EFFECT OF METAL IONS ON TRIPHENYLMETHANE DYE DECOLORIZATION BY LACCASE FROM *TRAMETES VERSICOLOR*

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Abstract: The aim of this study was investigate the influence of different metal ions on laccase activity and triphenylmethane dye decolorization by laccase from white-rot fungus *Trametes versicolor*. Laccase activity was inhibited by monovalent ions (Li^+, Na^+, K^+ and Ag^+) but the presence of divalent ions increased laccase activity at the concentration of 10 mmol/l. The effect of metal ions on decolorization of triphenylmethane dyes with different structures namely Bromochlorophenol Blue, Bromophenol Blue, Bromocresol Blue and Phenol Red was tested. The presence of metal ions (Na^+, K^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, Mn^{2+}, Zn^{2+}) slightly decreased triphenylmethane dye decolorization by laccase from *T. versicolor* except Na^+ and Mg^{2+}, which caused the increase of decolorization for all tested dyes. Decolorization of selected dyes showed that the presence of low-molecular-weight compounds is necessary for effective decolorization. Hydroxybenzotriazole (HBT) is the most frequently used. Although HBT belongs to most frequently used redox mediator and generally increase decolorization efficiency, so its presence decreased decolorization percentage of Bromophenol Blue and Bromochlorophenol Blue, the influence of metal ions to dye decolorization by laccase has the similar course with or without presence of redox mediator HBT.

Key words: laccase activity, *Trametes versicolor,* triphenylmethane dyes, decolorization, metal ions, hydroxybenzotriazole

1. Introduction

Synthetic dyes with different heterocyclic, anthraquinone, triphenylmethane or azo-based chemical structures are extensively used in industrial applications. Triphenylmethane dyes are widely used in various industrial sectors (dying of nylon, wool, silk or cotton, colouring of plastics, fats, oils or waxes, dermatological agents) and therefore are the main pollutants in wastewater (THAKUR, 2006; CASAS *et al*., 2009; YAN *et al*., 2014). Therefore, it is necessary to find ecologically effective biological removal of these solutions because chemical or physical methods are largely ineffective (ROBINSON *et al*., 2001). Biological methods have many advantages such as environmental friendly or production of less sludge (CASAS *et al*., 2009). Currently, one of the possible alternatives for biodegradation of synthetic dyes is the use of white-rot fungi which produce extracellular ligninolytic enzymes, especially laccases, which can oxidize wide spectrum of phenolic and non-phenolic substrates including synthetic dyes (CASAS *et al*., 2009; LEVIN *et al*., 2010; ASHRAFI *et al*., 2013; SAROJ *et al*., 2014).

Laccases (phenoloxidases, EC 1.10.3.2) are oxidases which belong to multi-copper oxidases family. These enzymes can oxidize various organic pollutants, using molecular oxygen as the final electron acceptor (BARRECA *et al*., 2003;

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CAMARERO *et al*., 2005). Laccases can be used for triphenylmethane dye degradation. However, in terms of the practical applications of laccases to degradation dyes, such as triphenylmethane dye-containing wastewater, there are still many problems to overcome. Wastewater typically contains high levels of salts, metal ions and organic compounds which could decrease laccase activity (GRASSI *et al*., 2011). Therefore, there is a need for laccase with high tolerance to different ions or compounds which are present in the industrial wastewater. Although several studies have been investigated on laccase/mediator system of synthetic dye decolorization (LIU *et al*., 2004; GRASSI *et al*., 2011; CHEN and TING, 2015), little attention has been focused on the effect of metal ions on laccase-catalysed dye decolorization (MURUGESAM *et al*., 2009).

Preliminary screening of laccase produced by different white-rot fungi was based on the literature. From the group of white-rot fungi, *Trametes* species are the most important sources for laccase with interesting properties (GRASSI *et al*., 2011; YAN *et al*., 2014). Namely *Trametes versicolor* is reported to be an excellent producer of laccase (BIRHANLI and YESILADA, 2010; SOUZA *et al*., 2011; PISCITELLI *et al*., 2011; WANG *et al*., 2013).

The aims of this study were to determine the effect of metal ions on laccase activity and to investigate triphenylmethane dye decolorization by laccase from *T. versicolor* with metal ions.

2. Materials and methods

2.1 Chemicals, dye and the enzyme

Hydroxybenzotriazole (HBT), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and four synthetic dyes were obtained from Sigma Aldrich (USA). All other reagents and chemicals were of the highest purity available and were obtained from MikroChem (Slovak Republic). The extracellular laccase from *Trametes versicolor* was purchased from Sigma Aldrich.

