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Kinetics of the enzymatic decolorization of textile dyes by laccase from *Cerrena unicolor*

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Abstract

This study deals with the laccase-catalyzed decolorization of azo and anthraquinone dyes. Both purified laccase (Lacc I and Lacc II) as well as the crude enzyme from the white-rot fungus *Cerrena unicolor* were used to convert the dyes at pH 3.5 (optimum of laccase activity) in aqueous solution. Biotransformation of the dyes was followed spectrophotometrically and confirmed by high performance liquid chromatography (HPLC). The results indicate that the decolorization mechanism follows Michaelis–Menten kinetic and that the initial rate of decolorization depends both on the structure of the dye and on the dye concentration. The saturation constants (K_m) of purified laccase isoforms (Lacc I, II) differ to some extend indicating different substrate affinities. Surprisingly, one recalcitrant azo dye (AR 27) was decolorized merely by purified laccase in the absence of any redox mediator.

Keywords: Laccase; *Cerrena unicolor*; Textile dyes; Decolorization; Kinetics

1. Introduction

There is currently a large interest in the removal of color from a wide range of wastewaters [1,2]. The dyestuffs disposed to the surface water can prevent its recreational and economic use and affect its aesthetic and sentimental value. As consequence, sunlight transmittance is reduced, photosynthetic processes are negatively influenced and the water's self-purification potential is affected. But removing dyes from textile wastewater with conventional methods is a difficult and expensive process [3]. However, the new EU environmental regulations on hazardous wastes force the necessity of finding innovative and environmentally friendly treatment technologies to prevent dye stuff damages. One approach is the use of enzyme-based methods, which have a minimal impact on ecosystems and low energy requirements. Moreover, enzymes can operate in a wide pH range, at moderate temperature or ionic strength and, to some extent, they are active in the presence of organic solvents [4].

Laccases (EC 1.10.3.2: benzenediol, oxygen oxidoreductase, *p*-diphenol oxidase) from plants and fungi have been known for decades [5]. These multi-copper oxidases contain four copper ions of two different types: one type I Cu, whose redox potential determines the substrates to be oxidised and other three Cu ions transferring electrons to O_2. The enzyme has different functions in the individual organism [6], and among others, it is thought to be involved in the synthesis of lignin by plants and the biodegradation of lignin by white-rot fungi [7]. As substrates, laccase can convert *o*- and *p*-diphenols, aminophenols, polyphenols, polyamines and lignin [8]. The interest in laccases as potential industrial biocatalysts has particularly increased after the discovery of their ability to oxidise recalcitrant nonphenolic lignin compounds [9].

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This capability has later been shown to be generally applicable to a number of biotechnological problems, all of them are related to the degradation or chemical modification of structurally diverse compounds, being either xenobiotic [10] or naturally occurring aromatic compounds [11]. Laccase is currently being investigated by a number of research groups, e.g. with respect to litter mineralization [12], dye detoxification and decolorization [13,14] or the bleaching of paper pulp [15,16].

Several reports have been published on the decolorization of industrial dyes by laccase and laccase-producing fungi, in the course of which also different groups of dyes were examined [17,18] as well as the environmental conditions of their bioconversion [19] and the kinetic characteristics of laccase oxidations [20]. Other studies dealt with the kinetics of bacterial dye decolorization [21,22] and with the kinetics of biosorption [23]. For the enzymatic reactions at constant temperature, the Michaelis–Menten equation is commonly in use. Many authors indicated the key role of mediators in decolorization [24]. The aim of the present work was to examine the ability of purified and crude laccase preparations from *Cerrena unicolor* to decolorize different dyes including two recalcitrant ones without any mediator and to investigate the kinetics of this process.

