Enzymatic oxidative polymerization of phenols by laccase from 
*Trametes* sp. Ha1

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**ABSTRACT**

Enzymatic oxidative polymerization of phenols with peroxidases and oxidases has been studied to produce phenolic resin without using toxic formaldehyde. We studied enzymatic oxidative polymerization of various phenols by laccase from *Trametes* sp. Ha1 (Daiwa Kasei) and showed the effects of reaction conditions and substituent groups on phenyl group on the polymerization. The laccase polymerized phenol in McIlvaine buffer (pH 4.5) containing 10-60% acetone although it slightly oxidized phenol in the buffer without organic solvents. The formed polymer was insoluble in both methanol and N,N-dimethylformamide (DMF) and considered to be cross-linked. The polymer yield was also very low (< 10%) in the enzymatic polymerization of alkylenphols ((o-, m-, and p-) cresols, (o- and p-) ethylphenols, and p-t-pentylphenol) in McIlvaine buffer and enhanced by the addition of 20-40% acetone into the buffer. On the other hand, the laccase polymerized methoxyphenols, such as o-methoxyphenol (guaiacol), in McIlvaine buffer without organic solvents. The polymerized methoxyphenols were soluble in DMF. The weight-average molecular weight of the polymers of o-methoxyphenol was 8,000-10,000. Double substituted methoxyphenol, 2,6-dimethoxyphenol, was also polymerized by the laccase in the buffer. The molecular weight of polymerized 2,6-dimethoxyphenol was very high and 80% of the polymer was insoluble in DMF. The reaction rates correlated with the ionization potentials of the methoxyphenols. The mono- and di- methoxyphenols would be suitable for production of phenolic resins by enzymatic oxidative polymerization by laccase without formaldehyde or organic solvent.

**KEYWORDS**
enzymatic oxidative polymerization, phenols, laccase

**INTRODUCTION**

The enzymatic oxidative polymerization of phenols was first performed to remove phenols in wastewater (Klibanov, et al., 1983). Then the reaction was applied to prepare formaldehyde-free phenol resins enzymatically (Dordick, et al., 1987; Kobayashi, et al., 2001). The use of formaldehyde in the chemical industry is desired to reduce because of its toxicity. Horseradish peroxidase (HRP, EC. 1.11.1.7) is the most widely used biocatalyst in the enzymatic oxidative polymerization of phenol, aniline or their derivatives. Akkara, et al. (1991) used HRP in an aqueous dioxane to prepare polymers and copolymers from different phenolic monomers. HRP is active in a number of organic solvents or solvent mixtures, and hydrogen peroxide is used as an oxidant. Laccase (EC. 1.10.3.2) is a copper protein that oxidizes phenols with oxygen molecules. The enzyme was originally found in the sap of lacquer trees (*Rhus verniciflua*, etc.). It oxidizes urushiol, a catechol derivative, to form lacquer naturally and industrially. White rot fungi (*Trametes versicolor*, *Pleurotus ostreatus*, *Pycnoporus coccineus*, etc.), which belong to basidiomycete, produce laccases. Fungal laccases also oxidize phenol and its derivatives (Tanaka and Taniguchi, 2003). Some researchers have used the enzymes to prepare phenol...
resins (Ikeda, et al., 2001; Kobayashi, et al., 2001) (Figure 1). In the polymerization by laccase, the molecular oxygen is used as an oxidant. Thus, hydrogen peroxide, which is toxic and necessary in the reaction of peroxidase, is unnecessary.

![Figure 1](image.png)

**Figure 1. Oxidation and polymerization of phenols by laccase (X = CH₃, C₂H₅, OCH₃, C₆H₅, etc.).**

In this paper we have studied enzymatic oxidative polymerization of phenol and its derivatives by an industrial fungal laccase from *Trametes* sp. Ha1. The effects of reaction conditions and the functional groups on the benzene rings on the reaction rate and the yield of polymer were discussed.

**MATERIALS AND METHODS**

**Enzyme**

A fungal laccase from *Trametes* sp. Ha1 (Laccase Daiwa) was kindly supplied by Daiwa Kasei Co. Ltd. (Osaka) and was used without purification as an enzyme preparation. One unit (U) of laccase activity was defined as the amount of enzyme that increased the absorbance at 440 nm by 1.0 per min in the reaction mixture (pH 4.5, McIlvaine buffer) containing 0.4 mM o-phenylenediamine at 30°C (Kofujita et al. 1991).

**Reagents**

All chemicals were commercial products of at least reagent grade.

