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Effect of metal ions on reactive dye decolorization by laccase from *Ganoderma lucidum*

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ABSTRACT

In this work, the influence of different metal ions on laccase activity and laccase-catalyzed dye decolorization was investigated under *in vitro* conditions using crude laccase obtained from a white rot fungus *Ganoderma lucidum*. Laccase activity was enhanced by metal ions such as Ca^{2+}, Co^{2+}, Cu^{2+} and Zn^{2+} at low concentrations (1 mM). Increasing the concentration of metal ions except that of Cu^{2+} and Zn^{2+} up to 5 mM and above decreased the enzyme activity. Among several heavy metals, Fe^{2+} highly inhibited the enzyme activity. Effect of metal ions was tested on decolorization of two reactive dyes, namely Remazol black-B (RB-5) and Remazol brilliant blue R (RBBR) at a concentration of 50 mg l^{-1}. The presence of heavy metals generally did not exert much influence on the decolorization except Fe^{2+}. Cu^{2+} and Cr^{6+} enhanced the decolorization of both dyes. In the presence of 1 mM Cu^{2+}, 94% of RB-5 and 35.5% of RBBR were decolorized during 1 h incubation. *G. lucidum* laccase was able to tolerate mixture of several metal ions. Treatment of simulated reactive dye effluent by laccase showed that the redox mediator system is necessary for effluent decolorization. Syringaldehyde, a natural redox mediator, was very effective than the synthetic mediator 1-hydroxybenzotriazole (HBT). The initial rate of effluent decolorization in presence of syringaldehyde (0.0831 h^{-1}) was 5.6 times higher than HBT (0.0152 h^{-1}). Although the rate of decolorization was markedly decreased in the effluent containing mixed metal ions, presence of syringaldehyde showed effective decolorization. This study indicates that *G. lucidum* laccase and natural redox mediator system could be a potential candidate for color removal from reactive dye effluent.

1. Introduction

Synthetic dyes are being extensively used in textile dyeing and printing processes. On the basis of dying process, textile dyes are classified as reactive dyes, direct dyes, disperse dyes, acid dyes, basic dyes and vat dyes. Reactive dyes are extensively used in dyeing process mainly due to their high reactivity and technical characteristics. Unfortunately, this class of dye is also the most unfavorable one from the ecological point of view, as the effluents produced are relatively heavily colored, contain high concentrations of salt and exhibit high BOD/COD values, and heavy metals [1]. Brightly colored, water-soluble reactive dyes are the most problematic, as they tend to pass through conventional treatment systems unaffected [2]. Different techniques including almost all the known physical and chemical and biological techniques were described for decolorization and the final conclusion was that each process alone might not be able to meet the requirements [3]. Because of the stable chemical structure, synthetic dyes are not evenly accessible to microbial degradation under aerobic conditions. In recent years, several microorganisms have been investigated for decolorization of reactive dyes [4,5]. The effectiveness of the microbial decolorization depends on the adaptability and the activity of selected microorganisms [6]. Since many reactive dyes are toxic to microorganisms, the decolorization of textile dye effluent is complicated and a serious environmental problem. White rot fungi are well-known organisms for decolorization of wide range of synthetic dyes due to their non-specific extracellular ligninolytic enzyme system consisting of lignin peroxidase, manganese peroxidase and laccase [7–9]. However, they require long incubation time in culture based method. On the other hand, enzymatic treatment method using isolated enzyme has been proposed as a potential alternative to conventional methods [9–12]. Enzymatic treatment falls between chemical and biological method since it involves chemical process based on the action of biological catalysts [13]. Thus, currently, dye decolorization using microbial enzymes, particularly laccase mediated decolorization, has received great attention due to its availability and potential decolorization ability against wide range of dyes [14–20].

Laccases are multi-copper containing enzymes which reduce molecular oxygen to water and simultaneously perform one electron oxidation of various aromatic substrates such as diphenols, methoxysubstituted monophenols and aromatic amines [21]. In

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addition, laccases oxidize a wide variety of non-phenolic aromatic compounds in the presence of redox mediators [22]. Laccases are abundant in white rot fungi and has been purified and characterized extensively from many white rot fungi. Recently, Baldrian [23] has comprehensively reviewed the occurrence and properties of the fungal laccases. One of the advantages associated with laccases is that they do not require H_2O_2 for substrate oxidation unlike peroxidases and, moreover, they have broad substrate specificity [24]. Further, the laccase production is constitutive in most of the white rot fungi and it can be easily enhanced and produced by solid-state fermentation (SSF) of agro-industrial by-products. Fungal laccases have received much attention from researchers in the last decades due to their ability to oxidize both phenolic and non-phenolic lignin related compounds as well as highly recalcitrant environmental pollutants, which makes them very useful for their application to several biotechnological processes [25]. Laccase mediated dye degradation has been described in liquid culture of white rot fungi and their purified laccases [12,16,26,27]. For the practical application, the use of crude laccase is the only cost effective way. Thus, the exploration of crude laccase for decolorization of dyes and effluents has been studied recently [15,17]. However, the presence of heavy metals and other cations in the textile effluents is a major obstacle which could potentially affect the activity of the enzyme.