2. Experimental section

2.1. Fungal strain and culture conditions

The *C. unicolor* (Bull. ex Fr.) Murr. strain 137 used in this study was obtained from the culture collection of the Department of Biochemistry, Maria Curie-Sklodowska University, Lublin, Poland. Stock cultures were maintained on 2% malt extract agar (MEA) slants at 4 °C, and inoculation material was pregrown on MEA plates at 25 °C for 10–14 days. For laccase production, two different liquid media were prepared. The modified Kirk medium (KM) contained per liter: glucose 13 g; KH_2PO_4 2 g; $MgSO_4$ 0.5 g; $CaCl_2$ 0.1 g; diammonium tartrate 0.5 g, yeast extract 0.25 g; to increase laccase production, $CuSO_4 \cdot 5H_2O$ was added to a final concentration of 50 μM Cu^{2+}. The pH of the medium was adjusted to 4.5 by addition of solid 2,2-dimethylsuccinic acid. Lindeberg–Holm medium (L–HM) comprised following compounds (per liter of distilled water): glucose 10 g; L-asparagine 1.5 g; KH_2PO_4 0.47 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; $Na_2HPO_4 \cdot 12H_2O$ 0.48 g; yeast extract 0.1 g and microelements: $Mn(CH_3COO)_2 \cdot 4H_2O$ 12 mg; $Zn(NO_3)_2 \cdot 6H_2O$ 3.14 mg; $CuSO_4 \cdot 5H_2O$ 3.19 mg; $Ca(NO_3)_2 \cdot 4H_2O$ 50 mg; $FeCl_3 \cdot 6H_2O$ 3.2 g; thiamine 50 μg. The pH of the medium was adjusted to 5.6 by addition of HCl. Liquid media were sterilized at 121 °C and 1.6 bar for 15 min. Cultures were inoculated with homogenized fungal mycelium from overgrown MEA plates and incubated on a rotary shaker (100 rpm) at 25 °C. Samples (1 ml of the culture liquid) were taken from each flask under sterile conditions every two days from day five onwards.

After 14 days of fungal growth, laccase activity reached its maximum and the cultures were harvested. The liquid of KM cultures was filtered through glass fiber filters (GF6, Schleicher & Schuell, Dassel, Germany) and the clear supernatant was used for further purification by FPLC as described previously [25]. Finally, we obtained two isoforms-laccase I (Lacc I) and laccase II (Lacc II), which were used for in vitro decolorization experiments. The concentrated culture liquid from L–HM was used as crude enzyme preparation.

2.2. Textile dyes

Following water-soluble dyes were selected as model compounds: C.I. Acid Blue 62 (AB 62), C.I. Acid Blue 40 (AB 40), C.I. Reactive Blue 81 (RB 81), C.I. Direct Black 22 (DB 22) and C.I. Acid Red 27 (AR 27) (Fig. 1); they are all market products of the Research Center of the Boruta Chemical Plant in Zgierz, Poland. Only in case of C.I. Acid Blue 62 pure substance was synthesized and purified by repeated crystallisation. Studied dyes belong to different groups regarding their functionalities and display totally different physical and chemical properties, but all of them have been widely used in textile industry.

2.3. Enzyme assays

Laccase activity was determined in the culture liquid measuring the oxidation of ABTS (2,2′-azino-bis(3-ethylthiazoline-6-sulfonate) (300 μM) in 50 mM citrate–phosphate buffer (pH 4.5; $\varepsilon_{420} = 36\ mM^{-1}\ cm^{-1}$), or using 0.5 mM syringaldazine dissolved in ethanol as the substrate buffered in 0.1 M citrate-buffer (pH 5,6, $\varepsilon_{525} = 65\ mM^{-1}\ cm^{-1}$). All spectrophotometric measurements were carried out using a Carry 50 spectrophotometer (Varian, Darmstadt, Germany) or a UV-300 spectrophotometer (Unicam, Cambridge, UK). Enzyme activities were expressed in units (U) defined as 1 μmol of product formed per min. All chemicals were obtained from Sigma-Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany).

2.4. Decolorization assays

Dye conversion was followed using a spectrophotometric method which recorded the UV–vis spectrum every 30 s for AB 62 and every 60 s for RB 81 in the wavelength range 250–750 nm. In case of AR 27, spectra were recorded after 0.5, 1, 2, 6, and 24 h due to its slower oxidation.

