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Modelling the transient kinetics of laccase-catalyzed oxidation of four aqueous phenolic substrates at low concentrations

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ABSTRACT

Laccase catalyzes the oxidation of aqueous phenolic substrates by oxygen. To assess the feasibility of using this enzyme to oxidize aqueous phenols at low concentrations, a five-parameter kinetic model was developed based on previous work to predict the transient kinetics of reactions catalyzed by laccase from *Trametes versicolor*. The model was calibrated against data collected at pH 5.0 and 25 °C for batch reactions of triclosan, cumylphenol, and estradiol. Phenol data arising from an earlier study was used for comparison. The kinetic model, which incorporated a term for enzyme inactivation, accurately simulated the time course of reactions of all four compounds for ranges of substrate and laccase concentrations that spanned up to 5 orders of magnitude. Furthermore, it was demonstrated that the model made accurate predictions far outside of the range of its calibration. The utility of the model for assessing the feasibility of using laccase to catalyze the oxidation of substrates over a range of reactant concentrations was demonstrated. Modelling and experimental results showed that estradiol, cumylphenol and triclosan can be oxidized through the catalytic action of laccase at aqueous substrate concentrations in the micromolar to nanomolar concentration range, while using substantially less enzyme than required for phenol.

1. Introduction

Oxidoreductase enzymes can be used to catalyze the oxidation of phenolic pollutants in wastewaters [1–4]. In particular, it has been claimed that, due to rapid rates of catalysis, laccase can oxidize compounds that are present in trace concentrations without suffering many of drawbacks facing traditional wastewater treatment processes [2,5]. However, before applications can be developed and implemented at the full-scale, it is necessary to be able to quantify the amount of enzyme required to achieve a given level of conversion of a trace-level pollutant in a wastewater in a selected timeframe. To do this, it is essential to develop an understanding of the kinetics of enzyme catalysis in the concentration range where such pollutants occur. Once the means to describe the kinetics of laccase reacting with different substrates has been established, it becomes possible to assess whether enzymatic treatment is feasible in terms of: (1) its ability to achieve the required effluent quality standard; (2) the quantity of enzyme required, with important implications on operating costs; and (3) the reaction time required, with important implications on the size of the reactor and, therefore, capital costs.

A kinetic model was recently reported [6] that described the transient kinetics of the laccase-catalyzed oxidation of phenol at low concentrations. The model was developed based on the reactions of laccase sequentially with oxygen, O_2, and an aqueous aromatic substrate, S, during the main catalytic cycle described with two successive Reactions (R1) and (R2):

$$E + O_2 \xrightarrow{k_1} E^* \quad \text{(R1)}$$

$$E^* + 4S \xrightarrow{k_2} E + 4S\cdot + 2H_2O \quad \text{(R2)}$$

where k_1 and k_2 are the rate constants for these reactions. In reality, such reactions are more complex and do not fully represent the series of reaction steps between different intermediate forms of enzyme, as described in an earlier study [6]. For example, in the reaction represented by (R1), laccase in its native state, E, is oxidized by O_2 resulting in the formation of a peroxide intermediate, E_p. This is followed by a rate-limiting two electron reduction step to form fully oxidized enzyme, E* [7]. From there, the reaction can take two parallel paths. The first path, which is part of the main catalytic cycle, as represented by (R2), involves four sequential single electron reduction steps in which four molecules of substrate, S, are oxidized to form reduced enzyme, E, substrate radicals, S·,

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Nomenclature

α	Exponent of $[E^*]$ in a kinetic equation describing the rate of substrate oxidation, where $0 < \alpha \leq 1$ (dimensionless)
β	Exponent of $[S]$ in a kinetic equation describing the rate of substrate oxidation, where $\beta \geq 1$ (dimensionless)
$[E]$	Concentration of laccase in the native reduced state (μM)
$[E^*]$	Concentration of laccase in the oxidized state (μM)
$[E_i]$	Concentration of inactive laccase (μM)
$[E_t]$	Total concentration of laccase initially added to reactions (μM)
k_1	Apparent second-order rate constant incorporating all steps governing the reaction between laccase in the reduced state, E, and oxygen (μM^{-1} min^{-1})
k_i	Laccase inactivation rate constant ($\mu M^{-0.5}$ $min^{-0.5}$)
ks	Apparent rate constant for substrate, S, reacting with oxidized laccase, E* ($\mu M^{1-\alpha-\beta}$ min^{-1})
k_La	First-order mass transfer coefficient for oxygen diffusing from ambient air into the batch reaction mixture (min^{-1})
$[O_2]$	Concentration of oxygen in the reaction mixture at time t (μM)
$[O_2]_{sat}$	Saturation concentration of the oxygen in the reaction media (μM)
$[S]$	Concentration of substrate in the reaction mixture at time t (μM)
$[S]_0$	Concentration of substrate in the reaction mixture at $t = 0$ (μM)
t	Time since initiation of reaction of substrate and laccase (min)

and two molecules of H_2O [7]. The second path, which is outside of the catalytic cycle (not shown), consists of the slow and reversible formation of a resting oxidized intermediate, E_{RO}, involving the addition of two protons and the release of one molecule of H_2O. E_{RO} is catalytically active and can react with the substrate resulting in the formation of reduced enzyme, E, and the release of substrate radicals and one molecule of H_2O [7]. It was also recently proposed, based on indirect evidence revealed through modelling, that another reversible intermediate, E_X, can be formed from E_P [8]. This intermediate is catalytically inactive and its formation also represents a diversion of the enzyme from the catalytic cycle.