Many heavy metals are highly toxic to microorganisms when present in excess and some of them are carcinogenic [6,28]. Several heavy metals are generally found in textile dye wastewater. The inhibitory activity of heavy metals on white rot fungal growth and dye decolorization has been reported previously [17,29–31]. As enzymatic bioremediation of industrial effluent is of current interest and emerging method, it is necessary to select enzymes which could potentially tolerate the inhibitory effect of the heavy metals. Although several studies have been investigated on laccase mediated reactive dye decolorization [15,17–20], very little attention has been focused to the effect of heavy metals on laccase-catalyzed enzymatic dye decolorization. Although several laccases are known, searching for potential laccase is being continued. The white rot fungus *Ganoderma lucidum* was identified as a dominant laccase producer [32,33]. Recently, we have shown a potential laccase from SSF culture of a white rot fungus *G. lucidum* for decolorization of chemically different groups of reactive dyes [15]. This laccase is a thermostable enzyme and effectively decolorized reactive dyes, Remazol black-5 (RB-5) and Remazol brilliant blue R (RBBR). Although this enzyme showed effective *in vitro* decolorization, it is necessary to study its decolorization efficacy in the presence of other inorganic pollutants such as metal ions. The aim of this study was to evaluate the effect of heavy metals and other metal ions, which are commonly present in the textile dying effluents, on the decolorization of reactive dyes and simulated effluent by crude laccase obtained from *G. lucidum*.

2. Materials and methods

2.1. Laccase production and activity

Enzyme preparation from the white rot fungus *G. lucidum* KMK2 was carried out in SSF using wheat bran as substrate as described previously [15]. Laccase (EC 1.10.3.2) activity was measured spectrophotometrically at 30 °C using 1 mM ABTS (2,2′-azino-di-[3-ethyl-benzothiazolin-sulphonate]) as the substrate. The assay mixture (1 ml) contained 880 μl of 100 mM sodium acetate buffer (pH 5.0), 100 μl of ABTS stock (final concentration 1 mM), and 20 μl of appropriately diluted crude enzyme. The enzyme activity was calculated using the molar extinction coefficient of oxidized ABTS ($E420 = 3.6 \times 10^4\ M^{-1}\ cm^{-1}$). One unit of activity was defined as the amount of enzyme that oxidized 1 μmol ABTS per minute.

Table 1
Effect of metal ions on laccase activity.

Metal ions	Metal salts	Relative laccase activity (%)[a]			
		0.5 mM	1.0 mM	5 mM	10 mM
Ca^{2+}	Calcium nitrate	104.6	104.5	106.3	99.0
Cd^{2+}	Cadmium chloride	100	93.4	82.9	80.3
Co^{2+}	Cobalt chloride	106.5	104.5	93.5	83.4
Cu^{2+}	Copper sulphate	105.5	116.5	136.1	172.3
Cr^{6+}	Potassium chromate	ND	ND	ND	ND
Fe^{2+}	Ferrous sulphate	0.002	0	0	0
Hg^{2+}	Mercury chloride	54.5	40	18	6.5
Li^{+}	Lithium chloride	91	90.5	85.3	70.4
Mn^{2+}	Manganese chloride	94	95.5	81.5	76.1
Ni^{+}	Nickel chloride	96.4	102.0	87.4	81.2
K^{+}	Potassium iodide	100	100	92.9	92.1
Na^{+}	Sodium chloride	90.6	75.21	64.23	9.6
Zn^{2+}	Zinc sulfate	106.6	118.5	123.7	137.0

Enzyme assays were performed in triplicates. The averages of triplicate activity measurements, varied <10%, were used to calculate. ND: not detected.
[a] Laccase activity relative to the control assay containing no metal ions.

2.2. Dyes and chemicals

Azo dye Remazol Black-B (RB-5), Remazol orange-16 (RO-16), and anthraquinone dye Remazol Brilliant Blue R were purchased from Sigma–Aldrich. All other reagents and chemicals are analytical grade obtained from Merck (Germany) and Sigma–Aldrich (USA).

2.3. Effect of metal ions on enzyme activity

The influence of various metal ions as listed in Table 1, normally found in dyeing effluents, was assessed against laccase activity. For this study, the enzyme was incubated with different metals ions (Table 1) at 0.5, 1, 5, and 10 mM concentrations for 1 h. Enzyme assay was performed with 1 mM ABTS as described above. All assays were performed in triplicates. Since the enzyme activity was completely inhibited by Fe^{2+} at 0.5 mM, the assays were performed with low concentration of Fe^{2+} range from 0.001 mM to 0.4 mM to find out minimum inhibitory concentration.

