Scientific paper

Oxidation of Coniferyl Alcohol Catalyzed by Laccases from *Trametes versicolor*

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Dedicated to the memory of the late Prof. Dr. Valentin Koloini

Abstract

Oxidation of coniferyl alcohol catalyzed by commercial laccase and crude laccase produced during the submerged cultivation of *Trametes versicolor* MZKI G-99 in a medium containing the waste from paper industry was investigated. pH of 6.6 and temperature of 35 °C was found to be optimal for coniferyl alcohol oxidation catalyzed by commercial laccase. Based on the initial reaction rate measurements, apparent Michaelis-Menten kinetic parameters for commercial laccase were determined in an aqueous media (V_m = 4.387 U mg^{-1}, K_m = 0.025 mmol dm^{-3}), as well as in 1:1 (v/v) methanol: phosphate buffer mixture (V_m = 0.979 U mg^{-1}, K_m = 0.019 mmol dm^{-3}). Inhibition of substrate was found for crude laccase and the following apparent kinetic parameters V_m = 9.272 U mg^{-1}, K_m = 0.045 mmol dm^{-3} and K_i = 0.002 mmol dm^{-3} were estimated. Mathematical model of batch process, which includes double-substrate Michaelis-Menten kinetics with oxygen as the second substrate and mass balances, has been developed and validated in experiments with or without additional aeration. 100 % conversions of up to 0.8 mmol dm^{-3} of coniferyl alcohol in batch experiment due to the high operational stability of enzymes was realized with both laccases.

Keywords: Coniferyl alcohol, laccase, *Trametes versicolor*, kinetic models

1. Introduction

Laccases (EC 1.10.3.2, *p*-diphenol: dioxygen oxidoreductases) are multi-copper glycoproteins ubiquitous in nature, which use molecular oxygen to oxidize various aromatic and non-aromatic compounds by a radical-catalyzed reaction mechanism.[1–4] Laccase is particularly attractive as a biocatalyst since it uses oxygen as a co-substrate and possesses relatively high reaction rates, as well as broad substrate specificity. Enzyme has been mainly described in lignolytic white-rot fungi, namely basidiomycetes *Trametes versicolor* and *Polysporus ostreatus*, and ascomycetes *Podospora anserine* and *Neurospora crassa*.[1] Their applications are numerous: food, textile, and pulp and paper industry, nanotechnology, soil bioremediation, synthetic chemistry, cosmetics, ethanol production, wine clarification, alkenes oxidation, herbicide degradation, diagnostic assays etc.[2,5,6] All of these applications are based on the ability of the laccases to oxidize a wide range of aromatic substances including phenolic substrates. Their efficiency is strongly dependent on the type and number of substituents on the aromatic ring.[7–9]

Oxidative coupling of phenolic compounds significantly influences the architecture of the plant cell wall. The cross-linking of hemicellulose-bounded feruloyl groups and of extension-bounded tyrosine groups in primary walls, as well as synthesis of lignin in wood are such reactions with a profound effect on the cell wall properties.[10,11]

The modeling of the enzyme reaction kinetics as a tool for enzyme reaction engineering plays an important role in the development of enzyme-catalyzed reactions

for large-scale productions. Knowledge of the enzyme kinetics enables us to find the optimal operation conditions, as well as facilitate the identification of the most effective mode of process operation. For the design of an enzyme reactor, detailed knowledge of the kinetic parameters of the catalyst under operational conditions is essential.[12,13] Relatively few studies have focused on the evaluation of the Michaelis-Menten kinetics of laccase-catalyzed oxidation of phenolic compounds.[6,14–16] A two-substrate Michaelis-Menten kinetic with substrate inhibition was identified as the best model for the characterization of the laccase-catalyzed oxidative polymerization of catechol.[17]

In this study, the oxidation of coniferyl alcohol catalyzed by crude laccase produced during the submerged cultivation of *Trametes versicolor* MZKI G-99 in medium containing industrial waste was carried out in aqueous media. The results were compared with commercial laccase from *Trametes versicolor*. Coniferyl alcohol was selected as a representative of phenolic compounds present in the cell wall. Laccase kinetics was investigated spectrophotometrically in aqueous and also for commercial laccase in organic medium. Effect of dissolved oxygen concentration in the reaction kinetics was evaluated by batch experiments performed with and without aeration. Different kinetic models were proposed and related apparent kinetic parameters were estimated. Model prediction results are compared with experimental results obtained during the batch oxidation. To the best of our knowledge, this work represents the first attempt to model laccase-catalyzed oxidation of coniferyl alcohol using the Michaelis-Menten kinetics.

