Modelling of L-DOPA Oxidation Catalyzed by Laccase

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Enzymatic oxidation of 3,4-dihydroxyphenyl-L-alanine (L-DOPA) with laccase from *Trametes versicolor* was investigated. The highest enzyme activity at pH 5.4 and at 25 °C was found. The reaction kinetics and the effect of dissolved oxygen concentration on the reaction rate were evaluated. A mathematical model, comprised of double-substrate Michaelis-Menten kinetics and mass balances for L-DOPA and dissolved oxygen concentrations, was developed in order to describe and predict the process of L-DOPA oxidation. Kinetic parameters, V_m, K_m^{L-DOPA} and $K_m^{O_2}$ were estimated and experimentally verified by a set of experiments with constant additional aeration for different initial concentrations of L-DOPA and dissolved oxygen. A significant increase in reaction rate was established at a higher oxygen concentration in the inlet gas. The developed model was used to investigate the influence of dissolved oxygen concentration on L-DOPA conversion.

Key words:
L-DOPA, laccase, enzymatic oxidation, modelling

Introduction

Laccases (EC 1.10.3.2, *p*-diphenol: dioxygen oxidoreductases) are multi-copper glycoproteins ubiquitous in nature, using molecular oxygen to oxidize various aromatic and non-aromatic compounds by a radical-catalyzed reaction mechanism.[1–3] The oxidation of substrates creates reactive radicals that can undergo non-enzymatic reactions of cross-linking of monomers, degradation of polymers or ring cleavage of aromatics.[1] They have been found in higher plants, fungi (especially in white-rot fungi), prokaryotes and insects.[2–5] The best known structures are the ones of laccases isolated from fungi,[1] where they often occur as isoenzymes that oligomerize to form multimeric complexes. The molecular mass of each monomer ranges from about 50 to 100 kDa.[1]

These enzymes play an important role in the polymerization of lignin-related substrates and depolymerization of lignin in nature.[6] They find their application in different industries: food industry (for enhancing or modifying the colour appearance of food or beverage), pulp and paper industry (delignification), textile industry (textile bleaching, dyes synthesis), nanotechnology, soil bioremediation, synthetic chemistry, cosmetics,[7] ethanol production, wine clarification, alkenes oxidation, herbicide degradation etc.[2]

Phenolic compounds belong to major pollutants,[5] their presence in drinking and irrigation water is a health hazard.[8] Mostly they originate from agricultural and industrial activities, including partial degradation of phenoxy herbicides, use of wood preservatives, generation of wastes by pulp and paper industry, petrochemicals, dyeing and other organic chemicals and textile industries.

Oxidative coupling of phenols is involved in some biological reactions, for example, formation of lignin or melanin.[6]

We focused our work on the enzymatic oxidation of 3,4-dihydroxyphenyl-L-alanine (L-DOPA), which is a simple and cheap representative of phenolic compounds with low solubility in water. It is a natural dietary amino acid, an intermediate in several metabolic pathways and a precursor of all catecholamine neurotransmitters and hormones, as well as of melanin.[9] L-DOPA has found a wide use for treatment of patients suffering from Parkinson's disease.[10]

Laccases[11,12] and tyrosinases[13,14] catalyze L-DOPA oxidation to dopaquinone, a highly reactive intermediate. In the absence of thiols, dopaquinone forms leucodopachrome, which oxidizes to dopachrome. Hydroxylation and decarboxylation yields dihydroxyindole, which can polymerize to DOPA-melanin.[13,15] Melanins are heterogeneous polymers of polyphenolic character and structure with colour varying from yellow to black. Melanins originate as the enzymatic browning in fruits and

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vegetables as well as the pigmentation of animals. They are also involved in malignant melanomas, carcinogenic tumors of the skin.[16] DHN-melanin and DOPA-melanin, the two most important types of melanins found in fungi, are both implicated in pathogenesis.[12,17–20] Fungal melanins have numerous functions: protection against UV irradiation, enzymatic lysis, oxidants and extreme temperatures.[11,15]

It is known that oxygen is a second substrate in the reaction of polymerization of phenolic substrates.[21] The objective of this work was to determine the kinetics of laccase-catalyzed L-DOPA oxidation and to evaluate the effect of dissolved oxygen concentration on the reaction rate. This is especially important for the enzymatic reaction occurring in closed systems such as microreactors and enzyme membrane reactors.