2.2 Effect metal ions on laccase activity

The effect of metal ions (Li^+, Na^+, K^+, Ag^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Sn^{2+} and Fe^{3+}) on laccase activity was determine in concentrations 1, 5 and 10 mmol/l with ABTS (1 mmol/l) in phosphate buffer (100 mmol/l; pH 5.0) after 10 minutes.

2.3 Decolorization of triphenylmethane dyes by laccase

Triphenylmethane dyes used for decolorization experiments were solubilized in 50 mmol/l phosphate buffer (pH 5.0). The reaction mixture (200 µl) contained phosphate buffer with dye (50 mg/l; 150 µl) and laccase solution (0.1 U/ml; 50 µl) with/without presence of inorganic ions (1 mmol/l) and redox mediator HBT (1 mmol/l). The reaction was initiated by enzyme addition. Reaction mixture was incubated at 20 °C and analysed every 24 hours. Absorbance was measured at

different wavelength depending on used synthetic dye. The percentage decolorization was calculated according to following formula:

$$Decolorization\ (\%) = \frac{Initial\ absorbance - Observed\ absorbance}{Inital\ absorbance} \times 100$$

2.4 Determination of laccase activity

Laccase activity was determined by oxidation of ABTS (SHIN *et al*., 1987). The assay mixture contained 150 µl of 50 mmol/l phosphate buffer (pH 5.0) with 1 mmol/l ABTS and 50 µl of enzyme extracts. Oxidation of ABTS was monitored by measuring of absorbance at 405 nm. Activity of laccase was expressed in unit (U) as the amount of enzymes able to oxidation of 1µmol of ABTS per minute.

2.5 Statistical analysis

All experiments were realized in triplicate. The obtained data were evaluated by Excel (Microsoft, 2010).

3. Results and discussion

3.1 Effect of metal ions on laccase activity

Metal ions are considered to be potential laccase inhibitors because these ions bind to the enzymes and de/stabilize the protein and they change the enzyme activity (ZHAO *et al*., 2012; HU *et al*., 2014). Textile wastewater usually contains a high concentration of metal ions such as Cu^{2+}, Zn^{2+} or Na^{+} which come from dye production process or the dye molecules (GRASSI *et al*., 2011; YAN *et al*., 2014). The first step was evaluation of the effect of some potential inhibitors such as Li^{+}, Na^{+}, K^{+}, Ag^{+}, Mg^{2+}, Ca^{2+}, Ba^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Sn^{2+} and Fe^{3+} ions on laccase activity. The influence of inorganic inhibitors to laccase expressed as percent of residual laccase activity is summarized in Table 1.

Laccase from *T. versicolor* seem to be tolerant to metal ions which are generally present in wastewater (Table 1). The results revealed that the most of the metal ions did not inhibit the laccase activity up to 1 mmol/l. However, laccase activity was strongly inhibited to various extents by the usual inhibitors, such as Fe^{3+}. Monovalent ions such as Li^{+}, Na^{+}, K^{+} and Ag^{+} inhibited laccase activity at the higher concentration (5 and 10 mmol/l). FANG *et al*. (2012) found that ions bind near T1 site of laccase and acts as competitive inhibitors of electron donors by blocking the access of substrate to the T1 site or inhibiting electron transfer.

Divalent ions have positive effect to residual laccase activity. However, several metal ions (Cu^{2+}, Mn^{2+}, Zn^{2+}, Mg^{2+}) can induct conformational modification of the enzyme and stimulate decomposition of the trimer complex (substrate, enzyme, metal ion) as evidenced by non-competitive inhibition model (DUGGLEBY, 1979). But

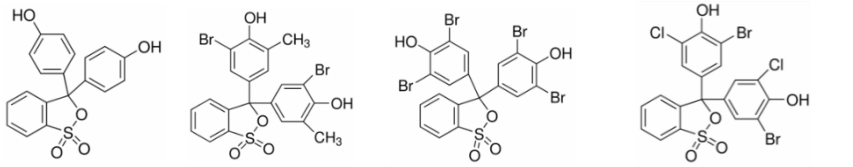
their competition with Cu^{2+} contained in the laccase catalytic site in the electron transport system probably changed into a cooperative relationship (LU *et al*., 2012). This synergistic effect on substrate had probably increased residual laccase activity. These results are better than the influence of metal ions on activity of laccases isolated from *Ganoderma lucidum* (MURUGESAM *et al*., 2009), *Trametes trogii* (GRASSI *et al*., 2011) or *Russula virescens* (ZHU *et al*., 2013). It can be assumed that these divalent metal ions can act as inorganic redox mediators.

