions are ubiquitous in soil systems, generally facilitated by such naturally occurring catalysts as iron and manganese oxides and a variety of extracellular enzymes[23,26]. Peroxidases and phenoloxidases are two enzyme groups that catalyze oxidative coupling reactions of a wide variety of substrates that contain the phenolic group, a group that is common in natural organic matter. Estrogens also contain a phenolic unit that makes them susceptive to oxidative coupling reactions. Earlier studies have shown evidence of enzyme-mediated oxidative coupling reactions of estrogens in aqueous systems[27,28], but studies in soil systems are limited. Laccases are an important class of phenoloxidases that have been widely studied and shown to catalyze the oxidative reactions of estrogens in aqueous systems leading to a reduction of estrogenic activity[29,30]. Laccases are multicopper-containing monophenol monooxygenases that catalyze one-electron oxidation of four substrate molecules simultaneously with four-electron reduction of molecular oxygen to water, and this enzyme is native to soil and has been studied for environmental remediation uses[31-33].

We systematically studied laccase-catalyzed transformation of E2 in water and that in soil at two different water content levels to compare the reaction behaviors. We also examined E2 transformation in soil using 14C-labeling in combination with soil organic matter (SOM) extraction and size exclusion chromatography to probe the interactions between E2 and SOM in laccase-mediated reaction systems. The results provide useful information for understanding and assessing the environmental fate of E2 and for development of enzyme-based remediation processes in soil.

MATERIALS AND METHODS

Chemicals

17β-Estradiol (> 98% purity), laccase (EC 1.10.3.2), and 2,4-dimethoxyphenol were purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade acetonitrile (ACN), methanol, dichloromethane (DCM), and ScintiVerse biodegradable cocktail were obtained from Fisher Scientific (Pittsburgh, PA). [4-^{14}C]-Labeled 17β-estradiol (55 mCi/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). The soil used in this study was collected from the Dempsey Research Farm, Griffin, GA, USA and was classified as a Cecil sandy clay loam. The soil sample was taken from the top 15 cm, sieved to 2 mm, and air dried. The physicochemical properties of the soil, including pH and organic matter content, were characterized by Agricultural and Environmental Services Laboratory, Athens, GA, and the details are given in section I of the Supporting Information.

Laccase solution was freshly prepared in deionized (DI) water before each experiment. The activity was determined spectrophotometrically by oxidation of 1 mM 2, 6-dimethoxyphenol in a citrate phosphate buffer (6.5 mM citrate/7 mM phosphate, pH 3.8), the absorbance of which was measured at 468 nm (Beckman Du 640-B spectrophotometer, Beckman Instruments Inc.).

One unit of laccase activity is defined as the amount of enzyme that causes a unit change per minute in absorbance at 468 nm in 3.4 mL of this solution in a cuvette with a 1 cm light path.

E2 Transformation in Aqueous System

Laccase catalyzed transformation of E2 was carried out in glass tubes at room temperature. Each reactor contained 0.2 µg E2, and 0, 2.5, 5.0, or 10 U mL^{-1} laccase in 2-mL DI water. The tubes were hand shaken first to mix the contents and then placed on a mechanical shaker to incubate (room temperature). Triplicate tubes were sampled at selected time intervals and acidified by adding 10 µL 1 N HCl. The sample was then immediately filtered through a 0.45-µm filter and loaded to a C18 (Restek, 6 mL, 500 mg) solid phase extraction (SPE) cartridge that was preconditioned with 5 mL of methanol followed by 5 mL of DI water at a flow rate of 10 mL/ minute. After loading, the SPE cartridge was eluted with 5 mL of methanol and the eluent was collected, blown dry by nitrogen, and then reconstituted to 1 mL with methanol, which was stored in an amber glass vial at 4 °C for HPLC analysis.

Incubation Experiments in Soil

Transformation of E2 in the presence of laccase was studied in sterilized soil under aerobic conditions at room temperature (25 ^{o}C) for 264 hours (11 days). Soil was autoclaved at 121 ^{o}C for 1 hour and left on the lab bench to incubate at room temperature for 24 hours. The autoclaving and incubation steps were repeated and followed by a final autoclaving to complete the sterilization of soil. To prepare E2 contaminated soil samples, 50 g of autoclaved soil was spiked with 1 mL of a 10-mg L^{-1} E2 stock solution in acetone, which was then left uncovered under a fume hood for 4 hours with vigorous mixing using a glass stir rod every 30 min to evaporate acetone and yield a sample containing 0.2 µg E2 g^{-1} soil.

