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# A Kinetic Model Development for Phenol Removal via Enzymatic Polymerization

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**Abstract**

Enzymatic oxidative polymerization of phenol catalyzed by laccase enzyme extracted from *Trametes versicolor* (ATCC 200801) in an aqueous solution containing acetone was investigated in a batch system. The effects of initial phenol and dissolved oxygen concentrations on the initial reaction rate of oxidative polymerization were experimented. An interactive kinetic model as a function of phenol and dissolved oxygen concentrations was developed for enzymatic polymerization and corresponding bio-kinetic parameters have been evaluated through a non-linear regression program so called SYSTAT 10.0 trial version. The bio-kinetic coefficient of the developed model, which are $V_{max}$, $K_{mm}$, $K_{mO_2}$ and $k_2$ were obtained as 0.029 mg DO /L.min, 66.58 mg/L, 0.89 mg/L, and 254 $s^{-1}$ respectively. The activation energy of oxidative phenol polymerization was calculated as 21.175 kJ/mol.

## INTRODUCTION

The enzymatic oxidative polymerization of aromatic compounds by catalytic effects of peroxidase enzymes has been extensively investigated with a strong environmental concern [1, 2]. Peroxidases, horse radish peroxidase, lipase, laccase, bilirubin oxidase, oxidize the aromatic compounds to form aromatic radicals, which in turn combine to form polymeric structures to precipitate spontaneously from solution due to their low solubility.

Phenolic polymers and resins have been used in industry as composite laminates, adhesives, fiber bonding, abrasives, foundry resins, and molding materials [3]. Because of the conventional polymerization methods, which are depend on toxic and precious inorganic catalysts, enzymes seem to be alternative catalyzes which catalyzes aqueous aromatic compounds in the presence of a water miscible organic solvent [4]. These enzymes are lipase [5], soybean peroxidase [2,6], bilirubin oxidase [7], horseradish peroxidase [8,9], tyrosinase [10] and laccase [11-13]. For the polymerization of phenol and its derivatives, horseradish peroxidase was widely used in the presence of $H_2O_2$ [14]. However, it is favorable to replace horseradish peroxidase with laccase because of the utilization of dissolved oxygen instead of $H_2O_2$, which causes inhibition and deactivation of the enzyme [13]. The usage of poly(phenol) has not been thoroughly investigated yet, just reported in few studies in literature [15-21].

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In this study, batch experiments were conducted to investigate the effects of substrate concentrations, phenol and dissolved oxygen, and temperature, on the initial reaction rate of oxidative phenol polymerization. An original multiplicative kinetic model for oxidative enzymatic polymerization was suggested as a function of initial phenol and dissolved oxygen concentrations. For model development, only the basic kinetic equations based on the quasi-steady-state approach for enzyme kinetics in the literature were employed. After finding the best-fit kinetic model, biokinetic parameters and activation energy of the oxidative polymerization reaction were evaluated accordingly.

## EXPERIMENTAL

### Materials

Solid malt extract-agar nutrient medium was purchased from Difco® (USA). Guaiacol and phenol were purchased from Sigma (USA) and BDH (UK), respectively, and were used as received. All organic solvents used in this study were obtained from Merck (Germany) and were of high purity and other chemicals purchased from various commercial suppliers were of analytical grade purity.

### Growth of Microorganism and Laccase Production

The culture *Trametes versicolor* (ATCC 200801) was obtained from the American Type Culture Collection. The microorganism, reactivated as described by ATCC, was first grown on agar slant tubes using malt extract-agar nutrient solid medium as described in the literature [13]. After 7 days of incubation, cultures in agar slant tubes were taken from the incubator and the culture on the slope was washed with 5 $cm^3$ of sterile distilled water to form a suspension. This suspended culture was then transferred aseptically into 100 $cm^3$ of liquid growth medium [22] in cotton-plugged erlenmeyer flasks of 250 $cm^3$. After inoculation, the cultures were incubated at 30°C with shaking at 150 rpm for 10 days. Extracellular laccase production was induced by adding 0.001 $cm^3$ p-xylidine after five days of incubation [23]. After 10 days, the fermentation medium was centrifuged at 5000 rpm for 5 min and clear supernatant dispensed into test tubes then stored frozen at -35°C. For use, frozen enzyme solution was thawed at room temperature, the activity was determined and the solution was used as required. This procedure enabled the use of standard enzyme activity. Laccase activity was determined according to literature [13].