2. Experimental

2. 1. Materials

2. 1. 1. Chemicals

Coniferyl alcohol was purchased from Fluka Chemie (Steinheim, Switzerland); glucose, Na_2HPO_4, K_2HPO_4, KH_2PO_4, HCl, $(NH_4)_2SO_4$, NaOH, $HClO_4$, CH_3COOH, citric acid, agar-agar and acetonitrile were purchased from Kemika (Zagreb, Croatia); glycine was purchased from Merck (Darmstadt, Germany); peptone, malt-extract agar and yeast extract were purchased from Difco – Becton, Dickinson and Co (New Jersey, USA). Solid waste with 53.5 % of moisture content was obtained from the chemo-mechanical treatment facility of a paper-manufacturing plant (Papirnica Vevče, Ljubljana, Slovenia). Elemental composition of the waste was (in %, based on the dry weight): C, 15.4, N, 0.33, S, 0.03, and different heavy metals in traces (mg/kg dry weight) (As <0.01, Ba <0.1, Cd <0.0005, Cr <0.025, Cu <0.008, Hg <0.001, Mb <0.1, Ni =1.24, Pb <0.005, Sb <0.01, Se <0.01, Zn <0.05). Waste contained 54.4 % (based on the dry weight) of $CaCO_3$.

2. 1. 2. Laccases

Commercial laccase (0.83 U mg^{-1}) from *Trametes versicolor* was purchased from Fluka BioChemie (Steinheim, Switzerland).

Crude laccase was produced by *Trametes versicolor* MZKI G-99, which was kindly donated by the Microbial culture collection of the National Institute of Chemistry, Ljubljana, Slovenia. Culture was stored at 4 °C and cultivated at 30 °C in the medium containing 2 % malt extract agar and 2 % agar-agar, and transferred at one-month intervals. Stock cultures were inoculated into 500 cm^3 shake-flasks containing 50 cm^3 medium with 10 g dm^{-3} glucose, 0.2 g dm^{-3} peptone, 0.3 g dm^{-3} yeast extract, 0.8 g dm^{-3} KH_2PO_4, 0.2 g dm^{-3} Na_2HPO_4, and 0.5 g dm^{-3} $MgSO_4 \times 7H_2O$. Cotton-stopped flasks were incubated in an orbital shaker (Innova 4330, New Brunswick Scientific, Edison, New Jersey, USA) at 140 rpm and 27 °C. After 72 hours of cultivation, cultures were harvested by filtration (5 H/N, Munktell & Filtrak Gmbh, Barenstein, Germany). 3 g dm^{-3} of mycelial pellets were transferred into 500 cm^3 shake-flasks containing 100 cm^3 medium with 20 g dm^{-3} of waste from paper industry, 0.6 g dm^{-3} peptone, 0.5 g dm^{-3} yeast extract, 0.8 g dm^{-3} KH_2PO_4, 0.2 g dm^{-3} Na_2HPO_4, 0.5 g dm^{-3} $MgSO_4 \times 7H_2O$, 0.05 g dm^{-3} $CaCl_2 \times 2H_2O$, and 0.15 g dm^{-3} citric acid. Flasks were incubated in an orbital shaker at 140 rpm and 27 °C. After 15 days of cultivation, the reaction mixture was harvested. Supernatant was separated from mycelia by centrifugation (Centric 150, Tehtnica, Železniki, Slovenia), stored at 4 °C and used in all experiments as crude laccase (1631 U dm^{-3}).

2. 2. Methods

2. 2. 1. Analytics

Laccase activity was determined spectrophotometrically (UV 1601, Shimadzu, Kyoto, Japan) with monitoring the oxidation of ABTS at 420 nm (ε_{420} = 0.036 dm^3 μmol^{-1} cm^{-1}).[18] 0.1 cm^3 sample was added to 0.9 cm^3 3 mmol dm^{-3} ABTS solution (pH = 3.5 glycine/NaCl buffer, kept at a constant temperature of 25 °C). Change in absorbance was monitored over 100 s. One unit of enzyme activity was defined as 1 μmol of ABTS oxidized per minute.

Protein concentrations were measured according to Bradford.[19] Specific activity of crude enzyme (U mg^{-1}) was calculated according to the concentration of total protein.

Concentration of coniferyl alcohol was determined using HPLC equipped with UV detector (Shimadzu, Kyoto, Japan) operating at 260 nm. C_{18} reverse phase column was used (LiChrospher® 100, 125 mm × 4 mm × 5 μm, Merck, Darmstadt, Germany). Mobile phase was water, acetonitrile and acetic acid at the ratio of 78:20:2. Analysis was performed at 30 °C and mobile phase flow rate of 1.0 cm^3 min^{-1}. Retention time of coniferyl alcohol was 4.32 min.

