Effect of an Electro-Fenton Process on the Biodegradation of Lignin by *Trametes versicolor*

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A synergistic effect was found between the electro-Fenton (E-Fenton) process and a white-rot enzyme (*Trametes versicolor*) system relative to the degradation of dealkaline lignin. The hydrogen peroxide produced by the E-Fenton process reacted with Fe²⁺ on the cathode to generate a large number of hydroxyl radicals. These hydroxyl radicals directly degraded various functional groups in lignin, which led to the quick initiation of lignin peroxidase (LiP) and manganese peroxidase (MnP) enzymatic hydrolysis and accelerated the progress of lignin biodegradation. In addition, the hydroxyl radicals produced by the Fenton reaction converted nonphenolic lignin into phenolic lignin, further promoting the ability of manganese peroxidase and laccase to degrade the lignin. Additionally, the Fe³⁺ secreted by white-rot fungi accelerated the regeneration of Fe²⁺ on the composite cathode, which sustained the lignin degradation system. In the synergistic system, mycelium growth was significantly improved, with the maximum growth amount reaching 2.3 g and the lignin degradation rate reaching 84.5%, the activity of the three enzymes increased with the increase of currents over 96 h. Among them, the activity of MnP increased significantly to 402 U/L.

Keywords: Lignin, E-Fenton; White-rot fungi; *T. versicolor*

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INTRODUCTION

Lignin is the second largest biopolymer in the earth’s biosphere after cellulose. It comprises approximately 10% to 30% of plant biomass and is an important component of biomass waste in the pulp and paper industry (Ragauskas et al. 2014). Lignin is also the largest biological source of aromatic compounds (Cadisch and Giller 1997). Its structural complexity, high molecular weight, and insolubility make it difficult to degrade (Pérez et al. 2002). Because of lignin’s resistance to degradation, chemical and physical degradation treatments can cause potential problems, including high energy consumption and environmental pollution (Dong et al. 2014). A number of fungi are able to degrade lignin. Although brown rot and soft rot fungi can also degrade lignin, the best degraders and most efficient ligninolytic organisms characterized thus far are white-rot fungi. Their capability to degrade lignin is due to their extracellular non-stereoselective and nonspecific enzyme systems (Tekere et al. 2001). White-rot fungi secrete phenol oxidases, including manganese peroxidase (MnP), lignin peroxidase (LiP), and laccase (Lac). These three enzymes have synergistic effects on lignin degradation. *Trametes versicolor* is a versatile white-rot basidiomycete showing a high efficiency in lignin degradation that leaves the cells either pierced with thinned secondary walls or with bore holes (Blanchette 1995; Faix 2002). With its ability to use lignin materials as substrates, *T. versicolor* is easily available and
fast-growing (Staïj et al. 2006). Previous studies have shown that T. versicolor is able to degrade polycyclic aromatic hydrocarbons, polychlorinated biphenyl mixtures, and artificial dyes (Novotný et al. 2004).

Composite cathode electro-Fenton technology (CCEF) is a combination of Fenton oxidation and electrochemical technology that can produce hydrogen peroxide and Fenton reagents in situ without Fe²⁺ addition. The oxygen in the solution is reduced by two electrons at the cathode, protonates to hydrogen peroxide, and then reacts with Fe²⁺ to produce hydroxyl radicals while Fe²⁺ is oxidized to Fe³⁺. The generated Fe³⁺ can be regenerated on the surface of the electrode to improve the efficiency of the ion catalyst. The cathode reaction equations are as follows (Eqs. 1 through 3) (Kurt et al. 2007; Ting et al. 2008):

\[
\begin{align*}
\text{O}_2 + 2\text{H}^+ + 2e^- & \rightarrow \text{H}_2\text{O}_2 \quad (1) \\
\text{H}_2\text{O}_2 + \text{Fe}^{2+} & \rightarrow \cdot \text{OH} + \text{OH}^- + \text{Fe}^{3+} \quad (2) \\
\text{RH} + \cdot \text{OH} & \rightarrow \text{Products} \quad (3)
\end{align*}
\]