Experimental

Materials

Chemicals

L-DOPA was purchased at Fluka Chemie (Steinheim, Switzerland); Na_2HPO_4, KH_2HPO_4, HCl, $(NH_4)_2SO_4$, citric acid, and perchloric acid were purchased at Kemika (Zagreb, Croatia); glycine was purchased at Merck (Darmstadt, Germany).

Enzyme

Laccase from *Trametes versicolor* was purchased at Fluka BioChemie (Steinheim, Switzerland).

Methods

Enzyme activity assay

The laccase activity was measured spectrophotometrically by the method of initial rates. The reaction mixture in a cuvette contained from 0 to 3.5 mmol dm^{-3} L-DOPA solution in 0.2 mol dm^{-3} phosphate buffer, pH 5.4, and laccase suspended in 3.2 mol dm^{-3} ammonium sulphate solution to give final volume of 1.01 cm^3. The mixture was pre-incubated at 25 °C for 5 min and the reaction was started by adding the laccase enzyme solution. The formation of dopachrome, a dark red compound, was measured with time at 472 nm on the spectrophotometer (UV 1601, Shimadzu, Kyoto, Japan) and the specific reaction rate (U mg^{-1}) was calculated. 1 U of enzyme activity was defined as 1 μmol of substrate oxidized per minute. Extinction coefficient was measured and calculated (ε_{472} = 3.37 cm^2 μmol^{-1}) and was in good correlation with literature data (ε_{472} = 3.6 cm^2 μmol^{-1}).[22] Furthermore, enzyme activity was measured in three different buffers (0.1 mol dm^{-3} glycine buffer, 0.2 mol dm^{-3} citrate-phosphate and 0.2 mol dm^{-3} phosphate buffers) with pH in the range from pH 3.0 to 6.9 and at different temperatures in the temperature range from 10 °C to 65 °C with 5 mmol dm^{-3} L-DOPA solution in order to find optimal conditions for the studied reaction. Each measurement was carried out twice. The confidence interval, presented as standard deviations, was calculated upon the results regarding the mean value.

Analytics

During the reaction in a reactor, the concentration of L-DOPA was followed by HPLC (Knauer, Berlin, Germany) at 30 °C with a reverse phase C_{18} 125 mm x 4 mm x 5 μm column (LiChrospher® 100, Merck, Darmstadt, Germany), and UV detection at 280 nm. The mobile phase used was water with the addition of perchloric acid until pH 2.10–2.15 was reached and sample elution was performed at the flow rate of 0.7 cm^3 min^{-1}. L-DOPA standards for the calibration curve and samples taken from the reactor were diluted with hydrochloric acid (0.1 mol dm^{-3}). Retention time of L-DOPA was 7.3 min.

Experiments in a batch reactor

Experiments were carried out in a 250 cm^3 reactor with the reaction volume of 50 cm^3. All reactions were carried out at 25 °C in 0.2 mol dm^{-3} phosphate buffer, pH 5.4. The reaction started with the addition of 0.1 cm^3 of laccase suspension to 5 mmol dm^{-3} aqueous L-DOPA solution to give final laccase concentration in the reactor 0.099 mg cm^{-3}. The reaction mixture was stirred with a magnetic stirrer and L-DOPA concentration in samples was followed by HPLC as described above. Experiments were done without additional aeration or by supplying a reaction mixture of air and oxygen, or by different mixtures of nitrogen and oxygen, in order to obtain varying initial concentrations of dissolved oxygen in buffer solutions. The gas was purged through the liquid with a plastic injector with a very narrow hole in order to obtain efficient mass transfer. An oxymeter (WTW pH/Oxi 340, electrode WTW Cellox 325, Weiheim, Germany) was used to measure dissolved oxygen concentration in the reaction medium.