2. 2. 2. Kinetic Measurements

Kinetics of oxidation of coniferyl alcohol catalyzed with commercial and crude laccase was measured with a spectrophotometer using the method of initial reaction rate to estimate apparent kinetic parameters, which are required for modeling the batch experiment.

Effect of pH (pH = 5.6 – 7.4, phosphate buffer, 0.2 mol dm^{-3}, T = 25 °C) and temperature (T = 20–70 °C, phosphate buffer, 0.2 mol dm^{-3}, pH = 6.6) on the activity of commercial laccase was investigated to find optimum conditions for the process. Kinetics of commercial and crude laccase was studied in aqueous medium. Coniferyl alcohol (0–0.2 mmol dm^{-3}) was suspended in phosphate buffer (0.2 mol dm^{-3}, pH = 6.6) in total volume of 0.9 cm^3 at a temperature of 35 °C. The reaction was initiated with addition of 0.1 cm^3 laccase solution (0.099 mg cm^{-3} for commercial and 0.94 mg cm^{-3} for crude laccase). Coniferyl alcohol consumption was monitored at 260 nm through time of 300 s.

Additionally, kinetic study of the commercial laccase-catalyzed coniferyl alcohol oxidation was carried out in 1:1 (v/v) methanol: 0.2 mol dm^{-3} phosphate buffer mixtures containing up to 0.1 mmol dm^{-3} of coniferyl alcohol and 0.099 mg cm^{-3} of laccase at pH 6.6 and at 35 °C. Reaction was monitored as described above.

2. 2. 3. Batch Experiments

Oxidation of coniferyl alcohol catalyzed by commercial and crude laccase was carried out in a batch reactor at a temperature of 35 °C. All experiments were performed in phosphate buffer (0.2 mol dm^{-3}) at optimal conditions for laccase activity. Experiments with commercial enzyme were carried out in a 50 cm^3 reactor with working volume of 10 cm^3. Experiments with crude laccase were performed in a 200 cm^3 reactor containing 40 cm^3 of reaction mixture. Reaction mixture was stirred with a magnetic stirrer. In experiments with aeration, the reaction mixture was aerated by air (0.4 dm^3 min^{-1}). The air was blowing through the reaction mixture with plastic injector with very narrow holes in order to obtain an efficient mass transfer. Dissolved oxygen concentration was measured during the experiments (12 mm OxyProbe, Broadley James Corporation, Irvine, USA). Experiments were started with the addition of laccase solution. Initial concentration of coniferyl alcohol, initial laccase concentration and initial laccase activity, which were tested, are summarized in Table 1.

Samples from the reactor (about 0.1 cm^3) were taken at irregular time intervals. Laccase activity in samples was determined using ABTS as a substrate. Coniferyl alcohol was analyzed by HPLC after the sample was filtered (0.2 μm, Macherey-Nagel GmbH and Co. KG, Duren, Germany).

Table 1. Initial conditions for the experiments carried out in a batch reactor

Laccase	$c_{S,0}$ [mmol dm^{-3}]	$\gamma_{E,0}$ [g dm^{-3}]	$A_{E,0}$ [U dm^{-3}]
Commercial	0.75	0.13	110
Crude	0.35[1] / 0.50[2]	0.94	153

1 – experiment without aeration
2 – experiment with aeration

2. 3. Modeling

Experiments with the laccase-catalyzed oxidation of coniferyl alcohol in cuvette of spectrophotometer were carried out at very low concentrations of substrate, and thus inhibition of substrate or limitation of oxygen are not taken into account that simplifies the model to single-substrate Michaelis-Menten kinetics (Eq. 1). Saturation concentration of oxygen in cuvette was about 0.234 mmol dm^{-3}, which corresponds to the saturation concentration of oxygen at 35 °C.

$$r_1 = V_m \cdot \frac{c_S \cdot \gamma_E}{K_m{}^S + c_S} \tag{1}$$

Since there are some reports of substrate-inhibited oxidative biotransformation of phenols in aqueous mixtures catalyzed by laccases[20], substrate inhibition effect on laccase-catalyzed oxidation of coniferyl alcohol was also investigated (Eq. 2).

$$r_2 = V_m \cdot \frac{c_S \cdot \gamma_E}{K_m{}^S + c_S + \frac{c_S^2}{K_I}} \tag{2}$$

In our previous work,[21] oxidation of L-DOPA catalyzed by laccase follows double-substrate Michaelis-Menten kinetics with oxygen as a second substrate. Therefore, kinetics of oxidation of coniferyl alcohol will be described with the same type of kinetic equations without or with substrate inhibition (Eq. 3 and Eq. 4, respectively) which will be used in the modeling of batch experiments in reactor. Oxygen concentration can be monitored in the batch experiments, but it is not possible in the cuvette of spectrophotometer.