### Experimental Set-Up and Procedure

Experiments were carried out in a pyrex flask with a net volume of 183 $cm^3$. A voltametric dissolved oxygen probe (CyberScan DO 100) with an o-ring was placed in the gas-tight neck to measure the dissolved oxygen concentration in the reaction medium with a precision of 0.01 g per $m^3$. The rate of dissolved oxygen consumption in the flask was taken as the base to monitor the reaction rate. The reactor was immersed in a constant temperature water bath operating at 25°C with a precision of ± 0.1°C. Reaction medium was mixed with a magnetic stirrer at 250 rpm. Prior to reaction, a prescribed amount of phenol was dissolved in a mixture of acetone (10% v/v) and sodium-acetate buffer (90% v/v) (50 mM, pH 5). When needed, high dissolved oxygen concentrations were maintained in the medium by sparging pure oxygen gas through the reaction solution. The polymerization started upon the addition of pre-determined laccase solution and dissolved oxygen concentration was monitored continuously. The reaction was terminated approximately after 60 min. depending on reaction conditions. Blank runs were also conducted under the same conditions without using phenol and without using enzyme in order to attribute all dissolved oxygen drop solely to consumption in oxidative polymerisation.

The initial reaction rate of polymerization was calculated in terms of initial consumption rate of dissolved oxygen, which was equal to one fourth of the consumption rate of phenol, due to a 1 to 4 stoichiometric ratio in the polymerization reaction [13]. Initial consumption rate of dissolved oxygen was estimated through linear regression of initial data of dissolved oxygen concentration.

**Model Development**

The laccase-catalyzed polymerization of phenol is an example of multi-substrate enzyme reactions. In those kinds of reactions the first substrate is oxygen which reduces at the active site of laccase enzyme to form oxidized enzyme-oxygen complexes which then abstract one or two hydrogen form aromatic substance as a second substrate. A series of reactions are necessary in laccase-catalyzed such reaction to achieve a polymer as final product.

Step 1: According to Lee and coworkers [19]; in the first step, oxygen binds to the copper centers of laccase and oxidizes those four copper atoms to form a laccase-oxygen intermediate. They reported that the intermediate occurs in two steps for laccase derived from *R.vernicifera*. i) Oxygen binds to the copper centers of laccase to form peroxy-intermediate with a rate constant of $2x10^6$ $M^{-1}s^{-1}$. ii) The obtained intermediate is not stable and simultaneously turns into native-intermediate yielding all of the copper atoms oxidized. The native intermediate is assumed as primary acceptor of reducing substrates under turnover conditions [19, 20]. This observation can be extended for fungal laccase, as Kurniawati and Nicell [21] applied, since all laccase utilize oxygen as primary oxidant.

In the model development those two steps of the oxidation of the enzyme can be combined in single step to yield laccase-oxygen complex. At the low concentration of laccase and oxygen the overall reaction can be represented as follow with a second-order rate constant, $k_1$.

$$E + O_2 \xrightarrow{k_1} OEO \qquad (1)$$

Step 2: Subsequently, *OEO* complex captures two substrate molecules into its active site in two steps and abstracts two $H^+$ per molecule of substrate generating substrate radicals as the reaction product. The enzyme turns its initial state after two cycle of the oxidation of the substrate. Those two steps are represented as follow.

$$OEO + S \longrightarrow OEOS \longrightarrow EO + P + H_2O \qquad (2)$$

$$OE + S \longrightarrow EOS \longrightarrow E + P + H_2O \qquad (3)$$

As in the model development one of these two reactions is assumed to be rate limiting step. Hence, the kinetics of the two-cycle of laccase can be modeled as a single step represented in reaction (4) with an overall reaction with a single apparent rate-constant, $k_2$.

$$OEO + 2S \longleftrightarrow SOEOS \xrightarrow{k_2} EO + 2P + 2H_2O \qquad (4)$$

Where *P* is represent phenol radical formed by the enzyme.

Step 3: In the final step; those formed radicals are released to the reaction mixture from the active site of the enzyme molecules and simultaneously linked to form polymer chains. The polymer chains precipitate whenever they achieve an insoluble molecular chain length.

Experimentally, the initial reaction rate data can be collected by monitoring either disappearance rate of *OEO* complex or disappearance rate of phenol in the reaction mixture. Disappearance rate of *OEO* complex in reaction (4) would be equal to the disappearance rate of dissolved oxygen (DO) in reaction (1) for the batch reaction. Hence, monitoring initial consumption rate of DO

concentration at a range of initial phenols and DO concentrations in the batch reactor was taken as the initial reaction rate for the laccase-catalyzed reaction system.