The incubation experiments in soil were conducted at field capacity (FC) and saturated conditions. The enzyme solutions added to the soil were prepared freshly using autoclaved distilled water prior to the experiments and the enzyme activity was added at 0, 2.5, 5.0, and 10 U/g soil for the saturated condition and 0 and 10 U/g soil for the field capacity experiment. Saturated conditions were achieved by adding 1.5 mL of an enzyme solution to 1 g of air-dried soil. The soil samples at field capacity were prepared by adding 0.294 mL of an enzyme solution to 1 g of soil and the total weight was measured several times a day to maintain the constant weight. Any loss of weight due to evaporation was adjusted using autoclaved distilled water. The method used to measure the water content of soil at field capacity is described in Supporting Information (Section II).

Each experimental setup was prepared in triplicate. All tubes were placed in the dark throughout the experiment, with tops covered with paraffin film to allow exchange of oxygen while restricting water evaporation. The contents of each tube were thoroughly mixed at the beginning of the incubation and then mixed several times a day during the entire incubation period. Triplicate set of tubes were sampled at 24, 96, 120, 192, and 264 hours for E2 analysis. The samples were first frozen at -18 ^{o}C and then freeze dried to extract E2.

Extraction of E2 from Soil

A dry soil sample (1 g) was extracted using a 5 mL mixture of dichloromethane (DCM) and methanol (v:v, 2:1). This mixture of soil and organic solvent was ultra-sonicated for 30 minutes, centrifuged (30 min, 250 g, room temperature) and the supernatant was collected. The extraction was repeated once, and the supernatant was collected and combined with the first extract. The extractant was evaporated to dryness under N_2 and then reconstituted in 1 mL of hexane. The sample was then loaded onto a normal phase silica cartridge (6 mL, 1000 mg) that was pre-

conditioned with 5 mL hexane. The silica cartridge was washed with 20 mL of hexane, dried under vacuum for 15 min, and then eluted with 5 mL of ACN. The eluant was dried under N_2 flow, reconstituted in 1 mL ACN and stored until analysis by HPLC.

Activity of Laccase in Soil and Water

The activity of laccase in soil and water over time was evaluated and the details are provided in Supporting Information Section III.

E2 Quantification

The concentration of E2 was quantified using a Shimadzu LC20AT HPLC equipped with a Shimadzu 20A photo diode array (PDA) detector (Shimadzu, Columbia, MD). The separation was performed on an Ascentic C18 reversed phase column (250 x 4 mm, Supelco, St. Louis, MO). Elution was conducted with a mobile phase gradient consisting of water (A) and ACN (B) at a flow rate of 0.7 mL min^{-1}. The gradient program spanned 45 min as follows: 10% B increased linearly to 40% B at 15 min and then held at 40% B for 30 min. Absorbance at 225 nm was used to quantify E2 concentration and a five point external calibration was established for the quantification. Estrone (E1) and estriol (E3) was also monitored.

Kinetic Analysis

A kinetic analysis was conducted to analyze the E2 degradation data and the enzyme inactivation data, and the details are presented in Supporting Information Section IV.

Incubation Experiments with ^{14}C-labeled E2

Experiments with ^{14}C-labeled E2 were performed for 264 hours under various conditions to examine the formation of bound residues and/or CO_2 during E2 transformation in soil, so as to compare the relative contribution of degradation and humification pathways. To this end, an approach similar to that described in incubation experiments was used. Each reactor (glass

beaker) contained 1 g of soil preloaded with 0.2 µg of ^{14}C-labeled E2, and 10 U laccase in 1.5 mL DI water. The reactor was kept in a 0.9-L capped Mason jar along with a 20-mL glass scintillation vial containing 2 mL of freshly prepared 1-M NaOH solution that was used as a CO_2 trap. Sampling was done at 24, 96, 120, 192, and 264 hour intervals that were selected in accordance with previous experiments carried out with non-labeled E2. For sampling, reactors containing ^{14}C-labeled E2 were removed from the Mason jar, frozen at -18 ^{o}C, freeze-dried, and then extracted for E2 following the same procedure as described above. The extract was then mixed with cocktail and analyzed for radioactivity using a model LS 5801 liquid scintillation counter (LSC, Beckman Coulter, CA). The inside of each reactor was also rinsed twice with ethanol (2 mL each time) and the rinsate was mixed with 18 mL ScintiVerse cocktail and was counted on LSC for any remaining radioactivity on glassware. Additionally, at each sampling 18 mL of ScintiVerse cocktail was added to the scintillation vial containing NaOH solution (CO_2 trap) and was counted on the LSC.