2.4. Effect of metal ions on enzymatic dye decolorization

The effect of metal ions on the dye decolorization process was studied using two different reactive dyes, RBBR and RB-5. Reaction mixture contained, 50 mg l^{-1} dye concentration, 20 U ml^{-1} crude laccase in 50 mM sodium acetate buffer (pH 5.0), and different concentration of heavy metals salt and other salts (1, 5 10 mM; as listed in Table 2) in a total volume of 1 ml in a test tube. The reaction mixture was incubated at 30 °C in dark for 60 min. and the dye decolorization was measured by monitoring the decrease in absorbance maximum (RBBR 592 nm and RB-5 597 nm) of each dye in a UV–Vis Spectrophotometer (Varian Cary-3 Bio) and the decolorization was expressed in percentage as described previously [15]. In parallel, control samples were maintained without addition of metal ions as well as without enzyme.

2.5. Effect of metal ions mixture on laccase activity

In order to determine the combined effect of all the metal ions on enzyme activity, the assay was performed by incubating the enzyme for 1 h in solution containing all the metal ions each at 1.0 mM concentration apart from Cr^{6+} and Fe^{2+}. In the case of Fe^{2+}, 0.4 mM was used. Since Cr^{6+} oxidizes the laccase substrates even in the absence of enzyme the mixed metal ion assays were performed in the

Table 2
Effect of metal ions on decolorization of RB-5.

Metal ions	Decolorization (%)		
	1 mM	5 mM	10 mM
Control	67.6 ± 4.4	67.6 ± 4.4	67.6 ± 4.4
Ca^{2+}	66.7 ± 3.0	69.0 ± 2.4	68.9 ± 3.5
Cd^{2+}	67.0 ± 2.8	58.9 ± 3.3	8.5 ± 0.5
Co^{2+}	65.2 ± 2.6	53.6 ± 1.7	23.6 ± 1.3
Cu^{2+}	94.0 ± 3.6	90.0 ± 4.8	72.0 ± 3.8
Cr^{6+}	68.4 ± 4.2	71.0 ± 5.3	69.0 ± 4.4
Fe^{2+}	0 ± 0	0 ± 0	0 ± 0
Hg^{2+}	40.3 ± 1.41	22.3 ± 1.6	9 ± 0.5
Li+	64.5 ± 2.2	61.2 ± 2.5	30.2 ± 1.8
Mn^{2+}	62.4 ± 3.9	44.2 ± 2.0	27.1 ± 0.7
Ni^{2+}	66.4 ± 2.5	47.7 ± 1.2	20.2 ± 0.48
K^{+}	44.4 ± 1.73	5.8 ± 0.3	0 ± 0
Na^{+}	69.2 ± 2.83	67.5 ± 3.5	61.5 ± 3.0
Zn^{2+}	67.1 ± 2.48	64.0 ± 1.8	60.6 ± 2.9

Control assay contained no metal ions. The results are average of triplicate assays and standard deviations.

presence and absence of Cr^{6+}. Decolorization of RB-5 and RBBR in the presence of mixed metal ions was also monitored as described above. The effect of redox mediator 1-hydroxybenzotriazole (HBT) on decolorization was monitored using 0.1–0.5 mM HBT.

2.6. Decolorization of simulated effluent by laccase

In order to check the efficacy of laccase for dye effluent decolorization, experiments were conducted with simulated reactive dye bath effluent. Simulated effluent was prepared as described by Vijayaraghavan et al. [34], which contains of RB-5 (0.768 g) and RBBR (0.369) Remazol orange 16, acetic acid (0.79 g), sodium chloride (41.0 g), sodium carbonate (13.0 g), and sodium hydroxide (0.51 g) per liter of deionized water. The dye bath mixture was boiled for 3 h and then cooled for 12 h. The initial color and pH of this effluent was dark blue and 10.4, respectively. Laccase did not show decolorization without pH adjustment or dilution. Therefore simulated effluent was diluted to 15-fold and adjusted to pH 5.0 before the treatment. Enzymatic treatment was conducted using laccase (20 U/ml) with and without addition of redox mediator and metal ions mixture as stated above. The effluent sample was scanned using UV–Vis Spectrophotometry, which showed the maximum absorbance at 593 nm.

3. Results

3.1. Effect of metal ions on laccase activity

Heavy metals in general are potent inhibitors of enzyme reactions. In this study, the effect of heavy metals and other salts on laccase activity was investigated by *in vitro* assay supplemented with individual metal salts at various concentrations as listed in Table 1. The results are shown in Table 1 as percent of relative activity to the control. The results revealed that most of the metal ions did not inhibit the laccase activity up to 1 mM. However, increasing the metal ion concentration decreased the laccase activity. The enzyme activity was completely inhibited in the presence of Fe^{2+} at all the tested concentrations. Interestingly, Ca^{2+}, Co^{2+}, Cu^{2+}, Cr^{6+} and Zn^{2+} enhanced the laccase activity at low concentrations (1 mM). In the case of Cu^{2+} and Zn^{2+} increasing the concentration of metal ion even up to 10 mM did not affect the laccase activity. However, in other cases apart from Fe^{2+} 10 mM metal ions did enhance the enzyme activity with little effect where there was complete inhibition of enzyme activity with Fe^{2+} (Table 1). Copper is a component of active site of laccases. It has been observed in previous studies that the addition of Cu^{2+} enhanced the laccase activity. Similarly, in the present study laccase activity was enhanced by 72% relative to the control in the presence of 10 mM Cu^{2+} (Table 1).