On the cathode surface, Fe³⁺ can be reduced to Fe²⁺ by E-Fenton reaction (Eqs. 4 through 6):

\[
\begin{align*}
\text{Fe}^{3+} + \text{H}_2\text{O}_2 & \rightarrow \text{Fe}^{2+} + \text{HO}_2 \cdot + \text{H}^+ \quad (4) \\
\text{Fe}^{3+} \cdot \text{HO}_2 & \rightarrow \text{Fe}^{2+} + \text{O}_2 + \text{H}^+ \quad (5) \\
\text{Fe}^{3+} + e^- & \rightarrow \text{Fe}^{2+} \quad (6)
\end{align*}
\]

The following side reactions are present in the solution (Eqs. 7 through 12):

\[
\begin{align*}
\text{Fe}^{2+} + \cdot \text{OH} & \rightarrow \text{Fe}^{3+} + \text{OH}^- \quad (7) \\
\text{H}_2\text{O}_2 + \cdot \text{OH} & \rightarrow \text{HO}_2 \cdot + \text{H}_2\text{O} \quad (8) \\
\text{Fe}^{2+} + \cdot \text{HO}_2 & \rightarrow \text{Fe}^{3+} + \text{H}_2\text{O}_2 \quad (9) \\
\cdot \text{OH} + \text{HO}_2 \cdot + \text{H}^+ & \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad (10) \\
2\text{HO}_2 \cdot + \text{H}^+ & \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad (11) \\
2 \cdot \text{OH} & \rightarrow \text{H}_2\text{O}_2 \quad (12)
\end{align*}
\]

In situ production of H₂O₂ can address the challenges of storage and shipment of concentrated H₂O₂ required for the traditional Fenton process (Moreira et al. 2017). H₂O₂ can be continuously synthesized from the two-electron electrochemical reduction of oxygen in acid media with E₀ = 0.695 V (vs. standard hydrogen electrode (SHE)) or in alkaline solutions with E₀ = 0.065 V (vs. SHE) (Liu et al. 2010). The advantage of H₂O₂ electrogeneration in acid media for the electro-Fenton (E-Fenton) process has been documented (Liu et al. 2010). The yield and efficiency of H₂O₂ electrogeneration are important factors affecting the E-Fenton process, which in turn depends on the type and properties of the cathode materials. Carbon cathodes are widely used for the E-Fenton reaction due to their characteristics, such as low cost, high surface area, and affordability in large-scale applications (Zhou et al. 2013; Le et al. 2015; Zhang et al. 2015; Le et al. 2016).

This investigation is based on the proposed synergy of E-Fenton and T. versicolor functionality. Electrochemical technology was used to promote the rapid growth of white-rot fungi under the action of applied voltage (Thrash and Coates 2008; Loghavi et al. 2009) and to provide a low-pH environment for Fenton reactions. Therefore, for the sake of environmental conditions and the convenience of experimentation, dealkaline lignin was selected for this study. Dealkaline lignin can be defined as the lignin resulting from alkaline
pulping of wood, followed by neutralization of the pH of the pulping liquor. The continuous Fenton reactions between H$_2$O$_2$ produced by the composite cathode and Fe$^{2+}$ on the cathode produced a large amount of hydroxyl radicals for the degradation of lignin. At the same time, H$_2$O$_2$ can quickly start LiP and MnP enzymatic hydrolysis reactions to accelerate the progress of lignin biodegradation. The synergetic effect of E-Fenton and T. versicolor on lignin degradation was studied, and the results provide the basis for a lignin degradation strategy.

**EXPERIMENTAL**

**Materials**

**Lignin and strain**

The dealkaline lignin was a mixture of different herbaceous plants, such as corn stover, bamboo, and straw, and was purchased from Shanghai Chemical Reagent Four Factory (Shanghai, China). *T. versicolor* (bio-66843) was purchased from the China Center of Industrial Culture Collection (Beijing, China).