Quantitative measurements were made at the absorption maxima of the individual dyes in the visible spectral range (584 nm for RB 81, 638 nm for AB 62, 480 nm for DB 22, 620 nm for AB 40, 520 nm for AR 27).

2.5. Optimum pH of decolorization

To estimate the optimum pH of the decolorization process, activities of Lacc I, Lacc II and crude laccase were measured with AB 62, AB 40, RB 81 and DB 22 in a citrate–phosphate buffer (50 mM) in the pH range 2.5–7.0. Following initial dye concentrations were used: AB 62, 125 μM; AB 40, 211 μM; RB 81, 124 μM; and DB 22, 92.25 μM.

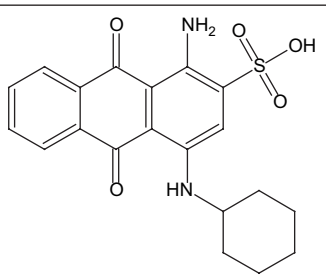
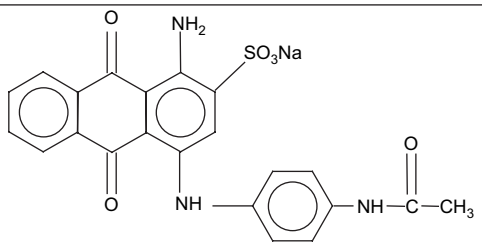
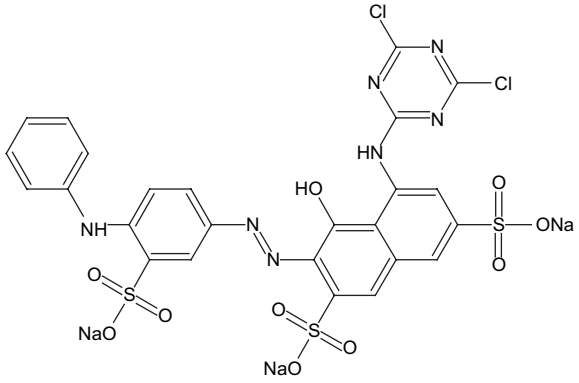
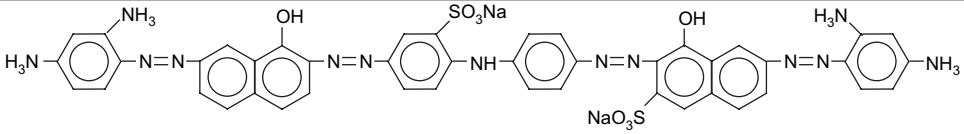
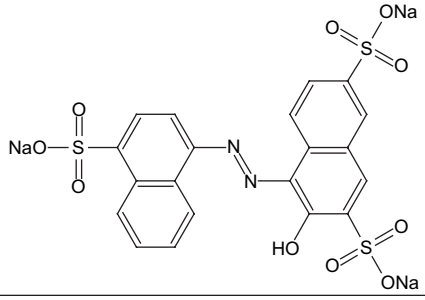
Acid Blue 62 C.I. 62 045 anthraquinone dye	
Acid Blue 40 C.I.62 125 anthraquinone dye	
Reactive Blue 81 C.I.18 245 reactive azo dye	
Direct Black 22 C.I.35 435 azo dye	
Acid Red 27 C.I.16 185 azo dye	

Fig. 1. The structures of the studied dyes.

2.6. HPLC analysis

Acid Blue 62 (45% pure dye) and its reaction products formed due to laccase catalysis were analyzed by high performance liquid chromatography (HPLC). The reaction mixture contained 50 mM citrate–phosphate buffer, 125 μM AB 62 and 0.25 U Lacc I. Reaction was performed at 23 °C, started by the addition of laccase and stopped after 20 min. The HPLC system (Agilent HP 1090, Waldbronn, Germany) was equipped with a diode array detector and a LiChroCART RP 18 column (Merck, Darmstadt, Germany). A mixture of methanol and 0.05% phosphoric acid (H_3PO_4) (60:40, vol/vol) served as solvent at a flow rate of 1 ml min^{-1} under isocratic conditions. Eluted substances were detected in the wavelength range from 190 to 700 nm.