**Enzymatic oxidative polymerization**

As a typical reaction, 1 cm³ of 2 U cm⁻³ laccase solution in a modified McIlvaine buffer (pH 4.5) was added to 4 cm³ of 250 mM phenol solution in the buffer containing 25% acetone in a 50-cm³ flask. The concentrations of laccase, phenol, and acetone were 0.4 U cm⁻³, 200 mM, and 20% in the reaction mixture, respectively. The flask was shaken at 45 strokes per minute and 30°C. For the measurement of initial reaction rates, 1 cm³ of 56.7 mM sodium sulfite solution and 4 cm³ of acetonitrile were added to the reaction mixture after 2 or 4 h of reaction to terminate the reaction. Then the mixture was diluted 100 times with 40% acetonitrile solution. To yield the polymer, the reaction mixtures were incubated for 24 h unless otherwise noted. Then the solvent in the mixture was evaporated under reduced pressure.
The residue was suspended in 30 cm$^3$ of methanol overnight and then filtered with a filter paper (No. 2, Advantec, Tokyo). The residue on the filter paper was washed with 30 cm$^3$ of water and 30 cm$^3$ of methanol. The residual solid after evaporation of methanol was designated as polymer product.

**Measurement of dissolved oxygen**

The concentration of dissolved oxygen during laccase reactions was measured at 30ºC with a biological oxygen monitor (Model 5300, Yellow Springs Instrument Co., OH) and a reaction cell containing 3 cm$^3$ of the reaction mixture. After 3 cm$^3$ of phenol solution containing 0% acetone were preheated in the reaction cell, 50 mm$^3$ of laccase solution was added to the cell with a microsyringe and then the oxygen concentration (the percent of saturation of oxygen to the reaction mixture) was monitored.

**Analysis**

The phenolic compounds were determined by high-performance liquid chromatography (HPLC) equipped with a reversed phase column (Shim-pack CLC-ODS ((M)), Shimadzu, Kyoto). The samples were eluted with 40% acetonitrile containing 30 mM potassium phosphate buffer (pH 3.7) at a rate of 1.0 cm$^3$ min$^{-1}$ (LC-6A, Shimadzu). Phenol, $o$-cresol, $m$-cresol, $p$-cresol, 2,6-dimethyphenol, $o$-ethylphenol, $o$-methoxyphenol, $m$-methoxyphenol, $p$-methoxyphenol, and 2,6-dimethoxyphenol were detected by a UV detector (Variable Wavelength Monitor, Hitachi, Tokyo) at 273, 273, 274, 279, 272, 273, 276, 275, 290, and 270 nm, respectively.

The weight average molecular weight of poly($o$-methoxyphenol) was determined at 50ºC by HPLC equipped with a gel permeation column (Shodex GPC KD-806M, Showa Denko, Tokyo) and a refractive index detector (RID-10A, Shimadzu, Kyoto). The mobile phase solution was $N,N$-dimethylformamide (DMF) containing 0.01 M LiBr. The column was calibrated with polystyrene standards (Showa Denko).

**RESULTS AND DISCUSSION**

**Enzymatic oxidative polymerization of phenol**

Figure 2 shows the enzymatic oxidative polymerization of phenol by laccase from *Trametes* sp. Ha1. The laccase slightly oxidized in McIlvaine buffer and no polymer was formed. When 20% of acetone was added to the reaction mixture, phenol was oxidized by the enzyme and methanol-insoluble polymer was formed. The formed polymer was insoluble in both methanol and DMF and considered to be cross-linked. Figure 3 shows the effect of content of acetone in reaction mixture on the initial reaction rate (the rate of decrease in phenol concentration) and the polymer yield. The laccase oxidized phenol and polymer was formed in McIlvaine buffer containing 20-50% acetone. The maximum yield was obtained at 20% of acetone. Uyama et al. (1996) shows the similar results in the enzymatic oxidative polymerization of phenol by horseradish peroxidase. The polymer yield was the maximum at 60% of 1,4-dioxane in the polymerization by the peroxidase. The difference would be due to the solvent or enzyme.
Enzymatic oxidative polymerization of alkylphenols

Figure 4 shows the enzymatic oxidative polymerization of o-cresol and o-ethylphenol by laccase. The oxidation rates and polymer yields in the both reactions were low in the buffer without acetone. The reaction rates were increased by the addition of acetone at 20-40%. The maximum rate and yield
were obtained at different acetone contents among the phenols. The maximum initial reaction rate was increased in the order of phenol < o-cresol < o-ethylphenol. The longer o-alkyl group is more electron donative to the hydroxyl group of the phenol and let the laccase oxidize the phenol more easily.