**Culture medium**

The white-rot fungi medium for lignin fermentation contained alkaline lignin (500 mg/L), KH$_2$PO$_4$ (2 g/L), MgSO$_4$ (0.25 g/L), CaCl$_2$ (0.1 g/L), MnSO$_4$ (5 mg/L), VB$_1$ (10 mg/L), ammonium tartrate (0.2 g/L), and trace elements (150 mL/L: NaCl, 1.0 g/L; FeSO$_4$•7H$_2$O, 100 mg/L; CoSO$_4$•7H$_2$O, 100 mg/L; CaCl$_2$, 100 mg/L; ZnSO$_4$•7H$_2$O, 100 mg/L; CuSO$_4$•5H$_2$O, 10 mg/L; KAl(SO$_4$)$_2$, 100 mg/L; H$_3$BO$_3$, 10 mg/L; and Na$_2$MoO$_4$, 10 mg/L). At a pH of 4.2 and 120 rpm, the samples were incubated in a constant temperature shaker incubator at 37°C for 5 d. All reagents were purchased from the Sinopharm Chemical Reagent Co., Ltd. (Beijing, China).

**Electrodes**

To prepare composite electrodes (Fe$^0$ and Fe$_3$O$_4$/GF), graphite felt was cut to a size of 4 × 13 cm$^2$, followed by ultrasonic cleaning for 12 min and drying in a convection oven at 60°C. In a 200-mL beaker, FeCl$_3$•6H$_2$O (9.66 g) was weighed and dissolved in 100 mL of 80% aqueous ethanol. The abovementioned pretreated graphite fiber was immersed in ferric chloride solution for 1 h, removed, and placed on a clean surface dish. NaBH$_4$ (3.54 g) was preliminarily dissolved in 100 mL of deionized water, and the NaBH$_4$ solution was dropwise added to the graphite fibers in the surface pan using a dropper. The dropping speed was 0.5 mL/s. The whole operation was completed at room temperature under a vacuum. During the dripping process, a large number of bubbles were produced, and black fluffy substances were generated on the graphite fibers. After dropwise addition for 1 h, residual NaBH$_4$ was removed, and the surface of the graphite fiber was rinsed with deionized water and ethanol. Infrared light drying was carried out under the protection of argon, and composite cathodes (Fe$^0$ and Fe$_3$O$_4$/GF) were obtained.

**Power supply**

A constant current was supplied using a DC power supply (Zhaoxin, China, 0-3 A, 0-5 V).

**Lignin Concentration Determination**

The sample was first hydrolyzed for 30 min at room temperature using 72% (w/w) H$_2$SO$_4$ and then hydrolyzed a second time for 60 min at 120°C with 4% H$_2$SO$_4$. The solid residue obtained after acid hydrolysis was determined. The ash content was determined in

an oven at 550 °C over 8 h. The lignin content on a free ash basis was the difference between the solid residue and ash (Ballesteros et al. 2004).

The lignin degradation ratio was calculated using the following formula: 
\[
R(\%) = 100 \times \frac{(m_0 - m)}{m_0},
\]
where \(R\) is the degradation ratio of the sample; \(m_0\) is the initial content of lignin; and \(m\) is the content of lignin in the sample.

**Mycelium Dry Weight Determination**

The lignin medium after fermentation was poured into a 500-mL beaker and placed in a lyophilizer for 24 h. The amount of residual solid after drying was weighed and recorded as the mycelium dry weight.

**Ligninolytic Enzyme Activity Determination**

**MnP**

A total of 3.4 mL of sodium lactate buffer (50 mmol/L, pH 4.5), 0.1 mL of MnSO\(_4\) solution (1.6 mmol/L), and 0.4 mL of crude enzyme solution were added to a colorimetric tube. After preheating at 37 °C, 0.1 mL of hydrogen peroxide solution (1.6 mmol/L) was added to initiate the reaction. The change in absorbance during the first 3 min of the reaction was measured at a wavelength of 240 nm (Glenn et al. 1986). One unit of activity was defined as the amount of enzyme releasing 1 mol of product per min per liter (U/L).