2.7. Kinetic studies

Substrate specificity towards the different dyes was kinetically analyzed in a cuvette system temperated at 25 °C using 450 μl dye solution (at varying concentrations, 10–120 μM) and 50 μl of laccase (0.05 U for pure enzyme, 0.2 U for crude

enzyme) in 500 µl citrate–phosphate buffer (pH 3.5; optimum pH for dye oxidation). Decolorization was monitored spectrophotometrically by following the initial slope at the dyes' absorption maxima (see Section 2.4). The initial rates of decolorization, Michaelis–Menten constants (K_m) and catalytic constants (k_{cat}) of purified and crude *C. unicolor* laccases were determined by linear regression and Hanes–Woolf plots [26].

3. Results

3.1. Laccase production

C. unicolor produced high amounts of laccase in both liquid media [4.000 U l^{-1} and 3.600 U l^{-1} measured with ABTS (KM) and syryngaldazine (L–HM), respectively]. Lacc I and Lacc II obtained after purification showed different physico-chemical and catalytic properties. Lacc I (M_w 64 kDa, pI 3.6) had a slightly higher affinity to the substrates ABTS, 2,6-dimethoxyphenol (DMP) and syringaldazine [25]. Lacc II (M_w 57 kDa, pI 3.7), on the other hand, was found to be more stable and did not lose any activity during 6 months' storage at 4 °C. The culture liquid from L–HM was not further concentrated and purified as we used it – for the purpose of comparison – as crude laccase preparation with all fungal metabolites.

3.2. Enzymatic decoloration of dyes

Purified and crude laccase from *C. unicolor* were used for decolorization studies with aqueous dye solutions in order to evaluate it's role in the conversion process. Except AR 27, all dyes were transformed to some extent by both purified and crude enzymes. This finding suggests that laccase is seemingly the key enzyme in dye degradation by *C. unicolor*. The results for purified laccase (Fig. 2A–C) demonstrate that the purified enzyme is able to transform both anthraquinone as well as azo dyes but not with the same efficiency. The decolorization was dependent both on the actual enzymatic activity and the chemical structure of the individual dye. The analysis of data indicate that AB 62 and RB 81 were more susceptible to enzymatic transformation than AR 27 in all reaction systems tested. For these two dyes, decolorization was observed almost immediately after adding the enzyme (12.5 min for AB 62; 20 min for RB 81). In contrast, the decolorization of AR 27 lasted much longer (24 h), but the final result was satisfactory since the solution had become transparent. In each case, the chromophore peak decreased with the reaction time (at wavelengths 638 nm for AB 62, 584 nm for RB 81, 520 nm for AR 27) which proves the transformation of the dyes. Simultaneously new peaks appeared in the shorter wavelength range of the spectra (320, 435 nm for AB 62; 350, 450 nm for RB 81) indicating the formation of conversion products. Some of these new peaks disappeared in the further course of the enzymatic treatment (data not shown) while others remained indicating the persistance of primary oxidation products.

Furthermore, decolorization of AR 27 was dependent on the enzyme activity applied. To achieve the effect shown in Fig. 2C, a 50-fold higher amount of the purified enzyme was required and using crude laccase, decolorization was not achieved at all. In contrast, AB 62 and RB 81 were efficiently decolorized both by the purified and crude enzyme preparations.

3.3. Optimum pH of decolorization

The effect of pH on laccase-catalyzed decolorization was similar for all dyes and enzyme preparations studied (Fig. 3A–C). The pH profiles show a maximum decolorization efficiency around pH 3.5. While the profiles of both laccase isoforms were almost identical for AB 62, Lacc I and Lacc II differed with respect to the oxidation of RB 81. Lacc I maintained about 75% of its maximum activity in the pH range from 2.5 to 5, whereas Lacc II showed a sharp activity maximum at pH 3 in the pH profile, which rapidly declined towards higher pH (40% activity at pH 5). At neutral pH, activity was drastically reduced in all cases reaching the best at 20% of its maximum value. No significant color change was observed in all control flasks containing the dyes in aqueous solutions at different pH without laccase.