The formed poly(o-cresol) was partially soluble in DMF while the poly(o-ethylphenol) was soluble in DMF. The yield of the latter was lower than that of the former in spite of the higher reaction rate of its monomer. The low yield of poly(o-ethylphenol) would be due to the high solubility of the polymer. The polymer yield was also very low (< 10%) in the enzymatic polymerization of alkylphenols (m-cresol, p-cresol, p-ethylphenol, and p-t-pentylphenol) in McIlvaine buffer and enhanced by the addition of 20-40% acetone into the buffer (data not shown). Uyama et al. (1995) reported the polymerization of cresols by HRP or soy bean peroxidase in a buffer containing 80% of 1,4-dioxane although they did not examine the effect of content of organic solvent. In contrast to other alkylphenols, 2,6-dimethylphenol was polymerized by laccase in the buffer. The two methyl groups let the phenol more easily oxidized than o-cresol (2-methylanilphenol). The yield of poly(2,6-dimethylphenol) was increased by the addition of acetone as well as the other alkylphenols and was the maximum at 30% of acetone. Ikeda et al. (1996) showed that the similar tendency in the polymerization of 2,6-dimethylphenol by laccase from _P. coccineus_ in a mixture of acetone and acetate buffer (pH 5.0). They also showed that 3,5,3',5'-tetramethyl-4,4-diphenoxquinone, which is soluble in methanol, forms as well as polymer in the oxidation of 2,6-dimethylphenol by laccase.

Enzymatic oxidative polymerization of o-methoxyphenol

The laccase polymerized methoxyphenols in McIlvaine buffer without organic solvents in contrast to cresols (methylphenols). Figure 5 shows the polymerization of o-methoxyphenol (guaiacol) by laccase in the buffer. The monomer was consumed in 8 h and then the polymer was formed. The formed polymer was soluble in DMF. The weight average molecular weight of the polymers of o-methoxyphenol was 8,000-10,000. The maximum yield was 40% although the monomer was
completely consumed. The polymer or oligomer was partially washed away with methanol or water due to its low molecular weight and high solubility.

![Graph showing enzymatic oxidative polymerization of o-methoxyphenol by laccase](image)

**Figure 5.** Enzymatic oxidative polymerization of o-methoxyphenol by laccase. Enzyme concentration = 0.4 U cm⁻³; content of acetone = 0%. Circles: monomer concentration; triangles: polymer yield; squares: weight average molecular weight of the polymer product.

The Michaelis constant calculated from oxygen consumption rate at 0.1-3.0 mM of o-methoxyphenol by Lineweaver-Burk plot was 0.4 mM. However, the initial reaction rate increased from 10 to 500 mM of the monomer concentration (Figure 6). Thus, the reaction mechanism was considered as in the case of laccase oxidative polymerization of 4-chloroguaiacol (Tanaka et al. 2003). At first, the monomer phenol (M) is oxidized by laccase (E) to form phenoxyl radical (M·). The reaction corresponds to the initiation in free radical polymerization of vinyl monomers.

\[
E + M \rightarrow M^\cdot \quad (1)
\]

The formed radical (M·) reacts with monomer to form a dimer radical (M₂·). Then the dimer radical reacts with another monomer. The reaction corresponds to propagation in free radical polymerization.

\[
M_{n^\cdot} + M \rightarrow M_{n+1}^\cdot \quad (2)
\]

The radical species disappear by recombination and disproportionation. The reactions are termination in free radical polymerization.

\[
M_{n^\cdot} + M_{m^\cdot} \rightarrow M_{n+m}^\cdot \quad \text{or} \quad M_n + M_m \quad (3)
\]

Assuming that the rate of monomer radical follows as Michaelis-Menten’s equation and that the propagation and termination proceed as those in the radical polymerization of vinyl monomers (Painter and Coleman 1997), the rate of decrease in the concentration of (substituted) phenols by the enzymatic oxidative polymerization \( R \) is expressed as,

\[
R = -\frac{d[M]}{dt} = \frac{k_{cat}[E][M]}{K_m+[M]} + k_p \sqrt{\frac{k_{cat}}{k_i}} \sqrt{\frac{[E][M]^2}{K_m+[M]}} \quad (4)
\]
where $k_{\text{cat}}$, $K_m$, [E], [M], $k_p$ and $k_t$ are maximum turnover number, Michaelis constant, enzyme concentration, monomer concentration, rate constant of propagation, and that of termination, respectively. The second term of Eq. (4) continues to increase linearly after the first term reaches close to the maximum reaction rate of the enzyme reaction (Eq. (1)) when the monomer concentration increases.