Extraction and Fractionation of Soil Organic Matter

Soil samples after E2 extraction were further extracted by a standard procedure to obtain humic acid (HA) and fulvic acid (FA) from the soil.[34] Details of the method for HA and FA extraction are given in section V of the Supporting Information. The solution containing FA (2 mL) was mixed with 18 mL of cocktail and then counted on LSC for radioactivity. The residual soil and the extracted HA were combusted in biological oxidizer (OX 500; R. J. Harvey Instrument Co. Tappan, NY) with CO_2 absorbed in cocktail and then counted on LSC.

Size Exclusion Chromatography (SEC)

The humic acids extracted from certain samples were first dissolved in 10 mL 0.1 M NaOH, and 100 µL was fractionated using size exclusion chromatography (SEC). The SEC

system consisted of a high pressure Shimadzu LC 20AT pump, and an UV/Vis variable wavelength detector set at 280 nm. The separation was performed on a Sepax column (300 mm, 4.6 mm i.d.; 5 µm particle size). The isocratic SEC procedure was performed using DI water (Millipore, MA, USA) as the mobile phase with a flow rate of 1 mL min^{-1}.

Statistical Methods

The experiments were arranged in a completely randomized design with three replications for each treatment. Analysis of variance (ANOVA) was performed to evaluate the effects of treatments, duration, and interaction of treatment and duration using a general linear model (GLM) (SAS Release 9.3, SAS Institute, 2001). Fisher's protected LSD test with $\alpha = 0.05$ was used for determining statistical differences among treatments and duration means following each ANOVA.

RESULTS AND DISCUSSION

Laccase Mediated Transformation of E2 in Water

Our experiments revealed effective removal of E2 in laccase-mediated aqueous systems as displayed in Fig 1. For the control systems, 87.3% E2 remained in water at the end of the 300-min reaction period, but in the system containing 10 U/mL laccase E2 was completely removed. The system containing 5 U/mL laccase showed over 98% reduction in E2 concentration while in the system with 2.5 U/mL laccase more than 93% reduction was observed. Estrone (E1) and estriol (E3) were monitored, but their formation was not observed, indicating that E2 was not converted into E1 or E3 in these systems. E2 removal might have resulted from oxidation by laccase to produce E2 free radicals that further participate in radical-radical coupling reactions leading to self-polymerization and precipitation of E2[27,30, 35,36]. E2 contains a hydroxylated aromatic (phenolic) group in its structure, which makes it a preferred laccase substrate to

generate nonspecific phenoxy radicals. In earlier studies conducted in aqueous phase with enzyme preparation from white rot fungus, near complete removal of E2, bisphenol A and nonylphenol (endocrine disrupting chemicals) via a radical polymerization mechanism was reported[28,37]. A direct association between disappearance of the chemicals from the reaction system and a decrease in estrogenicity was also observed[34].