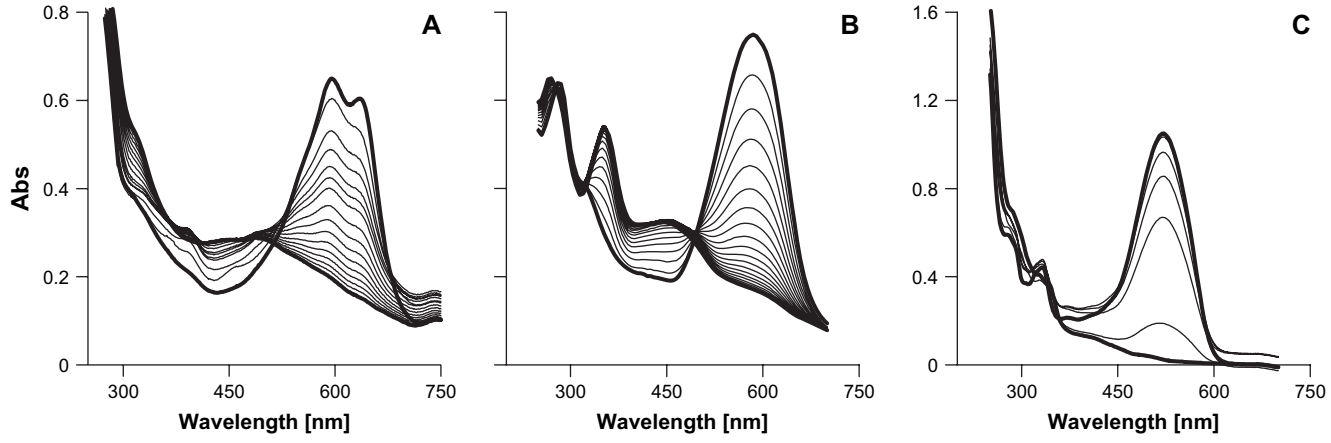


Fig. 2. UV–vis spectra recorded during the pure laccase-catalyzed decolorization of dyes: A – AB 62, spectra were recorded every 30 s over a total time of 12.5 min; B – RB 81, recording interval 60 s, total time 20 min; C – AR 27, recording intervals 0.5–6 h, total time 24 h.

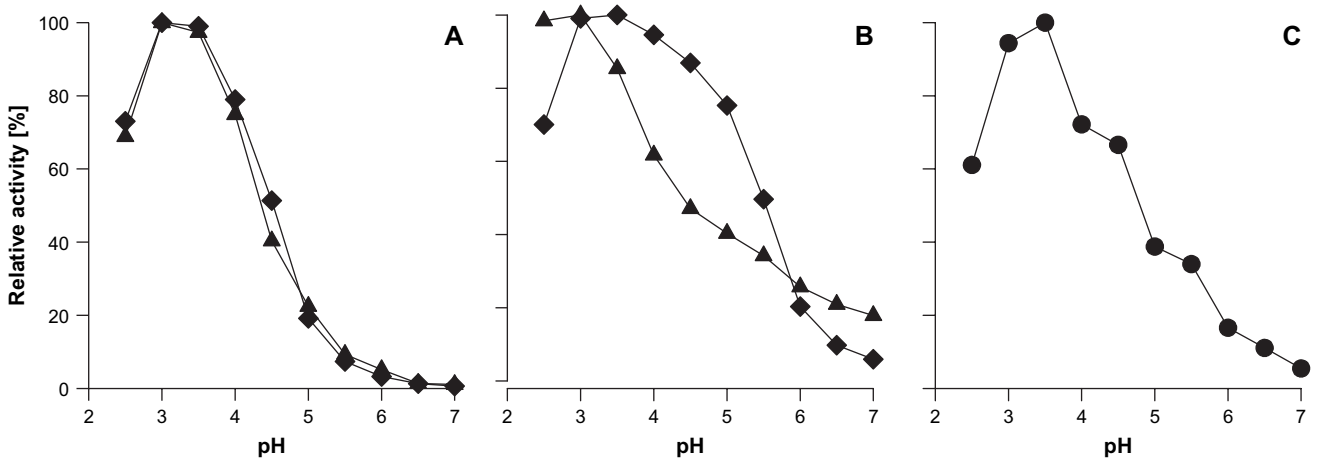


Fig. 3. Effect of pH on laccase-catalyzed dye decolorization: A and B: purified *C. unicolor* laccase isoforms I and II, A – with AB 62, B – RB 81; Lacc I (black diamonds), Lacc II (black triangles); C: crude *C. unicolor* laccase with DB 22. Measurements were carried out in triplicate (standard deviations < 5%).