$$r_3 = V_m \cdot \frac{c_S \cdot c_{O_2} \cdot \gamma_E}{(K_m{}^S + c_S) \cdot (K_m{}^{O_2} + c_{O_2})} \tag{3}$$

$$r_4 = V_m \cdot \frac{c_S \cdot c_{O_2} \cdot \gamma_E}{(K_m{}^S + c_S + \frac{c_S^2}{K_I}) \cdot (K_m{}^{O_2} + c_{O_2})} \tag{4}$$

Mass balances for coniferyl alcohol and for oxygen in batch reactor experiments are given in Eq. 5 and Eq. 6

$$\frac{dc_S}{dt} = -r_i \tag{5}$$

$$\frac{dc_{O_2}}{dt} = k_L a \cdot \left(c^*_{O_2} - c_{O_2}\right) - r_i \quad (6)$$

where r_i represents the corresponding kinetic expression (Eq. 1-4).

2. 3. 1. Data Handling

Model parameters were estimated by the non-linear regression analysis using the Simplex and Least Squares method implemented in the SCIENTIST software (MicroMath®, Salt Lake City, USA). They were evaluated by fitting the model to the experimental data from initial reaction rate experiments and from batch 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.

3. Results and Discussion

3. 1. Effect of pH and Temperature on the Activity of Commercial Laccase

Effect of pH and temperature on the activity of commercial laccase using coniferyl alcohol as substrate was carried out in accordance with standard procedure[22] to explore the influence of process conditions on the activity of enzyme (Figure 1). Activity of commercial laccase was determined in the range pH 5.4 to 7.6 in phosphate buffer. All experiments were carried out with 0.2 mmol dm^{-3} concentration of coniferyl alcohol and at 25 °C. Maximum activity was found at pH 6.6 and this pH was used for further experiments (Figure 1a). This result agrees with the report of Rogalski et al.[23], who claimed that the laccase from *Trametes versicolor* has an optimal pH range for oxidations of phenolic compounds in slightly acidic medium. Alternatively, laccase from *Rhus vernicifera* successfully catalyzed coniferyl alcohol oxidation in slightly alkaline medium.[24]

Commercial laccase activity was determined in the temperature range from 20 to 70 °C in phosphate buffer at pH 6.6, in order to determine the effect of temperature on the initial reaction rate. As is evident from Figure 1b, the optimal temperature is ranging from 30 to 40 °C, while maximum activity was found at 35 °C. This result agrees with the optimal temperature reported for oxidation of coniferyl alcohol using laccases from *Rhus vernicifera* and *Pycnoporus coccineus*.[24]

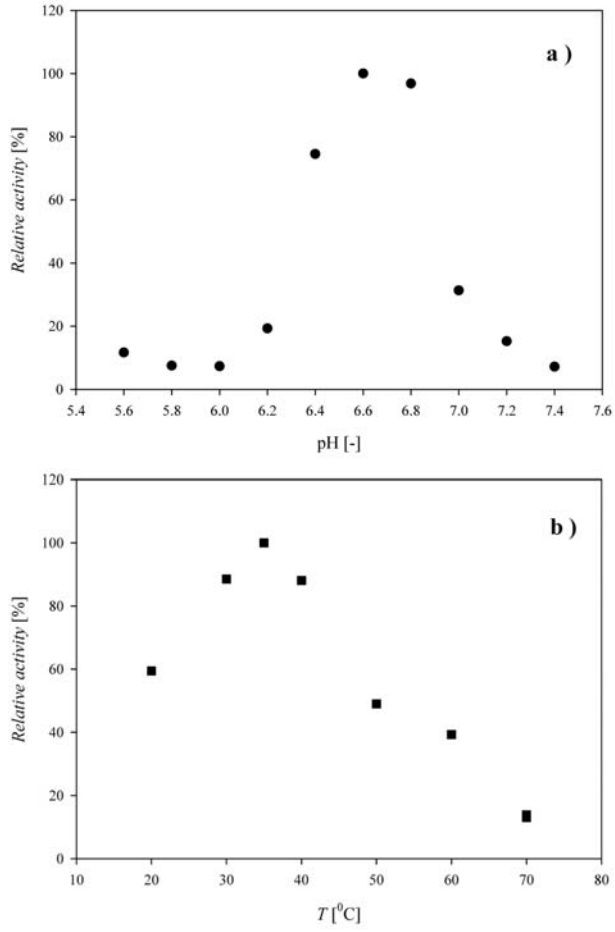

Figure 1. The influence of **a)** pH and **b)** temperature on laccase activity using coniferyl alcohol as a substrate.