Modelling

Although many kinetic studies with laccases considered enzyme reactions in terms of one-substrate kinetics, a second-order Michaelis-Menten kinetic model with dissolved oxygen concentration as

a second substrate was proposed and experimentally confirmed for oxidative polymerization of 1-naphtol,[21] while for laccase-catalysed polymerization of catechol, an enzyme kinetic model included a mixed inhibition expression for dissolved oxygen as a second substrate in the reaction.[23] Also in our system with L-DOPA oxidation, a double substrate model for laccase-catalysed reaction kinetics was suggested.

$$r = \frac{V_m \cdot c_{L-DOPA} \cdot c_{O_2} \cdot \gamma_{laccase}}{(K_m^{L-DOPA} + c_{L-DOPA}) \cdot (K_m^{O_2} + c_{O_2})} \quad (1)$$

where V_m, represents maximal reaction rate per mass of enzyme (U mg^{-1}), K_m^{L-DOPA} and $K_m^{O_2}$ represent Michaelis-Menten constants for L-DOPA (mmol dm^{-3}) and dissolved oxygen (mmol dm^{-3}), respectively, and c_{L-DOPA}, c_{O_2} and $\gamma_{laccase}$ denote concentrations of L-DOPA (mmol dm^{-3}), dissolved oxygen (mmol dm^{-3}) and laccase (mg cm^{-3}), respectively.

The mass balance for L-DOPA enzymatic oxidation in the batch reactor was:

$$\frac{dc_{L-DOPA}}{dt} = -r \quad (2)$$

while for oxygen, the mass balance was:

$$\frac{dc_{O_2}}{dt} = OTR - r \quad (3)$$

where OTR represents oxygen transfer rate:

$$OTR = k_L a \cdot (c_{O_2}^* - c_{O_2}) \quad (4)$$

and $k_L a$ represents volumetric mass transfer coefficient (min^{-1}), $c_{O_2}^*$ represents oxygen concentration in saturation (mmol dm^{-3}) and c_{O_2} represents oxygen concentration (mmol dm^{-3}).

Data handling

Model parameters were estimated by non-linear regression analysis using Simplex and Least Squares method implemented in the SCIENTIST software.[24] They were evaluated by fitting the model to the experimental data from initial reaction rate experiments. The calculated data were compared with the experimental data, recalculated in the optimization routine, and fed again to the integration step until a minimal error between experimental and integrated values was achieved. The residual was defined as the sum of the squares of the differences between the experimental and the calculated data. "Episode" algorithm for the stiff system of differential equations, implemented in the SCIENTIST software was used for simulations of batch reactor mathematical models.

Results and discussion

pH and temperature optimization

Activity of enzyme laccase was measured at a pH range from pH 3.0 to pH 6.9 in three different buffers (Fig. 1) to find the optimal pH. All the experiments were measured with 5 mmol dm^{-3} concentration of L-DOPA. Citrate – phosphate buffer (pH 4.2 – 5.0) was used as it was not possible to prepare the phosphate buffer with pH lower than 5.4. Crystallization of citrate-phosphate buffer at room temperature was noticed. The highest enzyme activity was determined in phosphate buffer at pH 5.4. According to the literature, high pH values are not favourable for the L-DOPA as it shows auto-oxidation in alkaline solutions.[25]

Furthermore, the activity of laccase was measured in a temperature range from 10 – 65 °C in phosphate buffer at pH 5.4 in order to establish the influence of temperature on the initial reaction rate. As evident from Fig. 2, the optimal temperature was 25 °C.