3.4. HPLC analysis

To ensure that the dye decolorization catalyzed by laccase is a real chemical transformation, we followed the conversion of AB 62 by Lacc I with HPLC. The dye was chosen because of its extensive decolorization. The HPLC elution profiles of AB 62 before and after laccase treatment show that the dye peak (retention time 2.6 min) disappeared while two new peaks and a shoulder emerged with considerably shorter retention times (1.18, 1.23, 1.42 min; Fig. 4) indicating the formation of more polar oxidation products. The major product peak (1.23 min) corresponds to a substance, whose absorption spectrum differs considerably from that of AB 62. Whereas the latter has three maxima at 260, 595 and 638 nm, the oxidation product is characterized by two absorption maxima in the shorter wavelength range (295, 465 nm). The data clearly demonstrate that laccase is capable of modifying the chemical structure of the dye AB 62 though a complete degradation (mineralization) did not occur as the metabolite got accumulated in the reaction solution.

3.5. Enzyme kinetic data (catalytic properties)

To investigate the mechanism of enzymatic conversion, a kinetic model has been used to fit the experimental data. The correlation between specific decolorization rate and dye concentration can be described by a Michaelis–Menten kinetics. The kinetic constants, Michaelis–Menten constant (K_m), maximum decolorization rate (V_{max}) and catalytic constant (k_{cat}) of purified *C. unicolor* laccases were determined for AB 62 and RB 81 (Table 1). Due to the persistence of AR 27, kinetic parameters could not be determined for this dye (neither using crude nor purified enzyme). Studies with crude enzyme were performed with AB 62, RB 81, AB 40 and DB 22 and allowed the estimation of K_m and V_{max} (Table 1). Hanes–Woolf plots were made from the initial rates obtained at varying dye concentrations while the amount of enzyme was held constant (Fig. 5A and B).

The results for AB 62 and RB 81 show that the K_m values of purified laccase were in most cases (except Lacc II with RB 81) lower than those of the crude enzyme, which can indicate a higher affinity of the purified protein to the dyes. This finding confirms again that laccase is mainly responsible for dye decolorization. In the case of the purified enzyme, differences were found between the two isoforms (Lacc I, Lacc II), which were not observed in an earlier study that dealt with the oxidation of ABTS, DMP and syringaldazine [25]. Thus, K_m values

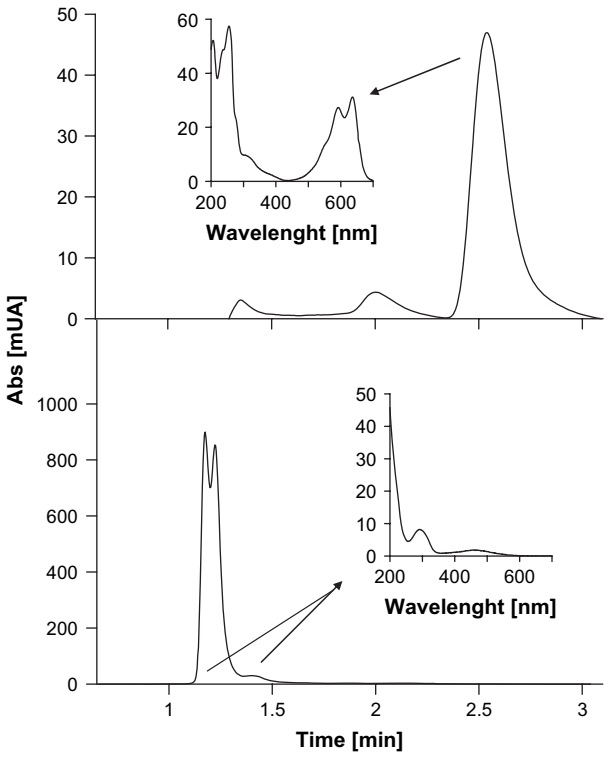


Fig. 4. HPLC elution profile of Acid Blue 62 and it's conversion products before (above) and after (below) treatment with purified *C. unicolor* laccase. The insets show the UV–vis spectra of AB 62 (above) and the conversion product at 1.23 min (below).