3. 2. Oxidation of Coniferyl Alcohol Catalyzed by Commercial Laccase in Various Media

Kinetic studies were performed in a cuvette of spectrophotometer using the standard initial reaction rate method. Enzyme kinetics was measured at optimal pH and temperature (pH 6.6 and T = 35 °C) with various initial concentrations of substrate. Since coniferyl alcohol is poorly soluble in aqueous medium, suitable aqueous/organic mixture, in which alcohol can still dissolve, and the laccase retains its activity/stability, could be used for successful oxidation. Therefore, the activity of laccase was determined also in 1:1 (v/v) methanol: 0.2 mol dm^{-3} phosphate buffer mixture at pH 6.6 and 35 °C. Oxidation rate of coniferyl alcohol catalyzed by commercial laccase in aqueous and in organic medium was described using monosubstrate Michaelis-Menten kinetics (Eq. 1, Figure 2).

Experimental data of kinetic measurements performed with commercial laccase in various media, shown in Figure 2, are in good agreement with the results of simu-

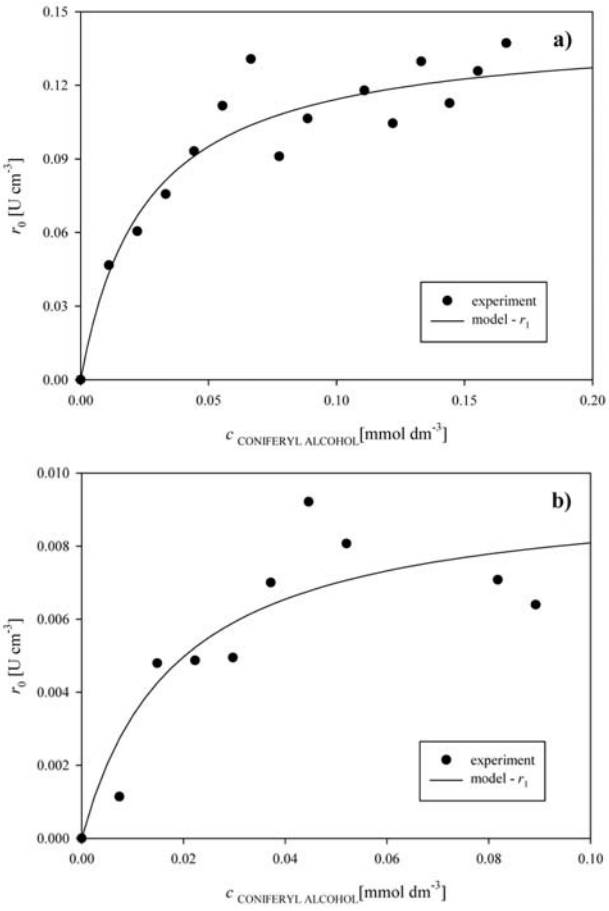

Figure 2. Activity of commercial enzyme in various media at pH 6.6 and 35 °C. Dependence of the initial reaction rate (r_0) on the concentration of coniferyl alcohol for **a)** 0.2 mol dm^{-3} phosphate buffer, $\gamma_{laccase}$ = 0.033 mg cm^{-3} and **b)** 1:1 (v/v) methanol: 0.2 mol dm^{-3} phosphate buffer mixture, $\gamma_{laccase}$ = 0.099 mg cm^{-3}

lation obtained with the proposed Michaelis-Menten kinetic model (Eq. 1).

Table 2. Estimated apparent kinetic parameters for oxidation of coniferyl alcohol catalyzed by commercial laccase in aqueous and organic medium

Media	Parameters	Unit	Value
Aqueous	$K_m^{CONIFERYLALCOHOL}$	mmol dm^{-3}	0.025 ± 0.007
	$V_m^{CONIFERYLALCOHOL}$	U mg^{-1}	4.387 ± 0.303
Organic	$K_m^{CONIFERYLALCOHOL}$	mmol dm^{-3}	0.019 ± 0.011
	$V_m^{CONIFERYLALCOHOL}$	U mg^{-1}	0.979 ± 0.182

Apparent kinetic parameters, which were estimated, are shown in Table 2. As can be seen, applied organic medium did not significantly affect the affinity of commercial enzyme. However, the estimated activity of enzyme in solution containing 50 % organic solvent was 5 times lower than in water media. This is consistent with previously reported results, where 10 times lower laccase activity on coniferyl alcohol was obtained in 50 % acetone – buffer mixture[24] and with well known fact that organic media strongly reduce activity of the enzyme.