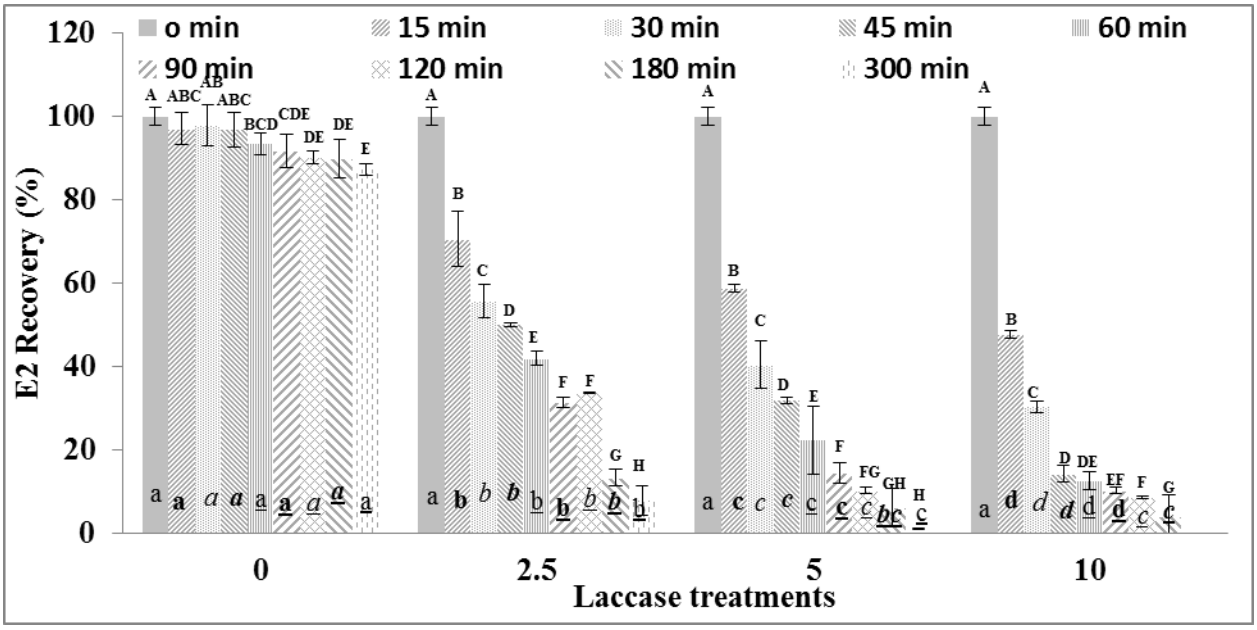

FIGURE 1. Recovery of 17β-Estradiol (E2) in aqueous systems with four different laccase activity levels, 0 (control), 2.5, 5, and 10 U mL^{-1}. Values are the means of three replicates and error bars are standard deviations. Small case letters are for comparison of laccase treatments within the same duration. Uppercase letters are for comparison of duration within a laccase treatment. Bars with the same letters are not considered to be statistically different according to LSD at $\alpha = 0.05$.

We have also measured the laccase activity in water system, both in the absence and presence of E2 over a 300-min reaction period and the details has been provided in Supporting Information III.

Laccase Mediated Transformation of E2 in Soil

The efficacy of laccase at different activity levels (2.5, 5.0, and 10 U g^{-1} soil) in mediating E2 removal in soil was compared. Effective transformation of E2 in laccase-mediated soil systems was evident (Fig. 2). In the control systems without laccase, the recovery of E2 by organic solvent extraction was reduced slightly during the first 24 hours, but no further reduction was observed for up to 264 hours of incubation, and the recovery at 264 hours was 80.2%. In a preliminary test, we have found that the E2 recovery from the control samples without laccase addition was not statistically different from those samples with the addition of laccase that had been heat inactivated (boiled in water for 30 min) at the dosage of 10 U/g soil. However, the treatment systems containing laccase at all three levels showed significant decreases in E2 recovery during the entire incubation period (Fig. 2). E2 recovery decreased significantly to 12.9%, 14.4%, and 5.4% after 264 hours of incubation with the application of 2.5, 5.0, and 10 U g^{-1} laccase, respectively. E2 that remained unextractable with organic solvents may have been incorporated into soil organic matter (SOM) via covalent bonds. This is consistent with the hypothesis that E2 is converted into free radicals upon reaction with laccase, and the radicals can subsequently participate in non-enzymatic coupling reactions leading to incorporation into SOM.[38]

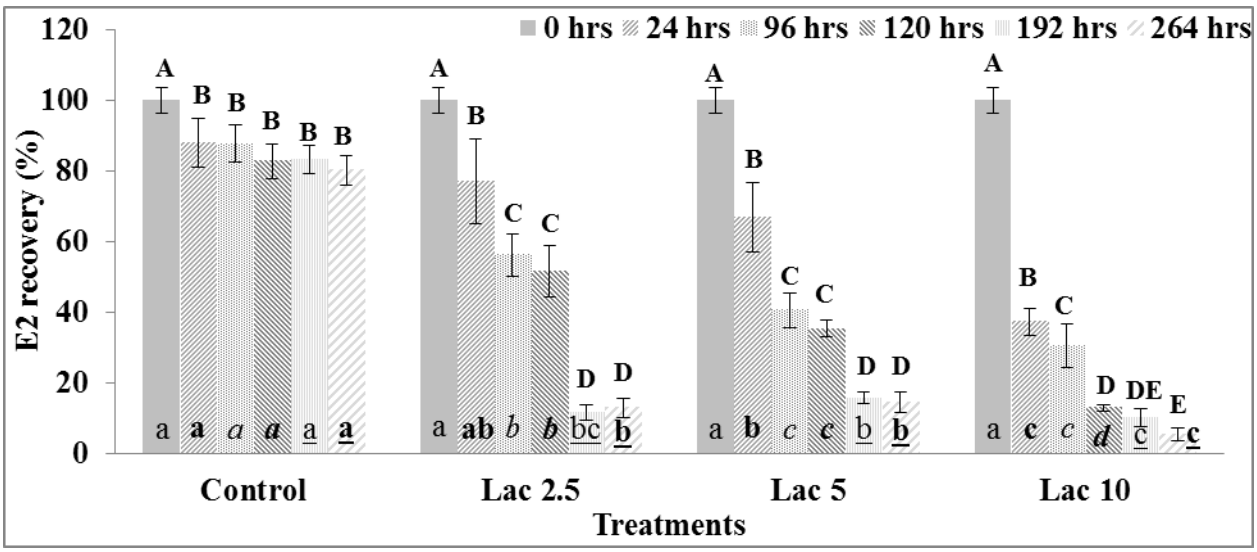

FIGURE 2. Recovery of 17β-Estradiol (E2) from soil with four different laccase activity levels, 0 (control), 2.5, 5, 10 U g^{-1} soil. Values are the means of three replicates and error bars are standard deviations. Small case letters are for comparison of laccase treatments within the same duration. Uppercase letters are for comparison of duration within a laccase treatment. Bars with the same uppercase letters are not considered to be statistically different according to LSD at $\alpha = 0.05$.