Table 1
Kinetic constants of purified and crude laccase from *C. unicolor*

Substrate		K_m (μM)	V_{max} (μmol min^{-1})	k_{cat} (s^{-1})
Acid Blue 62	Lacc I	42	0.142	366
	Lacc II	131	0.210	267
	Crude enzyme	306	0.373	–
Reactive Blue 81	Lacc I	18	0.032	16.4
	Lacc II	95	0.026	7
	Crude enzyme	79	0.012	–
Acid Blue 40	Crude enzyme	34	0.018	–
Direct Black 22	Crude enzyme	102	0.001	–

of Lacc I were lower than those of Lacc II (5,6-fold for AB 2, 2,5-fold for RB 81), which indicated a higher affinity of Lacc I to each of the dye-stuffs.

Surprisingly, the lowest K_m value (34 μM) was estimated for the oxidation of AB 40 by crude laccase, suggesting that this compound is well susceptible to laccase attack. A satisfactory result was also achieved with the crude enzyme for azo dye DB 22 ($K_m = 102$ μM), the value of which was in the range of that for RB 81 ($K_m = 79$ μM) and three times higher than that for AB 62 ($K_m = 306$ μM).

4. Discussion

Laccase from the white-rot fungus *C. unicolor* was found to be suitable for the decolorization of the different classes of dyes both using purified and crude enzyme preparations. This ability is seemingly connected with the earlier described capability of an efficient lignin destruction [27]. It must be emphasized that in our study, efficient decolorization was achieved without any mediator while a number of reports describe the necessity of redox mediators [24,28].

Maximum decolorization efficiency for all dyes tested was observed at a distinct pH value of 3.5, which is slightly lower than those reported for fungal cultures of *Phanerochaete chrysosporium* [17] or *Coriolus versicolor* laccase [19] (optimum pH range 4.0–5.0).

Laccase activity and the initial dye concentration are the parameters which determine the decolorization performance. In some cases, e.g. AR 27, the absolute enzyme activity can be crucial and below a certain level, decolorization did not occur. This result can confirm earlier reports [29,33] describing the resistance of azo dyes to microbial and enzymatic degradation. On the other hand, we were able to prove that an increased amount of purified laccase (50 U ml^{-1}) catalyzed the complete decolorization of recalcitrant AR 27 within 24 h. Futhermore, RB 81 which is also azo dye shows high susceptibility to decolorization. Again, it has to be pointed out that complete decolorization can be achieved without redox mediators, which confirms also the latest study [30] and contradicts the crucial role of mediators for azo dye conversion mentioned in earlier studies [24,29].

Our results show furthermore that the isoforms of purified laccase have different specificities towards the individual dyes. Thus compared to Lacc II, Lacc I showed a higher affinity to all substrates tested. This is in line with data reported for *Trametes versicolor* laccase [31], where also one isoform was found to possess a higher decolorization potential than the others.