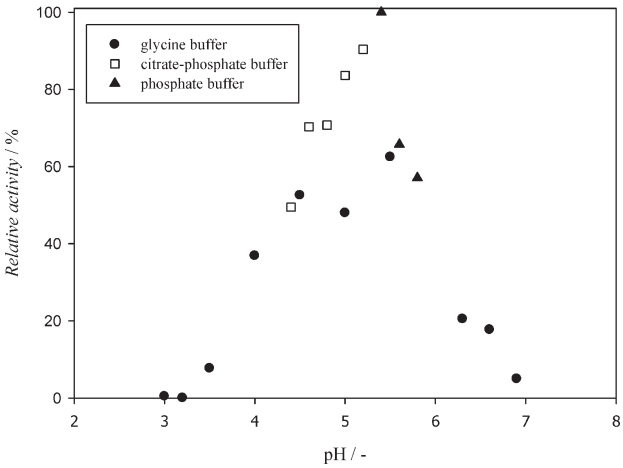


Fig. 1 – *Dependence of enzyme activity on pH (pH 3.0–6.9 0.1 mol dm^{-3} glycine buffer; pH 4.4–5.2 0.2 mol dm^{-3} citrate phosphate buffer; pH 5.4–5.8 0.2 mol dm^{-3} phosphate buffer)*

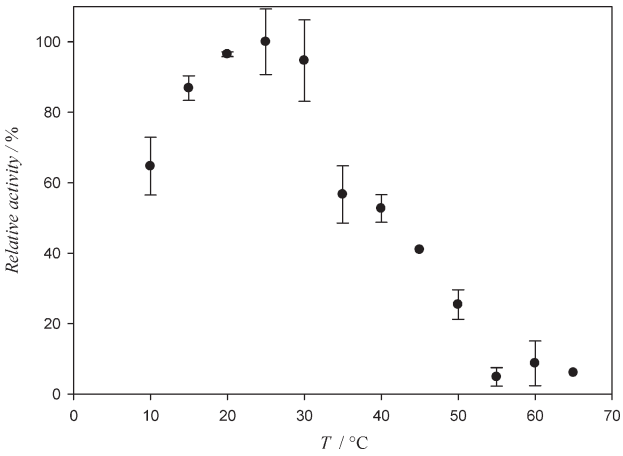
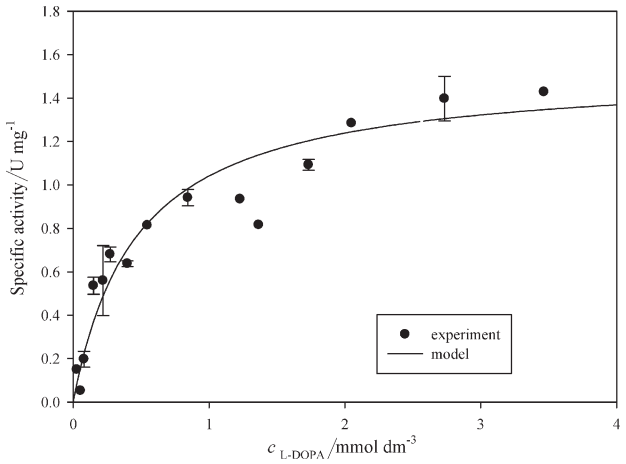


Fig. 2 – *Effect of temperature on enzyme activity (0.2 mol dm^{-3} phosphate buffer, pH 5.4, $\gamma_{laccase}$ = 0.033 mg cm^{-3})*

Laccase kinetics in a cuvette

Initial kinetic studies were performed in a cuvette by the standard initial reaction rate method using a spectrophotometer. Enzyme kinetics was measured at previously optimized conditions in phosphate buffer at pH 5.4 and 25 °C with different L-DOPA concentrations in the range from 0 to 3.5 mmol dm^{-3}. From the results, shown in Fig. 3, a Michaelis-Menten type of kinetics could be confirmed. By fitting the experimental data with monosubstrate Michaelis-Menten kinetics, the estimated apparent value for V_m^* was 1.529 ± 0.101 U mg^{-1}, while K_m^{L-DOPA} value was 0.469 ± 0.099 mmol dm^{-3} (Table 1).