**LiP**

A total of 0.5 mL of crude enzyme solution and 500 µL of Azure B solution (Beijing, China) (0.160 mmol/L) were added to 1 mL of 125 mmol/L sodium tartrate buffer (pH 3.0) (Sinopharm Chemical Reagent Co., Ltd., Beijing, China). The mixture was preheated to 30 °C, and 0.5 mL of 2-mmol/L hydrogen peroxide solution was added to start the reaction. The change in absorbance during the first 3 min of the reaction was measured at a wavelength of 651 nm (Asina et al. 2016). One unit of activity was defined as the amount of enzyme releasing 1 mol of product per min per liter (U/L).

**Laccase**

Take the crude enzyme solution 100 µL, laccase activity was tested in 1 mM sodium acetate buffer at pH 5 with 0.5 M 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) (Bourbonnais et al. 1995) and measured in a microplate reader (Shanghai Flash Spectrum Biotechnology Co., Ltd., Shanghai, China) with a distance of 0.29 cm at 420 nm (\(\varepsilon_{420} = 36,000\) M\(^{-1}\) cm\(^{-1}\)).

**Lignin Structure Analysis by Heteronuclear Single-Quantum Correlation Nuclear Magnetic Resonance**

Heteronuclear single-quantum correlation–nuclear magnetic resonance (HSQC–NMR) provides accurate and powerful data on the structure of lignin. Sixty milligrams of lignin sample were solubilized in 0.5 mL deuterated DMSO-6 reagent solution (Sinopharm Chemical Reagent Co., Ltd., Beijing, China). The specific conditions of the acquisition map were as follows: pulse turn angle of 30°, pulse time of 13.6 µs, acquisition time of 0.17 s, relaxation time of 1 s, spectral width of 2200 Hz, resolution of 1024 DPI (Dot Per Inch), transients of 128 times, and time increments of 256 times (i.e., 128 × 256).

**Statistics**

The experiment was repeated three times without special instructions. The data were analyzed by the one-way ANOVA using IBM SPSS Statistics 22 to determine whether had a significant effect. Statistically significant results were obtained when the P value was <0.05.
RESULTS AND DISCUSSION

Effect of Current on the Growth of T. versicolor

The growth of microorganisms under different currents varies (Thrash and Coates 2008). Under the appropriate current, microbial growth is promoted, but a current too large may hinder the growth of microorganisms and even kill cells. The dry weight of T. versicolor grown at different currents for 96 h was measured. The results showed that in the absence of an applied current in the control, T. versicolor grew slowly. However, under an applied current, the growth of T. versicolor rapidly increased with increasing current. Different currents had different effects; the higher that the current was in the range 0.05 A to 0.3 A, the more obvious the promoting effect. As shown in Fig. 1, mycelium growth was slow without E-Fenton treatment at 96 h, and the dry weight was approximately 1.2 g lower than that with E-Fenton treatment.

![Fig. 1. Dry weight changes of mycelium at different current intensities after 96 h. Each value is the mean value ± standard error mean of triplicate.](image)

However, the growth of mycelia increased gradually with increased current under E-Fenton treatment in the synergistic system at 96 h, and the maximum amount of mycelium reached 2.3 g at 0.3 A. Considering the complex structures of lignin, the hydroxyl radicals produced by E-Fenton treatment destroyed structures and functional groups, promoting their utilization by T. versicolor as a carbon source for its mycelia, which in turn promoted cell growth. In addition, a proper current can effectively change the permeability of the cell membrane and promote the growth of fungi in liquid medium (Loghavi et al. 2009), which was one of the reasons that the mycelia of T. versicolor increased under electrification. The results clearly revealed the synergy between E-Fenton treatment and T. versicolor, and this synergy was confirmed by further chemical analysis.