3.2. Effect of metal ions on decolorization of RB-5

Since the heavy metals affect the activity of extracellular lignin-degrading enzymes, from biotechnological point of view, presence of heavy metals is a serious problem for the use of lignolytic enzymes to decolorize textile dyes. Therefore, determination of their influence on the enzymatic dye decolorization is essential. In this study, we examined the effect of metals ions on laccase mediated enzymatic decolorization by supplementing metals ions in decolorization assay mixture. The results of RB-5 decolorization are shown in Table 2 as percent of decolorization and relative decolorization to control. Similar to the result on enzyme activity inhibition (Table 1), decolorization was not affected by all the tested heavy metals and other metal ions except Fe^{2+} at 0.5 mM. Decolorization was completely inhibited in the presence of Fe^{2+} as observed in enzyme activity. Increasing concentrations of Cd^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, and K^{+} showed significant inhibition on the decolorization. Only considerable level of decolorization was enhanced with Cu^{2+}. When 1, 5 and 10 mM Cu^{2+} was used, decolorization increased by 39%, 33% and 6.5%, respectively, over the control. This result also suggests that a slight increase in concentration of metal ions decrease the decolorization level. In the presence of 1 mM Cu^{2+} and 1 mM redox mediator (HBT), up to 94% of RB-5 was degraded within 1 h whereas it was only 67.6% in the absence of Cu^{2+}. Decolorization of RB-5 by laccase from *G. lucidum* can be observed in the UV–Vis kinetic spectrum (Fig. 1a) showing the decrease of

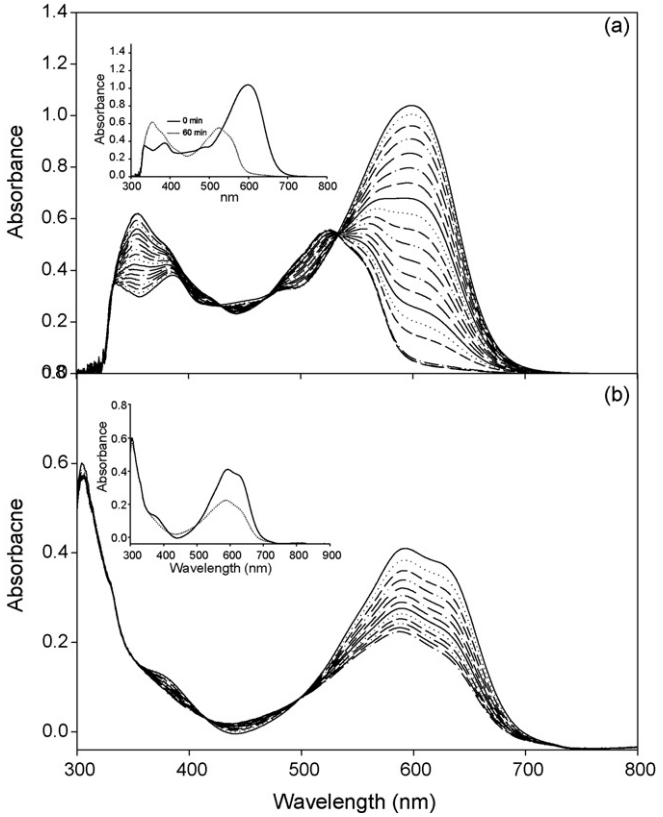

Fig. 1. Kinetic spectra of decolorization of RB-5 (a) and RBBR (b) by laccase from *Ganoderma lucidum* in the presence of 1 mM Cu^{2+}. The assay mixture contained the dye 50 mg l^{-1}, 20 U laccase, 1 mM Cu^{2+} in citrate-phosphate buffer at pH-4.0. Spectra were recorded at every 3 min for RB-5 and 5 min RBBR. Inset figures show 0 and 60 min spectra.

Table 3
Effect of metal ions on decolorization of RBBR.

Metal ions	Decolorization (%)		
	1 mM	5 mM	10 mM
Control	33.5 ± 1.5	33.5 ± 1.5	33.5 ± 1.5
Ca^{2+}	37.2 ± 1.3	37.0 ± 1.1	36.0 ± 1.7
Cd^{2+}	23.8 ± 0.7	28.3 ± 1.5	26.4 ± 1.2
Co^{2+}	18.5 ± 0.8	5.2 ± 0.2	3.3 ± 0.2
Cu^{2+}	35.5 ± 1.9	38.0 ± 2.4	41.4 ± 2.0
Cr^{6+}	41.4 ± 2.8	29.8 ± 2.2	23.0 ± 1.3
Fe^{2+}	0.0 ± 0	0 ± 0	0 ± 0
Hg^{2+}	15 ± 0.5	10 ± 0.8	4.5 ± 0.3
Li^{+}	28.0 ± 1.8	24.6 ± 2.1	18.5 ± 1.4
Mn^{2+}	13.6 ± 0.8	13.4 ± 0.7	12.4 ± 0.5
Ni^{2+}	18.8 ± 0.8	11.2 ± 0.5	6.3 ± 0.2
K^{+}	6.2 ± 0.3	0 ± 0	0 ± 0
Na^{+}	22.8 ± 0.8	12.5 ± 0.4	9.0 ± 0.6
Zn^{2+}	37.4 ± 2.5	46.5 ± 2.0	48.4 ± 2.2