Figure 6. Effect of monomer concentration on enzymatic oxidative polymerization of $\alpha$-methoxyphenol by laccase. Enzyme concentration = 0.4 U cm$^{-3}$; content of acetone = 0%. Circles: initial reaction rate; triangles: polymer yield; squares: weight average molecular weight of the polymer product.

Figure 7. Effect of enzyme concentration on enzymatic oxidative polymerization of $\alpha$-methoxyphenol by laccase. Monomer concentration = 200 mM; content of acetone = 0%. Circles: initial reaction rate; triangles: polymer yield; squares: weight average molecular weight of the polymer product.
Figure 7 shows the dependencies of the initial reaction rate and polymer yield on the enzyme concentration in the polymerization of \( \text{o-methoxyphenol} \) by laccase. The reaction rate increases less than proportionally to the enzyme concentration in contrast to general enzyme reactions where the reaction rate increases linearly. The tendency can be explained by the second term of Eq. (4), which increases proportionally to the square root of enzyme concentration. Figure 8 shows the dependence of the initial reaction rate on the content of acetone in the enzymatic oxidative polymerization of \( \text{o-methoxyphenol} \). The dependence was different from that in the polymerization of phenol and alkylphenols. The reaction rate decreased with increase of the content of acetone. The polymer yield was also nearly maximum value in the buffer without acetone. The methoxy group increased the reactivity of phenol more than methyl group in the buffer due to its higher electron donation.

![Graph showing the effect of acetone content on enzymatic oxidative polymerization of \( \text{o-methoxyphenol} \) by laccase.](image)

**Figure 8.** Effect of content of acetone on enzymatic oxidative polymerization of \( \text{o-methoxyphenol} \) by laccase. Enzyme concentration = 0.4 U cm\(^{-3}\). Circles: monomer concentration; triangles: polymer yield; squares: weight average molecular weight of the polymer product.

**Enzymatic oxidative polymerization of other methoxyphenols**

Figure 9 shows the initial reaction rate and the polymer yield in the polymerization of different methoxyphenols by laccase. The initial rate was the highest in the polymerization of \( \text{p-methoxyphenol} \) among the methoxyphenols. However, the yield of 2,6-dimethoxyphenol was the highest even in the buffer. The molecular weight of polymerized 2,6-dimethoxyphenol was very high and 80% of the polymer was insoluble in DMF. The initial reaction rate correlated well with the ionization potential of monomers (Figure 10). The lower ionization potential leads to the easier oxidation of the methoxyphenol by both laccase in initiation and phenoxyl radical in propagation. The values measured by Yoshida et al. (1997) were used as ionization potentials.

\( \text{o-Methoxyphenol} \) and 2,6-dimethoxyphenol will be suitable for production of phenolic resins by enzymatic oxidative polymerization by laccase without formaldehyde, hydrogen peroxide or organic solvent. Co-polymerization of \( \text{o-methoxyphenol} \) and 2,6-dimethoxyphenol will be possible to obtain a DMF-soluble and high-molecular-weight polymer at a high yield. These polymers have methoxy groups.
likewise lignin that is a natural phenolic resin.

Figure 9. Effect of content of acetone on initial reaction rate (a) and polymer yield (b) in enzymatic oxidative polymerization of methoxyphenols by laccase. Monomer conc. = 200 mM; enzyme conc. = 0.4 U cm$^{-3}$. Open circles: $o$-methoxyphenol; open triangles: $m$-methoxyphenol; open squares: $p$-methoxyphenol; open rhombi: 2,6-dimethoxyphenol; closed circles: phenol.

Figure 10. Effect of ionization potential on initial reaction rate in enzymatic oxidative polymerization of methoxyphenols by laccase. Monomer concentration = 200 mM; enzyme concentration = 0.4 U cm$^{-3}$; content of acetone = 0%.
CONCLUSIONS

Addition of organic solvent, such as acetone, was necessary to prepare phenolic resins via enzymatic oxidative polymerization of phenol or alkylphenols by laccase. Methoxyphenols can be polymerized by laccase in buffer without organic solvent. The dependence of reaction rate on concentrations of monomer and enzyme suggests that the enzymatic oxidative polymerization of o-methoxyphenol is initiated by the oxidation of the monomer by laccase and that a propagation reaction proceeds between the formed radicals and monomer molecules.

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