It is also noted in Fig 2 that the levels of E2 recovery at the end of 264 hours of incubation were similar for different laccase activity levels, although there was some differences in the initial period up to 120 hours. It is interesting that the treatment with relatively low laccase concentration (2.5 U g^{-1} soil) was almost equally effective as the high laccase concentrations when incubated for longer periods.

Our additional experiments to examine laccase activity in soil indicated that laccase remains active in soil for long periods of time (Supporting Information III). Nearly 40% laccase activity could be recovered from soil by simple water extraction after 264 hours (11 days) of incubation. This suggests a much better stability of laccase in soil than in water, where laccase activity appeared to diminish after a few hours of reaction (Supporting Information IV). Because of the prolonged laccase activity in soil, the initial difference in E2 removal at different enzyme dosages was reduced to insignificance as E2 removal was near complete towards the end of the

incubation period (Fig. 2). This result marked the importance of enzyme stability as a factor to consider in enzyme-based soil remediation design.

In order to assess the effect of moisture level on E2 transformation and laccase activity in soil, parallel tests were performed with the same E2 concentration and 10 U laccase per gram of soil at field capacity (0.294 g H_2O g^{-1} soil). The E2 removal under saturated soil conditions were compared with E2 removal at field capacity as displayed in Fig 3. After 24 hours of laccase treatment, 67.9% E2 recovery was observed in soil at field capacity compared to 37.4% recovery in saturated soil. The difference in E2 recovery between unsaturated and saturated soil conditions may result from enhanced E2 transformation under the saturated condition with water facilitating laccase contact with E2.

The activity of laccase in both saturated and unsaturated soil followed a similar trend (Fig. S1, Supporting Information III). This suggests that laccase activity may not necessarily decrease with a decrease in soil moisture content, consistent with an earlier finding.[36] Because of the sustained laccase activity in soil at both water content conditions, the difference in extractable E2 between saturated and unsaturated conditions decreased with incubation time (Fig. 3). At 264 hours, the extractable E2 in unsaturated soil was 9.0%, while in saturated condition was 5.4%.

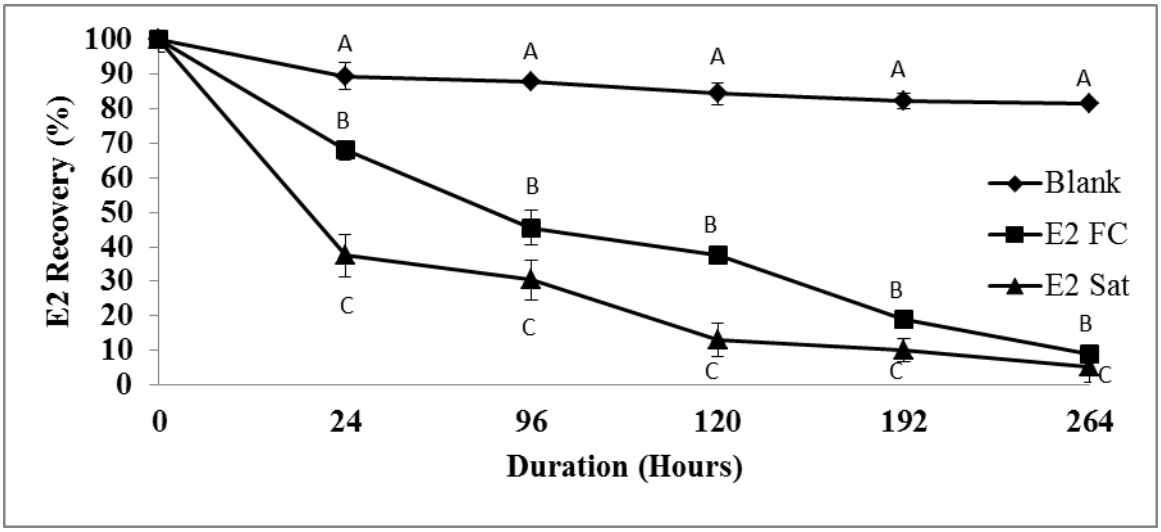

FIGURE 3. Recovery of 17β-Estradiol (E2) in soil at saturation (E2 Sat) and at field capacity (E2 FC) with 10 U g^{-1} laccase (Lac) application. Values are the means of three replicates and error bars are standard deviations. Small case letters are for comparison of E2 recovery within the same duration. Bars with same letters are not considered to be statistically different according to LSD at $\alpha = 0.05$.