The polymerization kinetics of phenol is based on expressions of enzyme kinetics. A double substrate interactive model for enzyme kinetics was suggested as a function of initial phenol and dissolved oxygen concentrations. Basic single substrate expressions in literature based only on quasi-steady state approach for enzyme kinetics were combined alternatively in the model to find the best fit by non-linear regression. The single substrate expressions used in the analysis are presented in Table 1 [22]. Combinations of contributing equations, exactly 25, were analyzed through Systat 10 [23], a package non-linear regression program, to find the expression giving the best fit.

Table 1. Single substrate expressions based on quasi-steady state kinetics for enzyme kinetics in literature [22].

| | | |
|---|---|---|
| $V = \frac{V_m S}{K_m + S}$ | (Michaelis-Menten) | (1) |
| $V = \frac{V_m S^n}{K_m + S^n}$ | (Moser) | (2) |
| $V = \frac{V_m S}{K_m(1 + \frac{S}{K_i}) + S}$ | (Competitive inhibition) | (3) |
| $V = \frac{V_m S}{(K_m + S)(1 + \frac{S}{K})}$ | (Non-competitive inhibition) | (4) |
| $V = \frac{V_m S}{K_m + S(1 + \frac{S}{K_i})}$ | (Mixed inhibition) | (5) |

## RESULTS and DISCUSSION

Initial rates of laccase catalyzed polymerization of phenol were calculated based on drops in dissolved oxygen concentration in the batch experiments. The initial rate of drop of dissolved oxygen concentration was determined by linear regression analysis of dissolved oxygen data. Figure 1 shows typical changes in dissolved oxygen over the course of a reaction, and the slope of the curve tracking the initial.

Figure 2 shows the variation of the initial reaction rate with monomer phenol and dissolved oxygen concentrations. The initial reaction rate data were measured with high correlation coefficients not smaller than 0.99. A blank run under applied conditions without phenol but with the enzyme did not show any dissolved oxygen drop more than 0.1 mg per L within an hour proving insignificant oxygen consumption of the electrode and/or the medium. Another blank run without the enzyme but with phenol again did not show dissolved oxygen drop more than 0.1 mg per L within an hour assuring non-

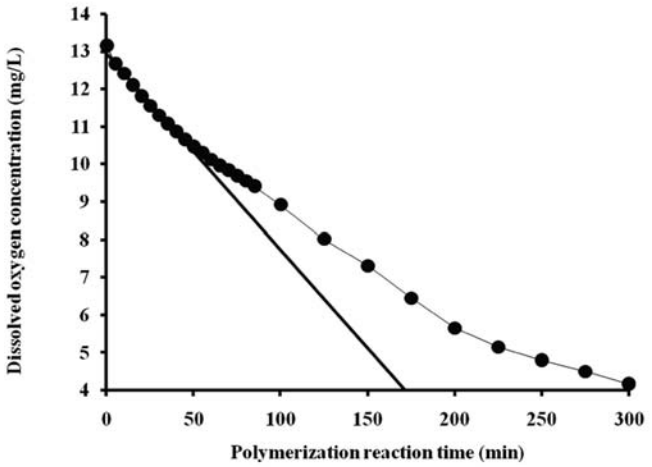


Figure 1. Drop in dissolved oxygen concentration during the batch polymerization reaction.

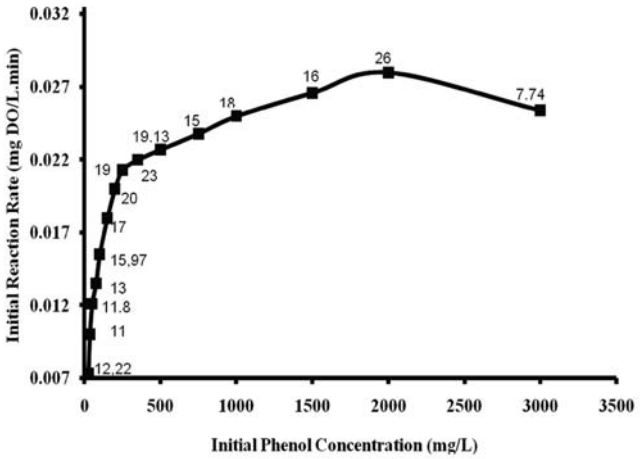


Figure 2. Variation of initial reaction rate by phenol and dissolved oxygen concentration.

existing self polymerization or oxidation of phenol in the medium.