Control assay contained no metal ions. The results are average of triplicate assays and standard deviations.

maximum absorbance with simultaneous increasing of two other peaks. In the case of Cr^{6+} no inhibition was observed up to 10 mM, in our study. The above results suggest that *G. lucidum* laccase is tolerable to the effect of the heavy metals normally present in wastewater.

3.3. Effect of metal ions on decolorization of RBBR

The results of the effect of metal ions and other salts on laccase mediated decolorization of anthraquinone reactive dye RBBR are shown in Table 3. As observed for RB-5, most of the tested metal ions did not inhibit the decolorization of RBBR. At 1 mM concentration, enhanced decolorization was found with Ca^{2+} Cu^{2+}, Cr^{6+}, and Zn^{2+} treated samples. Absorption spectrum of RBBR decolorization is shown in Fig. 1b. Enhanced decolorization was observed with increasing concentration of Cd^{2+}, Cu^{2+}, and Zn^{2+} whereas chromium Cr^{6+} showed contradictory effect. Interestingly, decolorization was enhanced with increasing concentration of Zn^{2+} and maximum decolorization of 48.4% was achieved at 10 mM which was relatively 44.5% higher over the control. *G. lucidum* laccase was able to decolorize RBBR without redox mediator. Nevertheless, addition of 1 mM HBT, as redox mediator, to the reaction mixture containing 1 mM metal ions showed enhanced decolorization than in the absence of HBT (Fig. 2). The laccase from *G. lucidum* in the present study was highly sensitive to the tested concentrations of Fe^{2+} and K^{+} (Table 3).

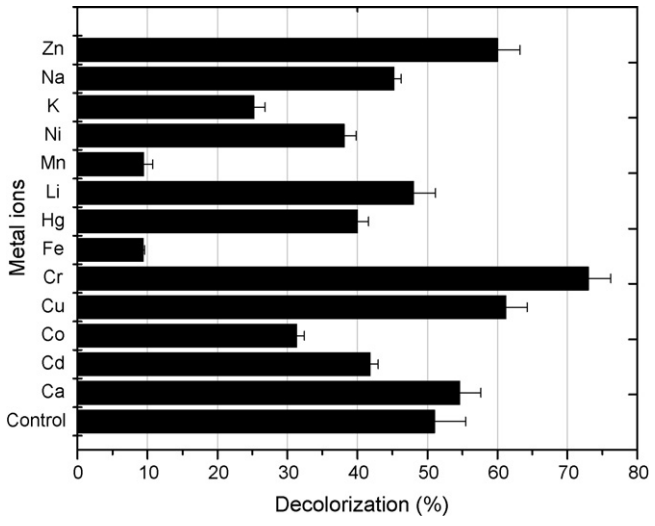

Fig. 2. Effect of HBT (1 mM) on decolorization of RBBR in the presence of metal ions.

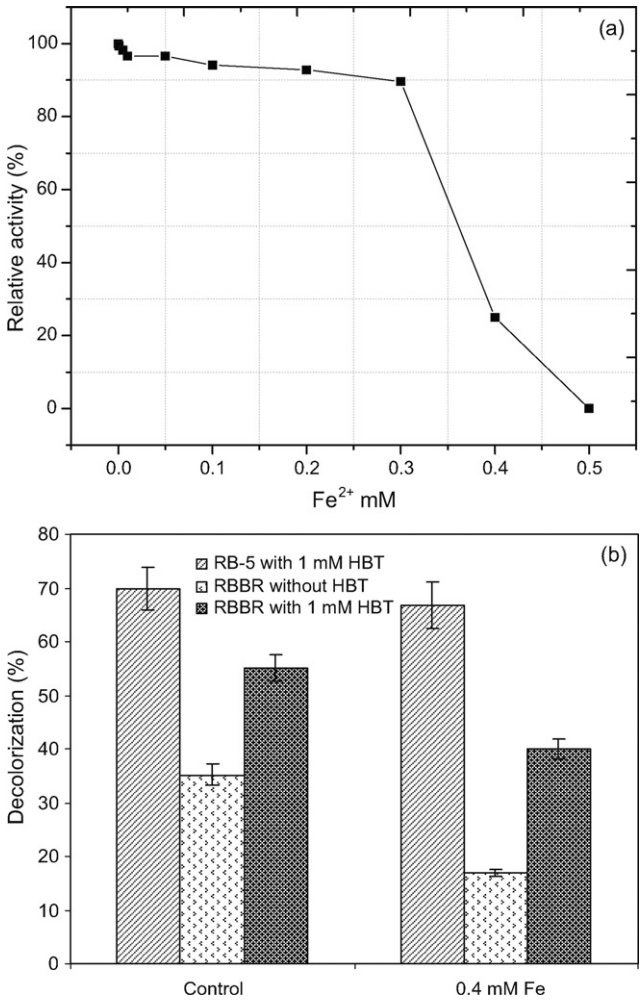

Fig. 3. Effect of Fe^{2+} on laccase activity (a) and decolorization of RB-5 and RBBR (b).