3. 3. Oxidation of Coniferyl Alcohol by Crude Laccase

Crude enzyme was separated from the production medium by centrifugation and kinetically characterized without any further purification and concentration step. Kinetic studies for oxidation of coniferyl alcohol by crude laccase were performed in a cuvette of spectrophotometer using the initial reaction rate method. Enzyme kinetics was measured at pH 6.6 and T = 35 °C with various initial substrate concentrations. Substrate inhibition of crude laccase was taken into account. Data from the initial reaction rate experiments is shown in Figure 3, from which could be verified monosubstrate Michaelis-Menten type of kinetics with substrate inhibition.

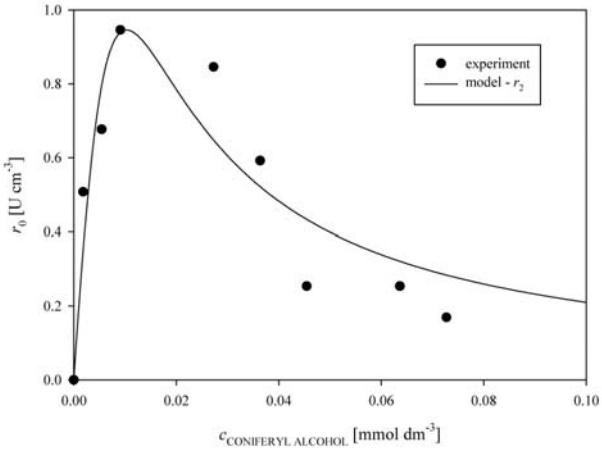

Figure 3. Kinetics of crude enzyme in a cuvette. Dependence of the initial reaction rate (r_0) on the concentration of coniferyl alcohol ($\gamma_{laccase}$ = 0.94 mg cm^{-3}, 0.2 mol dm^{-3} phosphate buffer, pH 6.6, T = 35 °C)

Apparent values of V_m, K_m and K_I (Table 3) were estimated by fitting the experimental data to the Michaelis-Menten equation with substrate inhibition (Eq. 2). There is not much literature data for estimation of K_m for lacca-

Table 3. Estimated apparent kinetic parameters for oxidation of coniferyl alcohol catalyzed by crude laccase in the cuvette of spectrophotometer

Laccase	Parameters	Unit	Value
Crude	$K_m^{CONIFERYLALCOHOL}$	mmol dm^{-3}	0.045 ± 0.014
	$K_I^{CONIFERYLALCOHOL}$	mmol dm^{-3}	0.002 ± 0.001
	$V_m^{CONIFERYLALCOHOL}$	U mg^{-1}	9.272 ± 0.938

ses from *Trametes versicolor* with regard to phenolic substrates and probably the K_m value for laccase was estimated for the first time in this work.

The result, shown in Tables 2 and 3 show that crude laccase has a lower affinity for coniferyl alcohol than commercial laccase and has greater activity. Estimated K_m values for both enzymes, are in the μmol dm^{-3} range, which is in line with previously published data for the oxidation of syringyl-type phenols catalyzed by peroxidases.[25] Furthermore, significant inhibition of the substrate was evident in the coniferyl alcohol oxidation catalyzed by crude laccase, but not with commercial enzyme, which indicate the likely structural differences between both enzymes.

3. 4. Laccase-catalyzed Oxidation of Coniferyl Alcohol in the Batch Reactor

To investigate the influence of dissolved oxygen concentration on the reaction rate, laccase-catalyzed oxidation of coniferyl alcohol was performed in a batch reactor with or without continuous aeration at process conditions listed in Table 1. Laccase activity using ABTS as substrate was measured during the process and did not change significantly during the reaction (data not shown), which means high operational stability of enzyme. Data for coniferyl alcohol oxidation catalyzed by commercial and crude laccase, where aeration is achieved only through the mass transfer across interfacial area, are shown in Figure 4. As can be seen, the commercial enzyme is much more efficient in coniferyl alcohol oxidation from crude enzyme, although the latter is used in higher concentration. This was the result of earlier detected substrate inhibition of crude laccase from *T. versicolor* (Figure 3). In any case, the efficiency of coniferyl alcohol oxidation catalyzed by this non-purified enzyme preparation is similar to previously published data obtained with purified laccase from white-rot fungus, *Rigidoporus lignosus*, trees *Acer pseudoplatanus* and *Populus euramericana*.[10]