Comparing the affinity of laccase to the dyes tested, one can conclude that for the crude enzyme, AB 40 is the most susceptible and suitable substrate. Moreover, it is interesting to note that AB 62 and AB 40 are both anthraquinone dyes, showing, however, different affinities to laccase. The crude enzyme has an almost 10-fold higher affinity to AB 40 (K_m of 34 μM) than to AB 62 (K_m of 306 μM). Comparing the azo dyes AR 27 and DB 22, we found that the crude enzyme can only decolorize DB 22 and not AR 27, which may be due to the limited accessibility of the –OH groups in AR 27. Interestingly, the reactive azo dye RB 81 was the preferred substrate for *C. unicolor* laccase (with the lowest K_m value of 18 μM). Unlike the findings in earlier reports [13,32], which

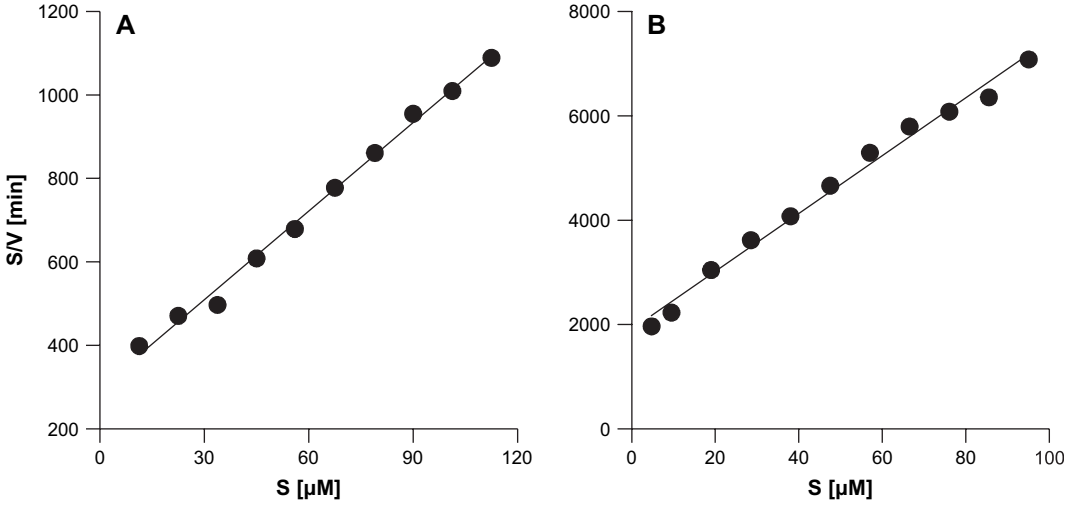


Fig. 5. Linearization plots: A – Hanes–Woolf plot of decolorization of Acid Blue 62 by pure *C. unicolor* laccase, B – Hanes–Woolf plot of decolorization of Acid Blue 40 by crude *C. unicolor* laccase.

indicated a higher affinity of laccase to anthraquinone dyes, RB 81 was oxidised more efficiently than anthraquinone dye AB 62.

Most reports on dye decolorization available in the literature [33] describe visual as well as spectroscopic changes and the formation of unknown, still colored reaction products. In our study, the final reaction solutions were also not colorless after decomposition of AB 62, RB 81 and DB 22. Typically, the absorbance maxima of the dye solution shifted towards lower wavelengths in the UV range as well as to the blue region in the visible range (around 400 nm). This phenomenon indicates the formation of new stable oxidation products which are not substrates for further laccase catalysis.

Furthermore, it was proved that the disappearance of the primary color of dye was not simply caused by protonation/deprotonation, since the HPLC analysis showed the formation of new, more polar products with shorter retention times while the pH of the reaction solution was not altered during the decolorization process [34].

The kinetics of the decolorization process can be sufficiently described by the Michaelis–Menten model which is in agreement with other literature data [17,20,35]. The K_m values for dye oxidation were found to vary between 18 and 306 μM which fits well with the range (about 10^{-5} M) reported by Yaropolov for the cosubstrate oxygen [36].

5. Conclusions

The capability of crude and pure laccase preparations from *Cerrena unicolor* for removing different classes of dyes was examined, including kinetic studies and HPLC analysis. The obtained results showed that enzyme activity and the initial dye concentration determine the decolorization performance and the process can be satisfactorily described by the Michaelis–Menten kinetic model. The most suitable pH for decolorization was 3.5. According to our study besides anthraquinone dyes laccase was able to decolorize also recalcitrant azo dyes all without any redox mediators. Last but not least, the results also suggest the possible industrial application of laccases from white-rot fungi for the bioconversion-complete decolorization of recalcitrant dye-stuffs.

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