However, in Fe^{2+} treated sample decolorization was enhanced by 8.8% in the presence of HBT (Fig. 2). Similar effect was also found in the samples treated with K^{+}, supplied in the form of KI. This result indicates that the addition of redox mediator diminish the effect of metal ions on decolorization activity. Fig. 1b shows the kinetic absorbance spectra of decolorization of RBBR in the presence of 1 mM Cu^{2+}.

3.4. Minimum inhibitory concentration of Fe^{2+}

Since the Fe^{2+} highly inhibited the laccase activity and decolorization at 0.5 mM its minimum inhibitory concentration was tested using concentrations less than 0.5 mM such as 0.001, 0.005, 0.01, 0.05, 0.1, 0.2, 0.3 and 0.4 mM. The results revealed that laccase could tolerate Fe^{2+} up to 0.3 mM. At this concentration only 10% of the activity was lost. As can be seen in Fig. 3a, with Fe^{2+} greater than 0.3 mM the laccase activity decreased sharply and 50% enzyme activity was lost at 0.36 mM. In further studies, 0.4 mM Fe^{2+} was tested for decolorization of RB-5 and RBBR both in the presence and absence of redox mediator. During 1 h incubation, about 50% of RBBR decolorization was inhibited in the presence of 0.4 mM Fe^{2+}. Whereas, in the presence of redox mediator the inhibition was diminished resulting in an enhanced decolorization. In the case of RB-5 no decolorization was observed without redox mediator and at the same time in the presence of redox mediator the decolorization was not much affected by the addition of 0.4 mM Fe^{2+} (Fig. 3b).

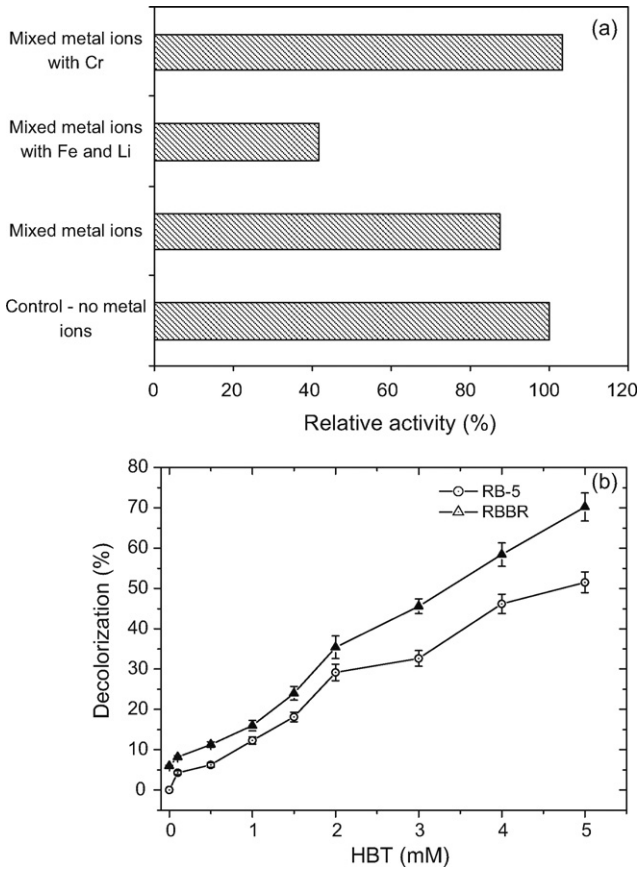

Fig. 4. Effect of mixed metal ions on laccase activity (a) and decolorization of RB-5 and RBBR in the presence of various concentration of HBT (b). Assay mixture contained all metal ions each at 1.0 mM concentration except Cr^{6+}.

3.5. Effect of mixed metal ions

The combined effect of all the metal ions on enzyme activity and decolorization was tested with mixture of metal ions each at 1.0 mM concentration excluding Cr^{6+} and Fe^{2+}. The result is shown in Fig. 4. About 13% of laccase activity was inhibited by mixed metal ions which indicate that laccase could tolerate the presence of mixed metal ions except Fe^{2+}. Addition of 0.4 mM Fe^{2+} with metal ions mixture further decreased the laccase activity by 59% than the control. Interestingly, addition of 1 mM Cr^{6+} with mixed metal ions enhanced the enzyme activity by 16% when compared with the mixed metal ions in the absence of Cr^{6+} and 3% enhanced activity over the control which had no metal ions (Fig. 4a). We observed that Cr^{6+} oxidized the laccase substrates viz., ABTS and 2,6-dimethoxy phenol (DMP) in the absence of laccase. This oxidation was subtracted from the oxidation rate obtained in the presence of laccase and we found that the presence of 1 mM Cr^{6+} did not have any inhibitory effect on laccase activity (data not shown).