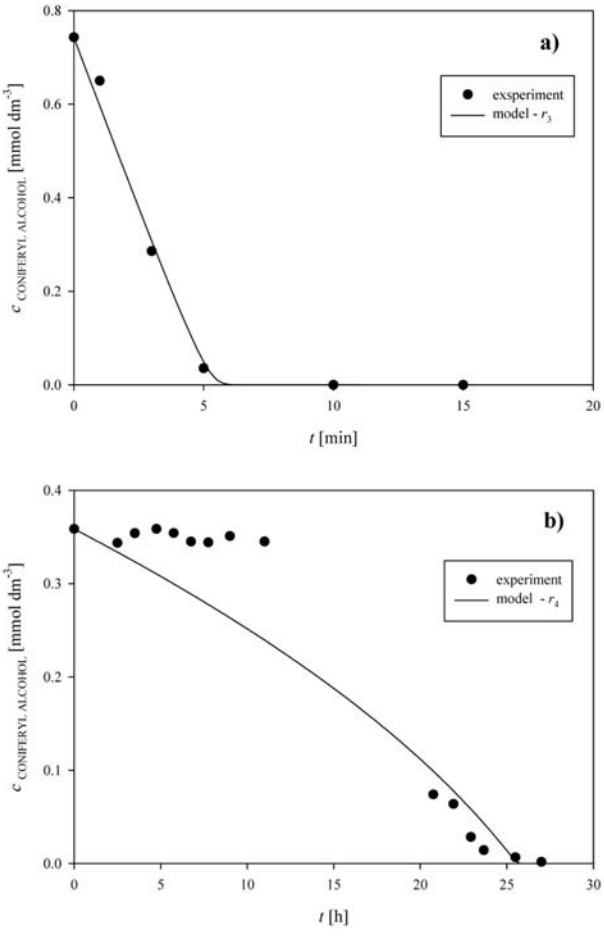

Figure 4. Coniferyl alcohol oxidation catalyzed by **a) commercial laccase**; $\gamma_{laccase}$ = 0.099 mg cm^{-3} and **b) crude laccase**; $\gamma_{laccase}$ = 0.94 mg cm^{-3} (0.2 mol dm^{-3} phosphate buffer, pH 6.6, T = 35 °C). Batch process was performed without additional air supply.

As seen from Figure 4a, a mathematical model consisting of mass balance for coniferyl alcohol in batch reactor (Eq. 5) and double-substrate Michaelis-Menten kinetics with oxygen as a second substrate (Eq. 3) can successfully describe the coniferyl alcohol oxidation catalyzed by commercial laccase. However, the description of the process catalyzed by crude laccase (Figure 4b), where substrate inhibition was taken into account (Eq. 4) is not appropriate, especially in the first phase of the reaction (about 10 hours) where the concentration of coniferyl alcohol is a constant. The estimated K_m^{O2} value of 0.099 mmol dm^{-3} for oxygen is used in double-substrate Michaelis-Menten kinetic model[21], but the value of V_m for commercial and crude enzyme must be re-estimated from data obtained in experiments in batch reactor (Table 4). Enzyme activity for crude laccase is significantly lower due to insufficient oxygen supply through the air-water layer, which agrees with previously reported data in the literature.[6,21]

Table 4. V_m values, estimated from the data obtained in batch reactor with and without aeration for commercial and crude enzyme

Laccase	Aeration	V_m [U mg^{-1}]
Commercial	Without	14.050 ± 1.220
Crude	Without	1.824 ± 0.037
	With	11.281 ± 0.257

Therefore, an additional experiment with crude enzyme preparation of laccase was carried out in the continuously aerated batch reactor. As seen from Figure 5, 100 % conversion of coniferyl alcohol was achieved after 10 h, which was significantly faster compared to the experiment without aeration (Figure 4b). A developed model that consists of double-substrate Michaelis-Menten kinetics with substrate inhibition (Eq. 4) and mass balances for coniferyl alcohol (Eq. 5) and oxygen (Eq. 6) describes the experimental data quite well. This confirms the multi-sub-

strate nature and the strong substrate inhibition of coniferyl alcohol oxidation catalyzed by crude laccase. $k_L a$ value of 0.4 h^{-1}, which is used for model simulations was estimated from independent measurements using the integral method.[26] It is interesting that the estimated value of the maximum reaction rate (V_m) for crude laccase, which is shown in Table 4, does not differ significantly from the value determined in the cuvette of spectrophotometer using the method of initial reaction rate (Table 3).