Effect of the Synergistic Action Between E-Fenton Treatment and T. versicolor on the Degradation of Lignin

Previous studies have shown that T. versicolor ATCC 20869 was grown on Pinus20 taeda wood chips under solid-state fermentation conditions to examine the wood-degrading 21 mechanisms employed by this fungus. Although no lignin-degrading enzyme activity was detected, the lignin loss reached 7.5% (m/m) (Aguiar et al. 2014). In this study, E-Fenton processes with different currents (0.05 A, 0.1 A, 0.15 A, 0.2 A, and 0.3 A) were used to degrade lignin together with T. versicolor. As shown in Fig. 2, the rate of lignin degradation reached 84.5% when fermented with both E-Fenton (0.3 A) and T. versicolor. When using E-Fenton alone, the lignin degradation extent was only 9.1%. The lignin degradation was quite obvious in the case of white-rot fungi, where the lignin degradation...
ratio increased linearly up to 31.1%. In contrast, for the synergistic action of E-Fenton treatment and *T. versicolor* treatment, the lignin degradation ratio increased with the current increase. This result further confirmed that there was a synergistic relationship between the E-Fenton process and white-rot fungi in degrading lignin. The hydrogen peroxide produced by the E-Fenton process reacted with Fe$^{2+}$ on the cathode, producing a large number of hydroxyl radicals that directly degraded various functional groups in lignin, which caused the quick initiation of lignin peroxidase (LiP), and the acceleration of lignin biodegradation by the manganese peroxidase (MnP) enzymatic hydrolysis reaction. In addition, the hydroxyl radicals produced by the Fenton reaction also converted non-phenolic lignin into phenolic lignin and further promoted the ability of manganese peroxidase and laccase to degrade lignin. Additionally, the Fe$^{3+}$ secreted by white-rot fungi accelerated the regeneration of Fe$^{2+}$ on the composite cathode, which sustained the lignin degradation system.

![Fig. 2. Changes of lignin degradation rates at different current intensities after 96 h. Each value is the mean value ± standard error mean of triplicate.](image)

In general, lignin degradation of the tested strains in the synergistic system was much greater than that reported in previous studies. A *Pleurotus ostreatus* strain degraded corn stover, but no lignin degradation was observed (Yang *et al.* 2010); some *P. chrysosporium* strains decomposed 20.7% of lignin in wheat straw in two weeks (Singh *et al.* 2011) and 39.4% of lignin in paddy straw in 60 days (Sharma and Arora 2010). These results of the present study indicated that the synergistic action of E-Fenton technology and white-rot fungi could have great potential for lignin degradation. The E-Fenton process and *T. versicolor* treatment could synergistically promote lignin degradation, which was consistent with the increased mycelium growth (Fig. 1) and the lignin concentration assay (Fig. 2).

**Change of Ligninolytic Enzymes Activity**

In this study, the activities of three enzymes of *T. versicolor* were determined under different currents of E-Fenton. As shown in Fig. 3, the activity of the three enzymes increased with the increase of currents over 96 h. Among them, the activity of MnP increased significantly (Fig. 3a). This is consistent with the work of Dinis *et al.* (2009); who reported that MnP activity was higher than that of laccase. Under conditions that included no addition of E-Fenton, 0.05 A, 0.10 A, and 0.15 A, the activity of MnP increased slowly over 96 h, giving values of 99 U/L, 141 U/L, 179 U/L, and 198 U/L.
Fig. 3. Production of peroxidase activity by T. versicolor at different currents: (a) MnP; (b) LiP; (c) Lac. Each value is the mean value ± standard error mean of triplicate.
However, it increased rapidly under 0.2 A and 0.3 A, and reached 298 U/L and 402 U/L at 96 h. The increasing trend of LiP activity was similar to that of laccase activity (Fig. 3b and 3c): the enzyme activity increased gradually with the increase of currents over 96 h, but it did not increase significantly under each current condition. It is worth noting that the enzymatic activity of the synergistic system was higher than that of white-rot fungi alone, and it increased with the increase of current in the synergistic system. These results differed from a previous study (Osma et al. 2011), which revealed that laccase activity remained consistent (around 400–500 UL⁻¹) over time, whereas LiP and MnP activity varied over time. The laccase activities of Phlebiopsis gigantea SPl0g6 and RM22b were low (enzyme activity was around 0 to 0.5 units per mL) within 28 days, whereas Ceriporiopsis subvermispora D98698 showed quite high activity, approximately 5 to 6 units per mL (Baker et al. 2015). The absence or low level of Lac activity and the presence of polyphenol oxidase (PPO) activity in P. chrysosporium PC2 and L. edodes LE16 (Dong et al. 2013) contrasts previous reports on the correlation between marked lignin degradation and Lac activity in wheat straw degradation (Isikhuenhen and Mikiashvilli 2009). In other studies, only Lac and MnP were considered as the main ligninolytic enzymes in white-rot fungi (Dinis et al. 2009). Ligninase activities in L. edodes and polyphenol oxidase (PPO) activity in P. chrysosporium have not been reported previously in sugar cane bagasse (SCB) decomposition (Camassola and Dillon 2009).