We also tested the effect of mixed metal ions on decolorization of RB-5 and RBBR using 100 mg l^{-1} dye concentration. No decolorization was observed during 1 h in the presence of mixed metals including Fe^{2+} even in the presence of redox mediator. This may be due to high amount of dyes. Therefore, reaction mixture was incubated with different concentration of HBT for 24 h. As seen in Fig. 4b, the hindrance in decolorization of the dyes in the presence of mixed metal ions which might be due inactivation of enzyme was diminished in the presence of HBT. Increasing the concentration of HBT from 0.1 to 5 mM enhanced the decolorization of both RB-5 and RBBR. During 24 h incubation decolorization rate of RBBR was higher than RB-5.

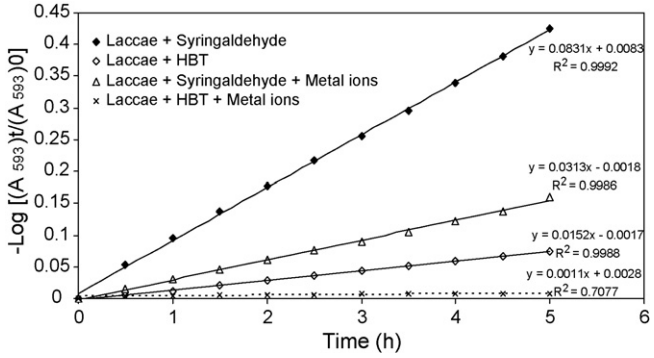

Fig. 5. Initial decolorization rate constant of simulated reactive dye effluent by *G. lucidum* laccase in the presence and absence of metal ions.

3.6. Decolorization of simulated effluent

Treatment of simulated dye bath effluent revealed that decolorization was observed only in the presence of redox mediator. During 24 h incubation, maximum of 82% and 80% decolorization of simulated effluent were observed in presence of HBT and syringaldehyde, a natural phenolic compound, respectively. In the presence of metal ions in effluent, the decolorization was suppressed in HBT added effluent whereas syringaldehyde added effluent showed 56% decolorization. The initial decolorization rate constant determined from the slope of $-\log(A_{593}t/A_{593}0)$ vs. t (min) during the first 5 h of decolorization reaction, where $A_{593}0$ and $A_{593}t$ are dye contents at zero and t times, respectively was shown in Fig. 5. Rate of decolorization in presence of syringaldehyde (0.0831 h^{-1}) was 5.5 times higher than HBT (0.0152 h^{-1}). Presence of metal ions decreased the above rate of decolorization by 2.6 and 13.8 times for syringaldehyde and HBT, respectively.

4. Discussion

Since the ligninolytic enzymes are highly non-specific and can effectively treat even dilute wastes [35], enzymatic treatment of industrial effluent has been considered as potential method. Further, enzymes are less likely to be inhibited by substrates which may be toxic to living organisms [13]. Application of laccases for dye decolorization has recently received much interest due to their decolorization potential towards wide range of dyes [9,11]. *Ganoderma* spp. are efficient white rot fungi that produce potential laccase [33,36]. Revankar and Lele [36] reported that dye decolorization rate by *Ganoderma* sp. WR-1 was very high compared to the most widely used strains of *Trametes versicolor* and *Phanerochaete chrysosporium*. Recently, we have shown that the strain *G. lucidum* KMK2 produced laccase as its major extracellular enzyme during solid-sate fermentation on wheat bran and it was identified as thermostable enzyme which effectively decolorized different reactive dyes [15]. In this study, we demonstrate the influence of metal ions on enzyme activities and dye decolorization. Among the metal ions tested, Ca^{2+}, Co^{2+}, Cu^{2+} and Zn^{2+} enhanced the laccase activity up to 1 mM concentration. Increasing the concentration of metal ions except for Cu^{2+} and Zn^{2+} up to 10 mM decreased the laccase activity. Kumari and Sirsi [37] reported that the purified laccase of *G. lucidum* was completely insensitive to heavy metals. In our study, among the tested metals ions *G. lucidum* laccase was highly sensitive to Fe^{2+}. This may be due to interaction of the Fe^{2+} with the electron transport system of laccase. The effect of laccase from our study was similar to the laccase purified from *Lentinula edodes* [30] and *Trametes trogii* [27]. In their study, although laccases isolated from these fungi were highly sensitive to Fe^{2+} other heavy metals did not influence the activity even at 20 mM. They

also found that decreased/increased enzyme production in liquid culture was caused by the presence of heavy metals in the media.