Table 1: The effect of potential inorganic inhibitors on laccase activity.

Potential inhibitors	Residual laccase activity (%) Inhibitor concentration (mmol/l)			
	0	**1**	**5**	**10**
Li^+	100.0	110.3 ± 3.1	105.1 ± 2.5	86.9 ± 3.1
Na^+	100.0	96.6 ± 7.5	78.4 ± 3.3	64.2 ± 0.9
K^+	100.0	104.8 ± 9.0	89.4 ± 3.7	47.6 ± 0.6
Ag^+	100.0	103.1 ± 1.5	95.5 ± 3.1	94.3 ± 4.7
Mg^{2+}	100.0	109.6 ± 4.9	103.8 ± 0.4	116.3 ± 1.1
Ca^{2+}	100.0	93.9 ± 0.9	119.7 ± 5.3	107.1 ± 5.2
Ba^{2+}	100.0	103.5 ± 1.8	149.0 ± 4.9	139.2 ± 6.3
Mn^{2+}	100.0	143.7 ± 4.0	123.9 ± 2.1	134.4 ± 2.1
Cu^{2+}	100.0	105.8 ± 3.7	93.4 ± 3.8	210.0 ± 4.0
Zn^{2+}	100.0	121.2 ± 1.5	104.1 ± 5.1	107.2 ± 2.1
Sn^{2+}	100.0	101.5 ± 1.9	99.7 ± 4.9	99.6 ± 2.6
Fe^{3+}	100.0	86.2 ± 2.2	34.8 ± 2.0	4.7 ± 3.4

3.2 Effect of metal ions on laccase-catalyzed decolorization of triphenylmethane dyes

Determination of metal ion influence on laccase dye decolorization is essential to potential negative effect on laccase activity and decolorization process. In this study, triphenylmethane dyes were decolorized by laccase from *Trametes versicolor* without or with metal ions. These dyes exhibit significant structural variability and are commonly used in textile or chemical industry (Fig. 1).



Phenol Red Bromocresol Purple Bromophenol Blue Bromochlorophenol Blue

Fig. 1. Structure of selected triphenylmethane dyes.

Purified commercial laccase ability of decolorization was expressed as percentage decrease of absorbance of selected triphenylmethane dye. Dye decolorization of laccase was carried out at pH 5.0 and 20 °C with respect to the conditions of laccase activity determination (data not shown) for 7 days. Decolorization of triphenylmethane dyes can be observed in VIS kinetic spectrum (Fig. 2) showing the decrease maximum absorbance by laccase oxidation.

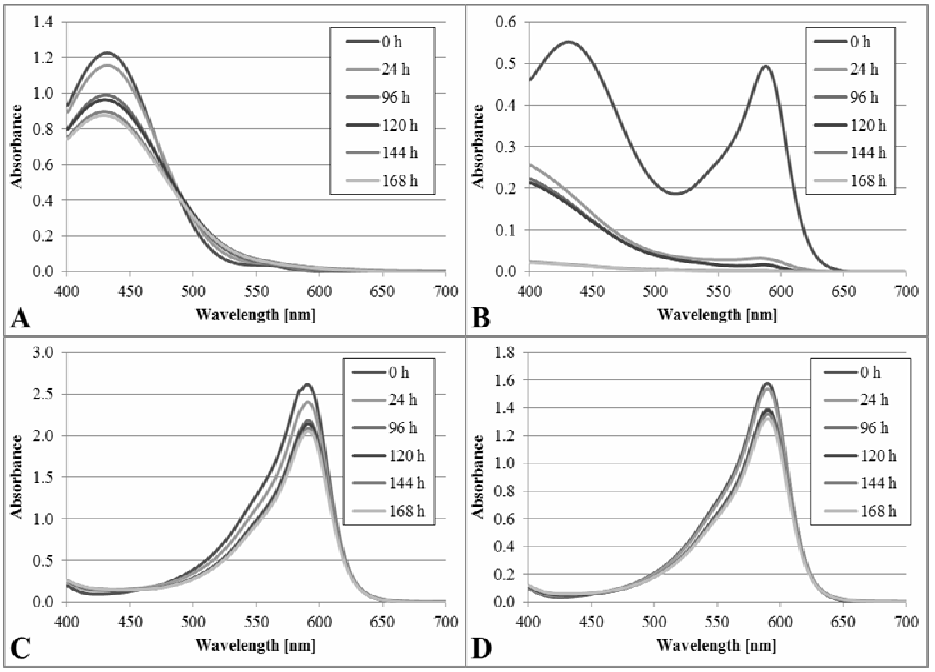


Fig. 2. Kinetics of selected triphenylmethane dye decolorization (A – Phenol Red, B – Bromocresol Purple, C – Bromophenol Blue and D – Bromochlorophenol Blue) by laccase from *Trametes versicolor* during 7 days at pH 5.0 and 20 °C.