F i g . 3 – *One substrate kinetics model. Dependence of initial reaction rate on the concentration of L-DOPA. (0.2 mol dm^{-3} phosphate buffer, pH 5.4, $\gamma_{laccase}$ = 0.033 mg cm^{-3}).*

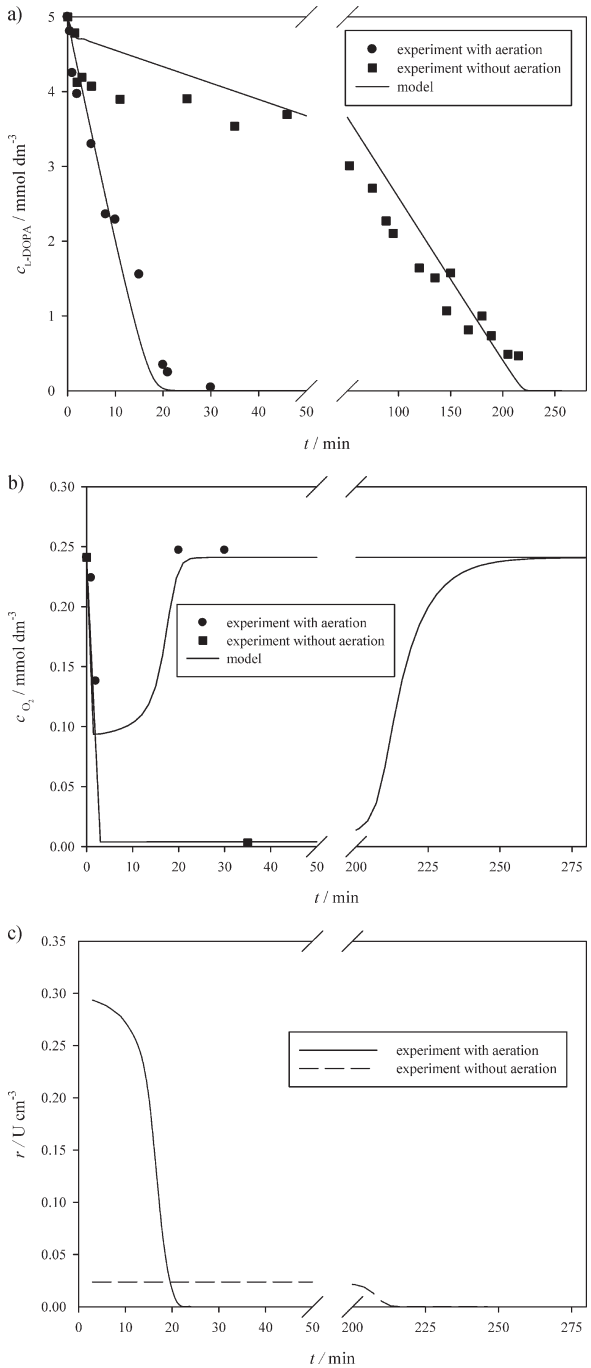
T a b l e 1 – *Estimated kinetic parameters for the experiments in cuvette, with and without additional aeration*

Parameters	Units	Experiment in a cuvette	Experiment without additional aeration	Experiment with additional aeration
K_m^{L-DOPA}	mmol dm^{-3}	0.469 ± 0.097	0.469 ± 0.097	0.469 ± 0.097
V_m	U mg^{-1}	1.529 ± 0.101*	6.897 ± 0.243	6.897 ± 0.243
$K_m^{O_2}$	mmol dm^{-3}	–	0.099 ± 0.015	0.099 ± 0.015
$k_L a$	min^{-1}	–	0.2	2.0

* – apparent value

Enzymatic oxidation of L-DOPA in a batch reactor

In order to obtain parameters for double-substrate kinetics, further experiments were performed in a batch reactor system at 25 °C using 0.2 mol dm^{-3} phosphate buffer at pH 5.4. Initial experiments of L-DOPA oxidation in the batch reactor were performed in two different ways: with and without additional aeration. In the experiment without additional aeration, oxygen supply was provided just through the boundary layer atmosphere/reaction mixture, while in the experiment with additional air supply the reaction mixture was constantly aerated by the air. Initial oxygen concentration was equal (c_{0,O_2} = 0.241 mmol dm^{-3}) in both experiments. Experimental results of L-DOPA and dissolved oxygen concentrations of both experiments are shown in Fig. 4a and Fig. 4b, respectively.