Kinetic analysis

E2 removal and laccase inactivation was much more rapid in water (Fig 1 and S2) than in soil (Figure 2 and S1). A kinetic analysis shown in Supporting Information IV indicated that laccase inactivation and E2 removal can both be well described as a pseudo-first-order rate process in both water and soil. The rate constant of laccase inactivation was 0.46 h^{-1} in water (Table S4) and 0.00031 h^{-1} in saturated soil (Table S5), indicating laccase inactivation in soil about 150 times slower than in water. The rate constant of E2 removal was 1.30 h^{-1} (Table S2) in water and 0.0122 h^{-1} in soil (Table S3) when 10 U laccase was used, showing E2 removal over 100 times faster in water than in soil.

The large difference in the rate constants of E2 removal in water and soil may reflect the different mechanisms of E2 reactions. E2 removal in aqueous systems are primarily due to the ultrafast self-coupling of E2 radicals[29,30], whereas in soil the radical self-coupling process is hindered by the presence of SOM[35] and E2 removal may have resulted mainly from covalent

binding between E2 radicals and the organic matter. The greater longevity of laccase in soil however compensated for the slower reactions, and the better stability of laccase in soil may be due to its immobilization in soil. It is known that an enzyme tends to be stabilized when is immobilized on a solid support material[39].

The concentration of E2 used in the soil experiment was 0.2 μg/g or 200 μg/Kg soil, close to a high E2 concentration found in manure-amended soil, as a report indicated that a land amended with poultry litter at the rate of 4 tons per acre contained 133 μg E2/Kg soil[40]. As E2 removal appeared to follow a pseudo-first-order rate behavior, a significantly lower reaction rate would be expected when E2 is present at a much lower concentration.

Characterization of E2 Removal in Soil Using ^{14}C Labeled E2

An experiment was performed using ^{14}C labeled E2 to quantify the formation of bound residues and CO_2 during E2 transformation in soil, and the result is shown in Fig 4. It is evident that ^{14}C recovery decreased significantly in laccase-treated soil systems (Fig. 4). Recovered ^{14}C (%) represented the amount of E2 and its transformation products that could be extracted from soil using the dichloromethane and methanol (v:v, 2:1) mixture, while the remaining non-extractable fraction was assumed to be soil bound. The increase in the soil bound fraction with time was indicative of E2 participation in cross coupling reactions with SOM. During the entire incubation experiment, the amount of $^{14}CO_2$ release was negligible, indicating minimal E2 mineralization. At each measurement point, the amount of extractable ^{14}C, measured by LSC, was always greater than the amount of remaining E2 as measured by HPLC. This is reasonable because LSC measurement of ^{14}C reflected the amounts of both E2 and its transformation products and the difference between the two measurements (Fig. 4) represents the relative

quantity of E2 transformation products that was extractable, probably those that have bound to dissolved soil organic matter.

Distribution of ^{14}C in the above mentioned soil systems after 264 hours of incubation were calculated (Table 1). The mineralized fraction (CO_2) was low for both the laccase-treated and the control samples. The fraction extractable by organic solvents significantly decreased from 85.4% to 21.1% upon laccase treatment, indicating a strong laccase-mediated humification effect on E2. We further extracted the soil organic matter and fractionated it into humic and fulvic acids using a conventional approach described in Supporting Information (Section V). In the laccase-treated sample, 75.5% ^{14}C was found in humic acid while radioactivity associated with fulvic acid was 4.3% (Table 1), indicating a favorable tendency of E2 coupling with humic acid[20]. The greater reactivity of humic acid in laccase-mediated reaction systems may be related to its higher aromaticity than fulvic acid. It has been noted that the reactivity of natural organic matter towards laccase is determined primarily by the abundance of phenolic functionalities[41]. The soil residue remaining after organic matter extraction, contained no ^{14}C, manifesting the lack of laccase-mediated E2 reactivity with soil minerals.