Decolorization efficiency may be attributed to the molecular structure of triphenylmethane dye (LIU *et al.,* 2004). The presence of electron-withdrawing groups such as -COOH, $-SO_3H$, $-NO_2$ groups can decrease dye degradation while the presence of electron-donating groups such as -OH, $-CH_3$, OCH_3, $-NH_2$, $-N(CH_3)_2$, $NH-COCH_3$ can increase dye degradation (SUZUKI *et al.*, 2001; CHEN and TING, 2015). The highest efficiency of laccase was observed at Bromocresol Purple decolorization (97.8 %), further Bromophenol Blue (19.9 %), Phenol Red (18.2 %) and Bromochlorophenol Blue (15.1 %) (Fig. 2).

The presence of halogen groups in the Bromophenol Blue (19.9 ± 0.4 %) and Bromochlorophenol Blue (15.1 ± 0.8 %) structure decrease of laccase-catalysed decolorization (CHIVUKULA and RENGANATHAN, 1995). The presence of chloro anions in Bromochlorophenol Blue structure led probably to decrease of the decolorization efficiency (Fig. 2). Although Bromocresol Purple similarly contains

halogen groups of the aromatic rings so the presence of electron-donating groups (-CH_3) makes this dye less resistant to oxidative degradation by laccase (97.8 ± 0.4 %). Surprisingly, Phenol Red degradation (18.2 ± 0.8 %) was comparable to Bromophenol Blue and Bromochlorophenol Blue, although Phenol Red is structurally similar to the monomers of lignin which are commonly laccase substrate and moreover, Phenol Red dye contains hydroxyl groups on its benzene rings.

The change in peak pattern for triphenylmethane dyes with the high percent of decolorization based on the adsorption spectrum (400-700 nm) suggest the biodegradation potential for Bromocresol Purple and Phenol Red (Fig. 3). These results suggest that the destruction of chromophoric groups would lead to dye degradation (CHEN and TING, 2015). Small structural differences between selected triphenylmethane dyes affect their decolorization.

Therefore, dyeing effluents are serious problem for environment, determination of metal ion influence on laccase dye decolorization is essential. The waste water contains different concentration of metal ions in the range from 0.5 to 100 ppm (SANCEY *et al.*, 2011; ANIRUDHAN and SREEKUMARI, 2011).

Table 2: The effect of metal ions (10 mmol/l) on laccase mediated decolorization of selected triphenylmethane dyes during 7 days at 5.0 and 20 °C.

	Phenol Red	**Bromocresol Purple**	**Bromophenol Blue**	**Bromochloro-phenol Blue**
Na^+	**20.2 ± 0.9**	**87.7 ± 0.7**	**34.9 ± 0.4**	**18.4 ± 1.5**
K^+	13.7 ± 1.1	83.3 ± 0.5	17.1 ± 0.2	6.6 ± 0.6
Mg^{2+}	9.9 ± 0.4	89.2 ± 0.2	16.5 ± 1.1	5.5 ± 2.4
Ca^{2+}	10.2 ± 0.4	87.5 ± 0.5	15.0 ± 0.7	5.8 ± 0.5
Ba^{2+}	8.6 ± 2.3	83.6 ± 0.1	15.8 ± 0.6	4.6 ± 0.3
Mn^{2+}	10.7 ± 0.3	87.5 ± 0.3	31.9 ± 1.5	6.3 ± 1.2
Zn^{2+}	**17.6 ± 0.9**	**84.6 ± 0.5**	**39.6 ± 0.5**	**28.7 ± 1.0**
control	**18.2 ± 0.8**	**97.8 ± 0.4**	**19.9 ± 0.4**	**15.1 ± 0.8**

Wastewater with higher metal ion concentrations is usually considered dangerous for environment and must be treated using physical or chemical methods. In our experiments, we have worked with lower limit of this metal ion concentration range (10 mmol/l). Based on the literature studying the influence of metal ion on enzymatic dye decolorization (LIU *et al.*, 2004; MURUGESAN *et al*., 2009; GRASSI *et al.*, 2011; CHEN and TING, 2015), the effect of selected metal ions on dye decolorization, namely Phenol Red, Bromocresol Purple, Bromophenol Blue and Bromochlorophenol Blue was observed and the results are shown in Table 2.