F i g . 4 – *L-DOPA oxidation catalyzed by laccase in a batch reactor without additional aeration and with continuous air aeration (25 °C, 0.2 mol dm^{-3} phosphate buffer, pH 5.4, $\gamma_{laccase}$ = 0.099 mg cm^{-3}, c_{0,O_2} = 0.241 mmol dm^{-3}). a) L-DOPA concentration; b) dissolved oxygen concentration; c) reaction rate.*

A significant increase in the reaction rate of L-DOPA oxidation was achieved (Fig. 4c) at aerated conditions as compared to the reaction rate without additional air supply. Obviously, in the experiment without additional aeration, oxidation of L-DOPA was controlled by oxygen transfer rate through the boundary layer atmosphere/reaction mixture, indicating the role of oxygen concentration for the reaction kinetics. In Fig. 4b dissolved oxygen concentrations for both experiments are presented. Instantaneous drop in oxygen concentration at the beginning of both experiments was observed as well as an increase of its concentration after L-DOPA depletion.

The proposed double-substrate kinetics (eq. (1)) was therefore used for the simulation of both experiments. The parameters were estimated by fitting the developed models (eq. (2) and (3)) with the experimental data. Estimated parameters are as follows: V_m = 6.897 ± 0.243 U mg^{-1}, $K_m^{O_2}$ = 0.099 ± 0.015 mmol dm^{-3}, $k_L a$ was estimated to be 0.2 min^{-1} for the experiment without additional aeration and 2.0 min^{-1} for the experiment with additional aeration (Table 1). K_m^{L-DOPA} value 0.469 ± 0.097 mmol dm^{-3} was taken from the kinetic measurements obtained in a cuvette.

As far as we know, no literature data is available for kinetic parameters describing L-DOPA oxidation by laccase from *Trametes versicolor*. However, kinetic studies of 1-naphtol polymerization by laccase from the same source revealed K_m value of 0.691 mmol dm^{-3} and $K_m^{O_2}$ value of 2.4 mmol dm^{-3},[21] while for catechol polymerization K_m value of 0.643 mmol dm^{-3} and $K_m^{O_2}$ value of 0.325 mmol dm^{-3} were reported.[23] These values of $K_m^{O_2}$ for laccase are higher than for tyrosinase ($K_m^{O_2}$ = 0.0046 mmol dm^{-3}).[14] Therefore, the high operational efficiency of laccase requires a continuous supply of oxygen, in fed batch or fluidized bed reactors with stirring and/or bubbling of air or pure oxygen.

Validation of the model

Validation of the model was performed for two different initial concentrations of dissolved oxygen and for two different initial concentrations of L-DOPA.

Experiments in batch reactor were performed with continuous additional aeration with a mixture of oxygen and nitrogen, where initial dissolved oxygen concentration was 0.94 mmol dm^{-3} and with additional aeration with pure oxygen, where initial dissolved oxygen concentration was 1.15 mmol dm^{-3}. Both experiments were carried out in batch reactor with the same initial concentration of L-DOPA ($c_{L\text{-DOPA}}$ = 5 mmol dm^{-3}) and the reaction mixture was continuously aerated. Mathematical model simulations were performed using eq. (2) and eq. (3) with previously estimated parameters shown in Table 1. Experimental and model simulation results