In general, the activities of three lignin degradation enzymes in T. versicolor were significantly affected by the current and increased with the increase of the current.

**Changes of Functional Groups in Lignin Degradation by E-Fenton and White-rot Fungi**

In order to understand the changes in the functional groups before and after lignin degradation by synergistic system, HSQC-NMR spectra of the untreated lignin (Fig. 4a) and treated lignin (Fig. 4b) were obtained. The signals were analyzed according to previous literature (Yang et al. 2016; De Carvalho Oliveira et al. 2018; Jin et al. 2019). The HSQC analysis showed that the synergistic system of E-Fenton and white-rot fungi degraded lignin by reducing the functional group content. As shown in Fig. 4, as compared to the untreated lignin, synergistic system treatment resulted in a substantial decrease of functional groups. In particular, functional groups in aromatic regions and aliphatic regions were degraded almost completely. In the aromatic region of untreated lignin (103 to 145/6.0 to 8.0 ppm), the spectra showed peaks at 130.28/7.54 ppm related with the C₂,₆–H₂,₆ in p-coumaroylated substructures (PC₂₆) and at 127.9/7.23 ppm corresponding to the C₂,₆–H₂,₆ in p-hydroxyphenyl units (H₂,₆). The two main functional groups were completely degraded after synergistic treatment, and the signal could not be detected. There were also a large number of functional groups in the aliphatic region that degraded. The main signals in the aliphatic region of untreated lignin at 50 to 90/2.5 to 6.0 ppm were from –OCH₃, A₅, and X₅. The signals of C–H from methoxyls (–OCH₃) were in the region at 55.7/3.69 ppm, the C₅–H₅ in γ-hydroxylated β-O-4 substructures (A₅) were in the region at 59.64/3.38 ppm, and the C₅–H₅ in β-D-xylopyranoside (X₅) were in the region at 70.18/3.52 ppm. The signals of these functional groups were barely detected in the samples treated by the synergistic system. Because of the Fenton reaction, the hydroxyl radical produced by the cathode degraded all functional groups without specificity. The activity of ligninolytic enzymes was induced and the efficiency of lignin degradation was improved. Therefore, in the coordinated degradation of lignin by Fenton and white-rot fungi, the functional groups in the aromatic and aliphatic regions were almost completely degraded. The results indicated the E-Fenton and T. versicolor could synergistically promote lignin degradation, which was consistent with the lignin concentration assay (Fig. 2).

Fig. 4. The HSQC analysis of the reaction system after different treatments: (a) untreated lignin and (b) treated lignin

CONCLUSIONS

1. This study unveiled an effective synergy between T. versicolor and E-Fenton treatment for lignin degradation at both chemical processing and mycelia growth levels. Compared with the traditional chemical method and biological method for lignin degradation, the synergistic effect of E-Fenton and white rot fungi can better explain the biodegradation process of lignin and provide a reasonable basis for the design of lignin degradation system.

2. At the current intensity of 0.05 to 0.3 A, the loading current had a clear and promoting effect on the growth of T. versicolor. Under the condition of receiving current, the dry weight of T. versicolor was obviously larger than that without current. The dry weight increased with the increase of current within 0.3 A.

3. The enzyme activity increased with increasing current and the effect of current on the activity of lignin degrading enzyme was significantly.

4. In this study, the enzyme activity and lignin degradation efficiency were improved by the electro-Fenton method combined with white rot fungus treatment, which established a promising strategy for lignin degradation and valorization.

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