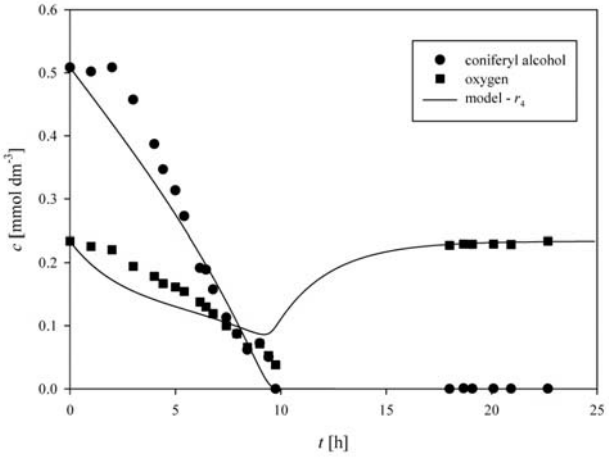

Figure 5. Coniferyl alcohol oxidation catalyzed by crude laccase at constant aeration ($\gamma_{laccase}$ = 0.94 mg cm^{-3}, 0.2 mol dm^{-3} phosphate buffer, pH 6.6, T = 35 °C)

4. Conclusions

The study of coniferyl alcohol oxidation catalyzed by commercial laccase revealed the highest enzyme activity at pH 6.6 and a temperature of 35 °C. Apparent values of K_m for commercial laccase in water medium (K_m = 0.025 mmol dm^{-3}) and for commercial laccase in 50 % methanol-phosphate buffer mixture (K_m = 0.019 mmol dm^{-3}), as well as for crude laccase in water medium (K_m = 0.045 mmol dm^{-3}) were estimated for the first time. 100 % conversion of laccase-catalyzed oxidation of coniferyl alcohol in a batch reactor with and without additional aeration was obtained. The proposed model that consists of a double substrate Michaelis-Menten kinetic model with oxygen as second substrate and the mass balances for the batch reactor, describes well the experimental data obtained from a series of experiments with a commercial enzyme, while the model for crude enzyme was not appropriate. Crude laccase showed significantly higher affinity and somewhat lower efficiency in comparison with commercial purified enzyme. It is important to emphasize that crude laccase used throughout this study was a supernatant of a submerged culture of *T. versicolor*, cultivated in media containing waste from paper industry without any additional synthetic inducer. Furthermore, crude laccase was kinetically characterized and successfully used for the oxidation of coniferyl alcohol without any purification and concentration steps. It represents an environmentally friendly and economically efficient alternative and the potential for oxidation of a wide range of phenolic compounds.

List of symbols and abbreviations

$c^*_{O_2}$	oxygen concentration in saturation, mmol dm^{-3}
c	concentration, mmol dm^{-3}
ε	molar extinction coefficient, dm^3 $mmol^{-1}$ cm^{-1}
K_i	substrate inhibition constant, mmol dm^{-3}
$k_L a$	volumetric mass transfer coefficient, h^{-1}
K_m	Michaelis-Menten constant, mmol dm^{-3}
r	reaction rate, U cm^{-3}
r_0	initial reaction rate, U cm^{-3}
S.A.	specific activity, U mg^{-1}
T	temperature, °C
t	time, min or h
V_m	maximal reaction rate, U mg^{-1}
γ	concentration, mg cm^{-3}
S	substrate
E	enzyme
0	initial conditions

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Povzetek

Proučili smo oksidacijo koniferil alkohola, katalizirano s komercialno lakazo in z neprečiščenim pripravkom lakaze, pridobljene s submerznim gojenjem glive *Trametes versicolor* MZKI G-99 v gojišču z odpadkom iz industrije papirja. Oksidacija koniferil alkohola, katalizirana s komercialno lakazo, je bila najuspešnejša pri pH 6,6 in temperaturi 35 °C. Na osnovi merjenja začetnih hitrosti smo komercialni lakazi določili navidezne kinetične parametre Michaelisa in Mentenove, tako v vodni raztopini (V_m = 4,387 U mg^{-1}, K_m = 0,025 mmol dm^{-3}), kot tudi v mešanici 1:1 (v/v) metanol:fosfatni pufer (V_m = 0,979 U mg^{-1}, K_m = 0,019 mmol dm^{-3}). Za neprečiščen pripravek lakaze smo ugotovili inhibicijo s substratom, ter ocenili naslednje navidezne kinetične parametre: V_m = 9,272 U mg^{-1}, K_m = 0,045 mmol dm^{-3}, K_m = 0,002 mmol dm^{-3}. Za šaržni proces oksidacije koniferil alkohola smo razvili matematični model, ki poleg snovnih bilanc vključuje kinetiko Michaelisa in Mentenove s kisikom kot drugim substratom. Model smo potrdili z eksperimenti, izvedenimi v šaržnih procesih z ali brez dodatnega prezračevanja. Z obema encimoma smo uspeli doseči 100 % pretvorbo koniferil alkohola v koncentracijah do 0,8 mmol dm^{-3}.