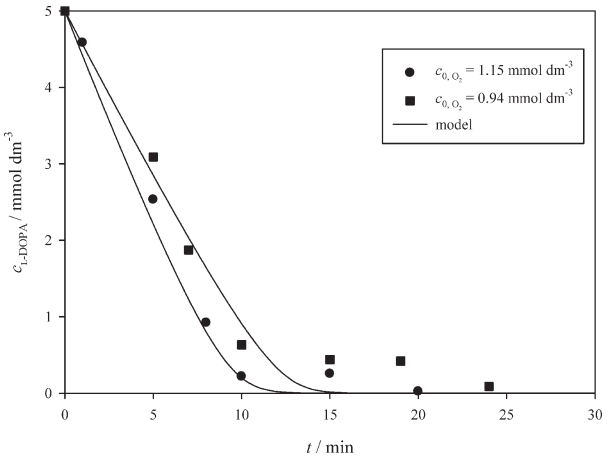


Fig. 5 – *Laccase-catalyzed oxidation of L-DOPA in a batch reactor (25 °C, 0.2 mol dm^{-3} phosphate buffer, pH 5.4, $\gamma_{laccase}$ = 0.099 mg cm^{-3}) with continuous additional aeration with a mixture of oxygen and nitrogen (c_{0,O_2} = 0.94 mmol dm^{-3}) and with additional aeration with pure oxygen (c_{0,O_2} =1.15 mmol dm^{-3})*

for both initial dissolved oxygen concentration are presented in Fig. 5.

As evident, the proposed model consisting of two substrate Michaelis-Menten kinetics shows good correlation with the experimental data, which confirmed the proposed model and revealed correct estimation of parameters.

In order to validate the proposed mathematical model for L-DOPA oxidation, experiments were conducted for different initial L-DOPA concentrations, 0.5 mmol dm^{-3} and 5 mmol dm^{-3}, respectively. Initial dissolved oxygen concentration was equal in both experiments (c_{0,O_2} = 0.241 mmol dm^{-3}). Previously estimated parameters (Table 1, experiments with aerations) were used for model simulations. Experimental results and model predictions for L-DOPA were in good correlation even for different initial L-DOPA concentrations (Fig. 6).

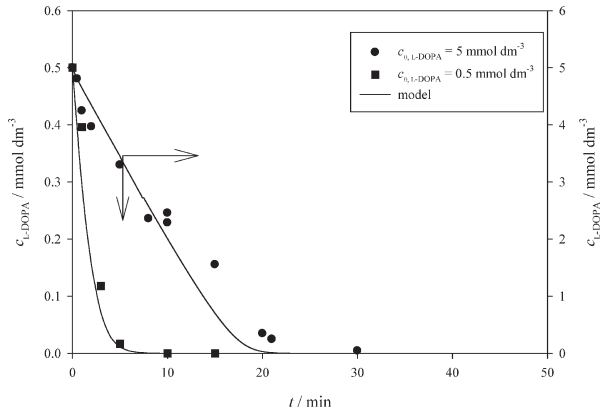
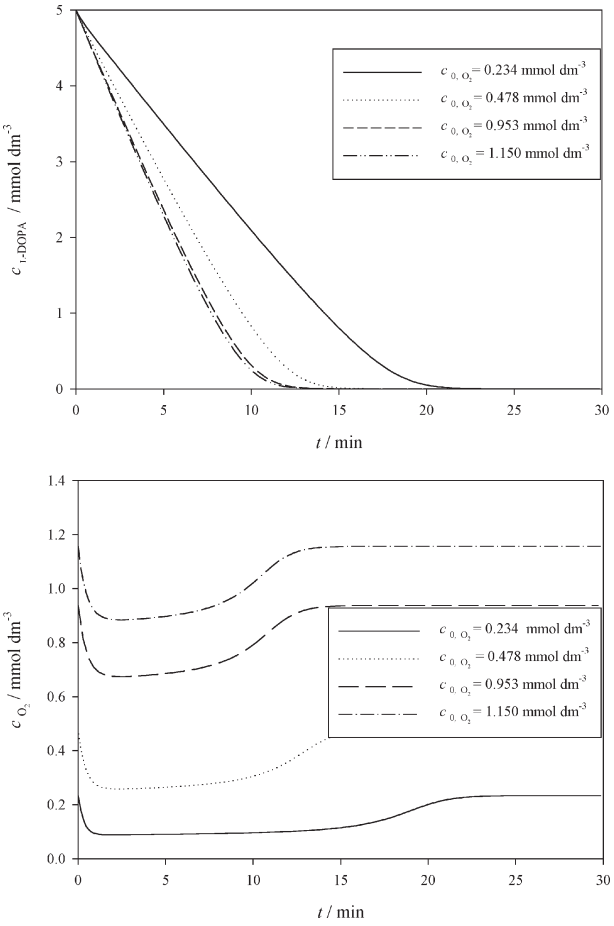