For the purposes of kinetic modelling, it was concluded that some of the side reactions described above will become particularly important at low substrate concentrations where laccase will tend to accumulate in its oxidized state, E* [6]. However, current analytical methods cannot be used to distinguish between the various enzyme intermediates, which make it impractical to develop a kinetic model that fully accounts for each of these individual reactions. Therefore, a semi-empirical approach was used in which impact of the side reactions were modelled using ordered differential equations that matched the trends observed in experimental data. As a result, the kinetic model developed to describe the laccase-catalyzed oxidation of phenol [6] consisted of three differential equations and one mass balance equation, as follows:

$$\frac{d[O_2]}{dt} = -k_1[E][O_2] + k_La([O_2]_{sat} - [O_2]) \quad (1)$$

$$\frac{d[S]}{dt} = -k_s[E^*]^{\alpha}[S]^{\beta} \quad (2)$$

$$\frac{d[E]}{dt} = -k_1[E][O_2] + k_s[E^*]^{\alpha}[S]^{\beta} \quad (3)$$

$$[E^*] = [E_t] - [E] \quad (4)$$

This model includes five calibration parameters including one mass transfer coefficient, k_La, and one kinetic parameter, k_1, both of which are substrate-independent, plus three other kinetic parameters, k_s, α, and β, which are substrate-dependent. Note that the latter three parameters can account for variations in reaction stoichiometry from the theoretical value of 4 expressed in (R2). The definitions of these and all other variables are provided in the Nomenclature. This model was calibrated and validated against transient batch reaction data collected over a 3 h period and over several orders of magnitude of initial concentrations of phenol ($0.5 < [S]_0 < 50$ μM) and laccase (i.e., $0.12 < [E_t] < 2.5$ μM) [6]. The model demonstrated excellent predictive ability outside of the range of its calibration and over extended time periods of up to 12 h.

Note that in much earlier work with laccase [9], a different kinetic model was developed to describe the laccase-catalyzed oxidation of phenol at initial concentrations ranging from 500 to 3000 μM. In this case, the orders of the variables $[S]$ and $[E^*]$ in rate equations were one (i.e., effectively $\alpha = 1$, $\beta = 1$), which differ from the more recent study involving substantially lower phenol concentrations where $\alpha = 0.48$, $\beta = 1.48$ [6]. From this, it was hypothesized that under conditions of high substrate concentration, where only a small fraction of enzyme would tend to be in the oxidized state, E*, the influence of side reactions will become negligible, resulting in kinetic exponents, α and β, that will tend toward unity [6]. It was hypothesized that the same would be true for substrates that are oxidized very rapidly, but this remains to be confirmed [6]. Moreover, in contrast to the findings reported for phenol at high concentrations [9], it was observed that the oxidation of phenol at low concentrations did not result in significant enzyme inactivation [6]. However, it has not been established whether the oxidation of other substrates at low concentrations will cause inactivation and whether their kinetics can be described by the model described above.

A sensitivity analysis showed that model predictions for phenol were very sensitive to changes in values of k_s, α and β but were mostly insensitive to k_1, except under conditions where the substrate concentration was high, and especially when also using low enzyme concentrations [6]. Since k_1 describes the rate of reaction between enzyme and oxygen, it is substrate-independent and should have a unique value for any given reaction pH and temperature. Because of the insensitivity of the model to k_1, this parameter could not be precisely determined during model calibration with phenol data and, as a result, had to be expressed as an inequality (i.e., $k_1 > 8.85 \times 10^{-3}$ μM^{-1} min^{-1}). However, it was hypothesized that the model would likely be more sensitive to k_1 for rapid substrates, where more of the enzyme would tend to be in the native state, E, thereby causing the reaction described by Eq. (1) to become rate limiting [6]. Thus, if the model were applied to faster substrates, the heightened sensitivity of the model should lead to a more precise determination of k_1 [6].

In light of the above, the primary objective of this study was to validate the general applicability of the kinetic model by extending its use to other phenolic substrates of interest and to refine the model, as necessary and then to use the insights gained to explore the questions and hypotheses raised above. A secondary objective was to apply the model to explore the feasibility of using laccase to oxidize the selected substrates at low concentrations. The compounds 17β-estradiol, 4-cumylphenol and triclosan were selected as substrates and data arising from the previous study with phenol [6] were used for comparison. Collectively, these four substrates were chosen to cover a diverse range of reaction rates in order to