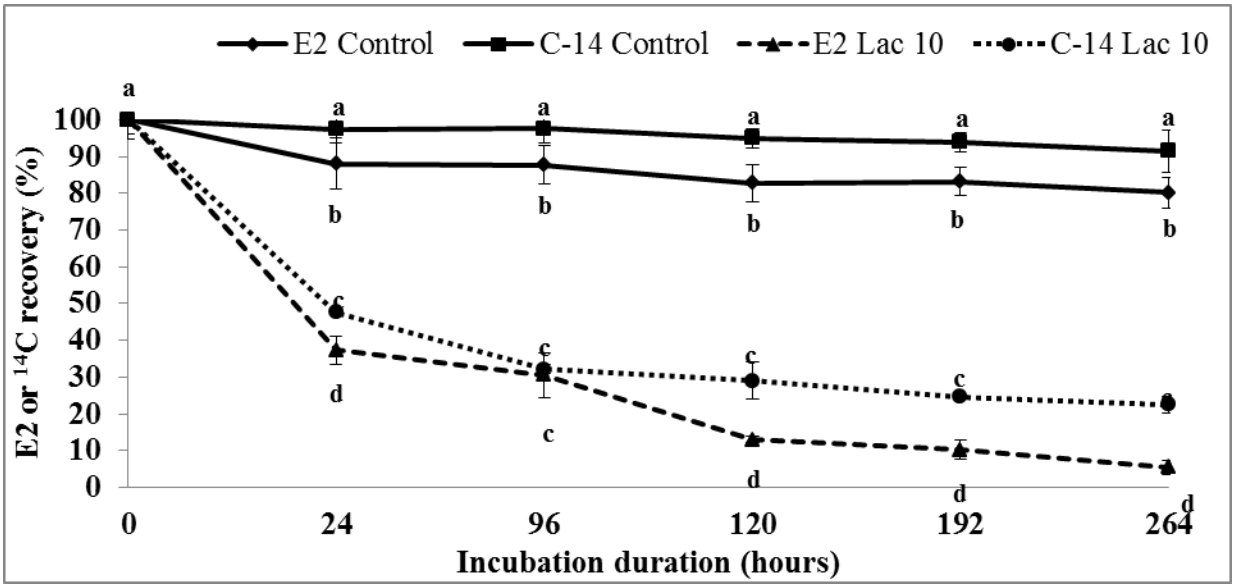

FIGURE 4. Recovery of E2 or ^{14}C from laccase-mediated reaction systems (Lac 10) in soil containing [4-^{14}C]-estradiol and laccase at 10 U g^{-1} soil and in the control systems (Control) that contained E2 but no laccase. Values are the means of three replicates and error bars are standard deviations. Small case letters are for comparison within the same duration. Bars with same letters are not considered to be statistically different according to LSD at $\alpha = 0.05$. $^{14}CO_2$ accumulated during the reaction for each sample was less than 2% of applied E2.

Table 1. Distribution of ^{14}C after 264 hours of incubation in a soil system containing ^{14}C-labeled 17 Beta-estradiol (E2)

Fractions	**(% of applied ^{14}C)**	
	Control	**Laccase treated†**
Extract‡	90.4 ±1.99**a**	21.1± 0.68**b**
CO_2	0.72 ± 1.21**b**	1.95 ± 3.38**a**
Humic acid	11.8 ± 1.82**b**	73.5 ± 2.80**a**
Fulvic acid	3.4 ± 1.62**a**	4.3 ± 1.99**a**
Soil residue§	ND	ND
Total	106.32	100.85

Same letters within fraction are not considered to be statistically different according to Fisher's protected LSD at $\alpha = 0.05$.
† Laccase activity level = 10 units g^{-1} soil; Values are means of three replicates
‡ The fraction extracted by the mixture of dichloromethane and methanol (v:v, 2:1)
§ The residue remaining after soil organic matter extraction

Changes in Humic Acids

Size exclusion chromatography (SEC) of the humic acids extracted from selected soil samples showed that laccase treatment of soil changed the chromatographic pattern of the humic acid (Fig. 5). The chromatogram of the humic acid from the control sample showed peaks eluting at approximately 7.5 to 9 min, but in the samples treated by laccase (5B) or by laccase and E2 (5C) those peaks disappeared. Elimination of these peaks having longer retention time in SEC, which corresponds to smaller molecular sizes, indicates a result of oxidative coupling reactions leading to the incorporation of small humic molecules to larger moieties. Another distinct difference between the control (5A) and the laccase-treated soil sample (5B) was the onset of the peaks shifted from ~2.8 min to 4 min and the peak intensity (measured by UV absorbance)

increased. In a study by Lu et al.[38], a similar increase in UV absorbance was observed for laccase-treated fulvic acid. Another previous study[42] reported significant conformational changes of natural organic matter (NOM) during oxidative coupling reactions, featuring cross-linkage of NOM moieties primarily via converting aromatic hydroxyl groups into ether bonds. Such conformational changes have increased the hydrophobicity of the humic molecules by capping the hydroxyl groups. This may have caused the humic molecules that were extracted by the SOM fractionation procedure used in this study shifting to a smaller size ranges, thus leading to shift of the onset of the SEC peak in a laccase-treated sample to a slightly longer retention time (5B). In Fig. 5C, which indicates the soil system incubated with both E2 and laccase, a decrease in UV absorbance peak was observed compared to the soil system only with laccase (5B). Another difference was the appearance of a small shoulder peak approximately at 6.5 minutes. These differences may be reflective of the E2 reaction with humic acid and thus conformational changes brought about by oxidative coupling reactions[43].