As a general trend, the initial reaction rate reached a plateau value above 500 mg/L phenol concentration.

**Kinetic Model Assessment**

For non-linear analysis of data in Figure 2, different interactive forms of quasi-steady-state equations in Table 1 were employed. Out of 25 combinations, the conventional Michaelis-Menten kinetics expression for phenol and dissolved oxygen, were determined as the best fit model with a loss of $0.80406 \times 10^{-3}$ after 29 iterations, $R^2$=0.962. The loss is defined as the summation of squared error i.e. the error was the difference between the experimental and estimated reaction rates. The best-fit equation is represented as,

$$V = V_{max}\left[\left(\frac{S_m}{K_{mm} + S_m}\right)\left(\frac{S_{O_2}}{K_{mO_2} + S_{O_2}}\right)\right] \quad (5)$$

where $V_{max}$, $K_{mm}$, and $K_{mO_2}$ were estimated as 0.029 mg DO/L.min, 66.58 mg/L.min, 0.891 mg /L.min, respectively, at a fixed initial enzyme activity (10 U in total). $K_{mm}$ found for phenol in this work was close to that of pyrogallol, denoting strong monomer-enzyme affinity by its low value. $K_{mO_2}$ for phenol was smaller than $K_{mm}$, again showing higher affinity of the laccase enzyme for the substrate dissolved oxygen. In most kinetics model only monomer concentrations were monitored and taken into account for kinetic models [24]. Substituting initial concentrations of both substrates into Equation 1 with calculated coefficients, theoretical initial reaction rates were estimated and plotted against experimental reaction rates in Figure 3 to visualize the fit. As it is clear from the graph and statistical parameters, the proposed double substrate model fits the data fairly well.

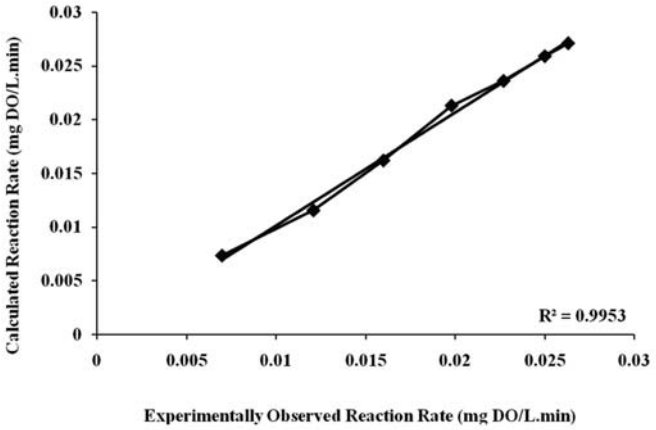


Figure 3. Theoretically calculated reaction rates vs. experimentally observed ones.

**Determination of Activation Energy**

For elucidating the temperature effect on the reaction rate alone, experimental initial reaction rate values at fixed 1000 mg/L phenol, 11 mg/L dissolved phenol concentrations and 10 U total enzyme activity.

A series of runs were carried out for the variations of initial reaction rates with temperature. Employing Equation 1, $V_{max}$ values were evaluated at five different temperatures of 15, 25, 35, 40, 45 and 50°C. Equation 2 is the equation to relate the maximum enzyme reaction rate, $V_{max}$, of Michaelis-Menten kinetics to enzyme concentration, $E_0$, at quasi-steady state [22].

$$V_{max} = k_2 [E_0] \quad (6)$$

where

$$k_2 = k_0 e^{-E_a/RT} \quad (7)$$

which leads to Equation 6 in logarithmic form,

$$\ln k_2 = \ln k_0 - E_a/RT \quad (8)$$

Five different reaction rate values were evaluated for temperature and lnV versus 1/T was plotted in Figure 4 where activation energy, $E_a$, was

calculated, with a high correlation coefficient, from the slope. Activation energy for the enzymatic oxidative polymerization of phenol was determined as 21.175 kJmol$^{-1}$. This value is almost in the range of 30-60 kJmol$^{-1}$ for different laccase catalyzed oxidation reactions [24].