Fig. 6 – *Laccase-catalyzed oxidation of L-DOPA in a batch reactor (25 °C, 0.2 mol dm^{-3} phosphate buffer, pH 5.4, $\gamma_{laccase}$ = 0.099 mg cm^{-3}, c_{0,O_2} = 0.241 mmol dm^{-3}) with continuous air aeration for different initial L-DOPA concentrations, 0.5 mmol dm^{-3} and 5 mmol dm^{-3}*

In order to more clearly present the influence of different dissolved oxygen concentrations on L-DOPA consumption rate, the simulations of oxidation reaction were performed for the same initial L-DOPA concentration ($c_{\text{L-DOPA}} = 5$ mmol dm^{-3}), with continuous aeration, and for different initial dissolved oxygen concentrations. Mathematical model simulations (using previously estimated parameters from Table 1, experiments with aeration) for L-DOPA (eq. 2) are presented in Fig. 7a, while for dissolved oxygen (eq. (3)) they are shown in Fig. 7b. Obviously, initial dissolved oxygen concentration and continuous aeration are important parameters for L-DOPA oxidation catalyzed by laccase.



F i g . 7 – *Mathematical model simulations of a) L-DOPA concentration (eq. (2)) and b) dissolved oxygen concentration (eq. (3)) at continuous aeration for different initial dissolved oxygen concentrations, ($\gamma_{\text{laccase}} = 0.099$ mg cm^{-3}, $c_{0,\text{L-DOPA}} = 5$ mmol dm^{-3})*

Conclusion

The study of enzymatic oxidation of 3,4-dihydroxyphenyl-L-alanine (L-DOPA) with laccase from *Trametes versicolor* revealed the highest enzyme activity at pH 5.4 and at 25 °C. A remarkable influence of oxygen on the enzymatic oxidation of L-DOPA by laccase from *Trametes versicolor* was established with batch experiments, performed without aeration or with constant aeration. Therefore, dissolved oxygen was treated as a second substrate in the mathematical model consisting of double-substrate Michaelis-Menten kinetics and mass balances for both reactants. Kinetic parameters V_m, $K_m^{\text{L-DOPA}}$ and $K_m^{O_2}$ were evaluated as 6.897 ± 0.243 U mg^{-1}, 0.469 ± 0.097 mmol dm^{-3} and 0.099 ± 0.015 mmol dm^{-3}, respectively. The proposed mathematical model can simulate well experimental results for different initial dissolved oxygen concentrations and different initial L-DOPA concentrations.

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List of symbols and abbreviations

c – concentration, mmol dm^{-3}
$c_{O_2}^*$ – oxygen concentration in saturation, mmol dm^{-3}
$k_L a$ – volumetric mass transfer coefficient, min^{-1}
$K_m^{\text{L-DOPA}}$ – Michaelis-Menten constant for L-DOPA, mmol dm^{-3}
$K_m^{O_2}$ – Michaelis-Menten constant for oxygen, mmol dm^{-3}
OTR – oxygen transfer rate, mol dm^{-3} min^{-1}
r – reaction rate, U cm^{-3}
t – time, min
T – temperature, °C
V_m – maximal reaction rate, U mg^{-1}
V_m^* – apparent maximal reaction rate, U mg^{-1}
γ – mass concentration, mg cm^{-3}
0 – initial conditions
L-DOPA – 3, 4-dihydroxyphenyl-L-alanine

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