Among the seven metal ions normally found in dyeing effluents, Na^+ and Zn^{2+} have caused slightly increase of decolorization efficiency of selected synthetic dyes, except Bromocresol Purple with the comparable decolorization ability to the control (Table 2). Although the presence of monovalent ions such as Li^+, Na^+, K^+ and Ag^+ rapidly

decreased laccase activities (Table 1), the presence of these ions, especially Na^+ had slightly stimulatory effect on triphenylmethane dye decolorization (Table 2). These results suggest that laccase from *T. versicolor* is resistant to sodium chloride, which is usually primary salt found in wastewater (ZILLY *et al.*, 2011) Similarly, MURUGESAN *et al.* (2009) described that decolorization of laccase from *Ganoderma lucidum* was positively enhanced with Zn^{2+} ions in concentration 10 mmol/l. In the presence of other metal ions, Bromocresol Purple and Bromophenol Blue decolorization were minimally affected. Decolorization of Phenol Red and Bromochlorophenol Blue was partially inhibited in the presence of metal ions, except Na^+ and Zn^{2+}. MURUGESAN *et al*. (2009) found that the increasing the concentration of metal ions from 1 to 10 mmol/l negatively enhanced decolorization efficiency of laccases isolated from *G. lucidum*. The above results suggest that *T. versicolor* laccase is useful for synthetic dye degradation from wastewater.

Based on the results obtained above, we have verified the possibility of the increasing of decolorization efficiency by laccase in the presence of low-molecular-weight compounds known as redox mediators. The most commonly used synthetic mediator is hydroxybenzotriazole (HBT) which appears to be very effective mediator (GRASSI *et al*., 2011; ASHRAFI *et al.*, 2013; SHANKAR and NILL, 2015).

Table 3: The effect of metal ions (10 mmol/l) on laccase mediated decolorization of selected triphenylmethane dyes in the presence of hydroxybenzotriazole (1 mol/l) during 7 days at 5.0 and 20 °C.

	Phenol Red	**Bromocresol Purple**	**Bromophenol Blue**	**Bromochloro-phenol Blue**
Na^+	33.1 ± 1.8	89.9 ± 0.5	14.5 ± 2.0	14.0 ± 1.2
K^+	13.2 ± 0.2	87.4 ± 0.5	5.9 ± 0.3	6.3 ± 0.6
Mg^{2+}	7.4 ± 2.9	87.7 ± 0.8	2.9 ± 0.5	3.1 ± 0.1
Ca^{2+}	4.4 ± 0.8	88.0 ± 0.1	2.3 ± 0.4	2.9 ± 0.4
Ba^{2+}	3.1 ± 1.5	85.9 ± 0.5	3.9 ± 0.5	2.9 ± 0.3
Mn^{2+}	15.6 ± 3.7	90.8 ± 0.3	6.5 ± 0.2	7.1 ± 0.1
Zn^{2+}	22.0 ± 1.4	90.6 ± 0.4	13.1 ± 1.1	11.6 ± 0.3
control	**28.2 ± 0.7**	**99.6 ± 0.2**	**15.1 ± 0.3**	**12.0 ± 0.7**

The effect of HBT on dye decolorization by laccase from *T. versicolor* showed an increase of decolorization efficiency of Phenol Red (28.2 %) and Bromocresol Purple (99.6 %). Bromophenol Blue (15.1 %) and Bromochlorophenol Blue (12.0 %) decolorization was inhibit by the presence of HBT in reaction (Table 3). Laccase oxidizes redox mediators and creates unstable radicals. In the case of HBT, putative N-OH radicals are generate (MOROZOVA *et al.*, 2007; MOREIRA *et al.*, 2014). These radicals act by mechanism different from enzymatic one, namely radical mechanism. It is possible that radicals forming by mediator oxidation by laccase, cause triphenylmethane dye decolorization. The effect of metal ions on decolorization efficiencies of selected triphenylmethane dyes had the similar course as in the reaction without redox mediator HBT (Table 2).

4. Conclusion

In this work, we studied the influence of metal ions on laccase activity and enzymatic dye decolorization of selected triphenylmethane dyes. The results showed that monovalent ions had negative effect on laccase activity while divalent ions positively affected laccase activity. Experimental data indicated that the triphenylmethane dye degradation was depended on dye structure and the presence of methyl group of aromatic rings positively enhanced dye oxidation by laccase from *Trametes versicolor*. The presence of Na^{+} and Zn^{2+} stimulated laccase decolorization efficiency and caused the increase of decolorization percent.

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