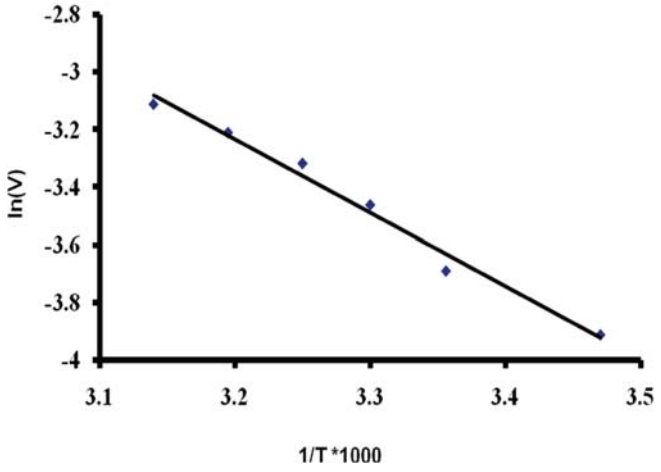


Figure 4. Variation of lnV with reciprocal temperature.

### Calculation of rate constant, $k_2$

A series of experiment was carried out with different enzyme concentrations to evaluate rate constant of the enzyme, $k_2$. The variation of initial reaction rates with initial enzyme concentrations was monitored at fixed temperature, phenol and dissolved oxygen concentrations, 30°C, 1000 mg/L and 10 mg/L, respectively. The molar concentration of the enzyme in reaction medium was determined according to the relationship between laccase activity per volume of the enzyme solution and mg purified enzyme published in the literature [18]. Then the rate constant of the enzyme, $k_2$, was calculated from the slope as 254 s$^{-1}$, being the same order of magnitude as previously reported values (Table 2) for different monomers catalyzed by laccase in the literature.

## CONCLUSIONS

In this study enzymatic-oxidative polymerization of phenol in a aqueous solution of sodium acetate buffer-acetone was studied. Kinetics of the reaction mechanism was investigated by employing a mathematical model presenting a multisubstrate character for phenol and dissolved oxygen. A non-linear regression program evaluated kinetic parameters $V_{max}$, $k_2$, $K_{mm}$, and $K_{mO_2}$ as 0.029 mg DO/L.min, 256 s$^{-1}$, 66.58 mg/L, 0.891 mg/L, respectively. The activation energy of the polymerization reaction was evaluated as 21.175 kJ/mol, being almost in the typical range of 30-60 kJ/mol for laccase catalyzed polymerization reactions.

Table 2. Polymerization reaction rate constant, $k_2$, values for different monomers catalyzed by laccase enzyme from various sources.

| Compound | Laccase source | Rate constant $k_2$ (s$^{-1}$) |
|---|---|---|
| Guaiacol | *Trametes versicolor* | 200 |
| Guaiacol | *Fusarium graminearum* | 660 |
| Vanillic alcohol | *Trametes versicolor* | 182 |
| Vanillin | *Trametes versicolor* | 90 |
| Vanilic acid | *Trametes versicolor* | 160 |
| Eugenol | *Trametes versicolor* | 150 |
| Dihydroeugenol | *Trametes versicolor* | 160 |
| Hydroquinone | *Coriolus hirustus* | 404 |
| Pyrophenol | *Coriolus hirustus* | 492 |
| Phenol | *Trametes versicolor* | 254 |

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## NOMENCLATURE

DO : Dissolved oxygen concentration in the reaction medium (g per m$^3$)

$E_a$ : Activation energy of laccase catalyzed polymerization of phenol (kJmol$^{-1}$)

$E_o$ : Initial enzyme concentration in the reaction medium (M or U per dm$^3$)

$k_0$ : Frequency factor of Arrhenius equation

$k_2$ : Reaction rate constant of enzymatic polymerization (s$^{-1}$)

$K_m$ : Michaelis-Menten saturation constant (mg/L)

$K_{mm}$ : Michaelis-Menten saturation constant with respect to phenol (mg/L)

$K_{mO_2}$ : Michaelis-Menten saturation constant with respect to dissolved oxygen(mg/L)

n : Constant in Moser equation

R : Universal gas constant (J/molK)

$R^2$ : Index of determination in regression analysis

S : Substrate concentration (mg/L)

$S_m$ : Initial concentration of phenol in reaction medium, (mg/L)

$S_{O_2}$ : Initial concentration of dissolved oxygen (mg/L)

T : Absolute temperature (K)

V : Initial reaction rate of polymerization in terms of dissolved oxygen consumption rate (mg DO/L.min).

$V_{max}$ : Maximum reaction rate of polymerization (mg DO/L.min)

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