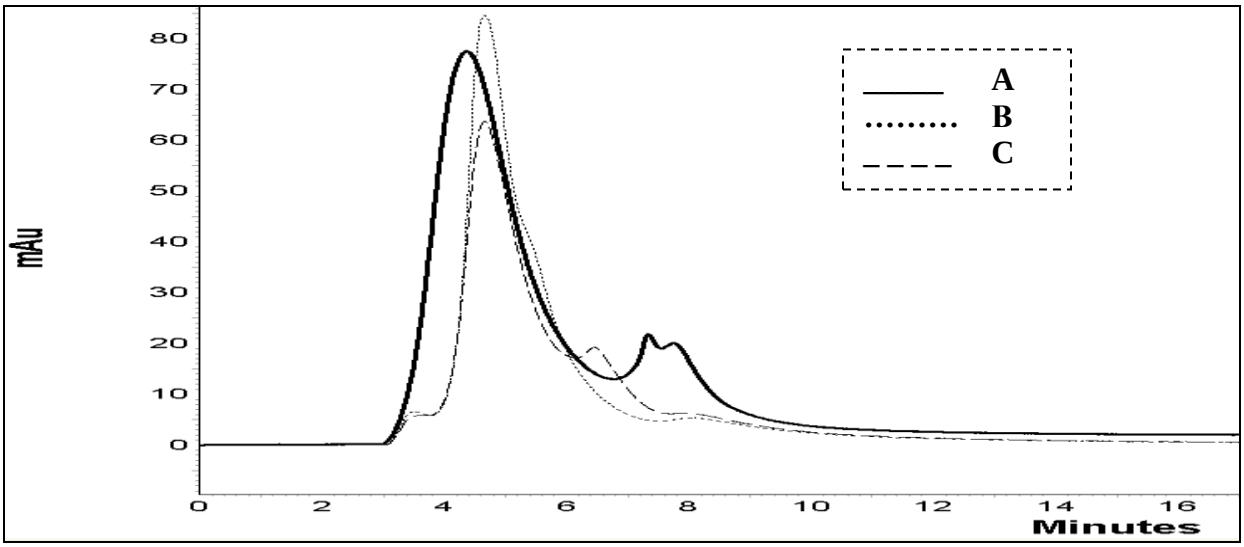

FIGURE 5. Size exclusion chromatograms of the humic acid extracted from a control sample (A), from a laccase-treated soil (B), or from a laccase-treated soil system containing 17β-Estradiol (E2) (C). Laccase concentration = 10 U g^{-1} soil, E2 concentration = 0.2 µg g^{-1} soil, treatment duration = 264 hours.

CONCLUSIONS

This study systematically investigated laccase-mediated oxidative coupling reactions in a soil system; while most previous studies were performed in aqueous phase. The results verify that laccase is very effective in mediating E2 transformation in soil, but also indicates that certain reaction behaviors appear to differ from those in water. For example, laccase activity remains present in soil after a prolonged reaction time (264 hours), whereas it lasts only a few hours in aqueous phase during oxidative coupling reactions. This has significant ramification in potential soil remediation applications. Because of prolonged laccase activity, E2 under different laccase dosages (2.5, 5, and 10 U g^{-1} soil) approached similar levels of nearly complete removal when incubated for a long period of time (264 hours). It is a significant topic of future study to further elucidate the mechanism leading to greater laccase stability in soil. The result suggests that enzyme-catalyzed oxidative coupling reactions may play a significant role in the environmental transformation and fate of E2, a factor that needs to be paid greater attention in the environmental risk analysis. It should be noted that this study focuses on the behaviors of laccase-mediated E2 reactions in soil by batch experiments, while a soil column study is necessary to fully elucidate the reactive transport behavior in natural soil environment.

The result from ^{14}C-labeled E2 study revealed that laccase-mediated E2 reactions in soil lead to a significant fraction of E2 being incorporated into the humic fraction of the soil organic matter. The SEC study revealed significant differences in the structures of humic acids that were obtained from soil sample with and without laccase addition.

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SUPPORTING INFORMATION AVAILABLE

Physico-chemical properties of soil (I), measurement of water content at field capacity (II), activity of residual laccase in soil (III), activity of laccase in water system (IV), and extraction of soil organic matter (V) are available in the Supporting Information. This information is available free of charge via the Internet at http://pubs.acs.org.

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