Several heavy metals are generally found in dyeing effluents [6,17]. This may potentially affect the efficiency of enzymatic treatment under natural conditions since many metal ions inhibit the activity of oxidative enzymes. It has been reported that the mycelial growth and lignolytic enzyme production by white rot fungi were highly sensitive to Cd^{2+} and Hg^{2+} but less sensitive to Zn^{2+}, Cu^{2+} and Pb^{2+} [30,31]. Pointing et al. [31] also reported that dye decolorizing activity of white rot fungal cultures was reduced in the presence of heavy metals. Thus, considering the toxic effect of heavy metals to mycelial growth of fungi and their biological activities, the use of isolated enzymes for enzymatic decolorization would be effective. This has been proved for decolorization of various dyes [15–17,37]. As laccase from *G. lucidum* in the present study is able to tolerate high concentrations of metal ions, the presence of heavy metals in effluent under natural condition would not affect the decolorization process. The decolorization experimental results also proved that except Fe^{2+}, decolorization process was not much affected by the presence of other metal ions. In addition, increased concentration of Cu^{2+} and Cr^{6+} exhibited positive effects and mainly decolorization was enhanced considerably with increasing concentration of Cu^{2+} for both reactive dyes. Positive effects of Cu^{2+} on laccase production and activities have been reported in other white rot fungi [38,39].

Cr^{6+} is an extremely toxic heavy metal. Aksu et al. [6] observed that the addition of Cr^{6+} in to the culture medium inhibited the RB-5 removal by *T. versicolor* to a greater extent. Interestingly, in our study no inhibition was observed with Cr^{6+} even at 10 mM concentration. Further, the decolorization was enhanced in the presence of Cr^{6+}. Fe^{2+} was found to inhibit the enzyme activity strongly even at very low concentrations. About 50% inhibition of enzyme activity was found at 0.4 mM Fe^{2+} concentrations. Similar level of inhibition was also found during the decolorization of RBBR in presence of Fe^{2+} at the above concentration. In general, actual industrial effluents contain several metals ions together and normally textile wastewater contains Cu^{2+}, Cr^{2+}, Co^{2+}, Zn^{2+}, and Na^{+} [10]. Therefore, the influence of mixed metal ions on enzymatic reaction was tested in simulated condition in the present study. Our results showed that laccase from *G. lucidum* could oxidize its substrate even in the presence of mixed metals. This result was further confirmed in dye decolorization studies with mixed metals ions. It was however observed that prolonged incubation was required to achieve decolorization.

Some dyes are resistant to laccase mediated decolorization due to steric hindrance of functional groups in the dye molecule or they do not serve as a substrate to laccase. In these cases, redox-mediated decolorization using various redox mediators has been reported [14,15,19]. HBT was shown to increase the range and rate of decolorization of different reactive dyes [40]. Previously, we found that HBT was most effective redox mediator for *G. lucidum* laccase and was essential for RB-5 decolorization and, also enhanced the decolorization for RBBR [15]. In this study, in order to check the influence of redox mediators in the presence of heavy metals, we tested the effect of HBT on decolorization. It was found that HBT enhanced the RBBR decolorization both in the presence and absence of metals ions (Fig. 3). In the presence of Fe^{2+} and Hg^{2+}, HBT enhanced the decolorization of RBBR suggesting that negative effects of heavy metals were diminished by HBT. Previously, Couto et al. [17] also observed similar effect with Hg^{2+} for dye decolorization by crude laccase from *Trametes hirsuta*; where presence of HBT is essential for dye decolorization in the presence of Hg^{2+}. In the case of mixed metal ions, HBT is necessary to achieve increased rate of decolorization. Earlier, we found that beyond 1 mM HBT no significant decolorization was achieved [15] whereas in the presence of mixed metal ions a positive correlation was found with decolorization and HBT concentration up to 5 mM. However, high concentration of HBT may be toxic to microorganisms when applied in the real effluent system and further the redox mediators effect may vary in the real dye bath effluent system due to high complexity of the effluent. To study this, we also conducted experiment using simulated reactive dyes effluent. Laccase alone failed to decolorize the effluent. Thus addition of redox mediator is required for effluent decolorization. Although HBT and syringaldehyde differed in decolorization rate, both were found to be effective for dye effluent decolorization. However in presence of mixed metal ions, HBT addition did not effectively remove the effluent color. This result is in contrast with the result observed for pure dyes (Fig. 4b). Interestingly, syringaldehyde showed significant color removal even in the presence of mixed metal ions. This result suggests the efficiency of laccase to decolorize the recalcitrant dyes the complex effluent system and the inhibitory effect of heavy metals could be diminished by the addition of natural redox mediators.

5. Conclusion

The influence of various metals ions on laccase-catalyzed reactive dye decolorization was investigated. The results of this study clearly showed that the laccase produced from *G. lucidum* by solid-state fermentation was not affected to a great extent by the heavy metals. It was found that several metal ions normally present in textile effluent (Cu^{2+}, Co^{2+}, Zn^{2+}) enhanced the decolorization activity. Interestingly Cr^{6+}, a highly toxic metal ion, did not affect the enzyme activity. *G. lucidum* laccase could tolerate the presence of mixture of several metal ions together and effectively decolorized the complex reactive dye effluent in presence of natural redox mediator. This study suggests that laccase from *G. lucidum* could be a potential enzyme for the removal of color from reactive textile dye effluent.

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