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ORIGINAL PAPER

Laccase-mediator system in the decolorization of different types of recalcitrant dyes

Mei Rong Hu · Ya Peng Chao · Guo Qing Zhang · Zhi Quan Xue · Shijun Qian

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Abstract Phloroglucinol, thymol, and violuric acid (VIO) were selected as laccase mediators after screening 14 different compounds with indigo carmine (indigoid dye) as a substrate. With the presence of these three mediators, a nearly complete decolorization (90–100%) was attained in 1 h. Thus, these three compounds were used as mediators for the decolorization of other four dyes. The results indicated that VIO was effective mediator in decolorization of Remazol brilliant blue R (RBBR, anthraquinoid dye) and Coomassie brilliant blue G-250 (CBB, triphenylmethane dyes), and Acid red (diazo dye). In presence of VIO, the four dyes described above attained 70% decolorization. Thymol was able to mediate decolorization of RBBR and Azure A (heterocyclic dye). Phloroglucinol has no mediating capability in decolorization of the four dyes analyzed. Mediator concentration, pH, and copper ion have an effect on the decolorization of the RBBR. Our data suggested that the decolorization capabilities of laccase/mediator system were related to the types of mediator, the dye structure and decolorization condition.

Keywords Laccase · Decolorization · Mediator · Copper ion · Dye

M. R. Hu · Y. P. Chao · G. Q. Zhang · Z. Q. Xue · S. Qian (✉)
State Key Laboratory of Transducer Technology,
Institute of Microbiology, Chinese Academy of Sciences,
P. O. Box 2714, 100101 Beijing, People's Republic of China
e-mail: Qiansj@sun.im.ac.cn

M. R. Hu · Z. Q. Xue
Graduate Schools of the Chinese Academy of Science,
100039 Beijing, China

Introduction

There are more than 100,000 commercial dyes available to textile industries, and approximately 10–15% of these were released into the environment during manufacturing and usage [26]. The majority of these dyes are either toxic to flora and fauna or mutagenic and carcinogenic [21]. Conventional treatments of textile effluent are either ineffective, costly, complicated or have sludge disposal problems. Therefore, other alternatives such as biodegradation attract attention. The biodegradation of dyes is carried out mostly by fungi and by extracellular enzymes produced by white-rot fungi [9, 12]. Laccases are oxidoreductases that belong to the multinuclear copper-containing oxidases and are able to decolorize and detoxify industry dyes [1, 6, 9, 22]. However, some of the dye or lignin cannot be oxidized, or just partly oxidized by laccase, because they are too large to penetrate into the enzyme active site or have a particularly high redox potential [2, 4, 23]. Laccase mediators, such as 1-hydroxybenzotriazole (HBT), were found to extend or permit the oxidation of non-specific substrate by laccase. Thus, laccase mediators have been widely studied in recent years. Three types of mediators have been proposed: (1) an electron transfer route for mediators such as 2,2′-azino-bis 3-ethylbenzothiaoline-6-sufonic acid (ABTS); (2) a radical hydrogen atom transfer route for mediators of the -NOH-type, such as 1-hydroxybenzotriazole (HBT), violuric acid (VIO), first, laccase extract hydrogen, and $NO^{\cdot -}$ radicals are formed. Then the $NO^{\cdot -}$ radicals extract a hydrogen atom from the substrate [3, 8, 19, 20, 24]; (3) phenolic compound mediators, such as phenol compounds syringaldehyde, acetosyringone, whose mediating mechanism was same as HBT, and except that intermediate was a phenoxy radical.

Both the NOH-type and phenol-type mediators were found to be effective in the decolorization of dyes. Decolorizations of

88% for Sella Solid Red and 49% for Luganil Green were attained by laccase in the presence of HBT [23]. Syringaldehyde and acetosyringone were found to be efficient laccase mediators in the decolorization of recalcitrant dyes [4, 5]. Moreover, a laccase-mediator system (LMS) has been used in bleaching indigo carmine. In 1996, Novozyme (Novo Nordisk, Denmark) launched a new industrial application of laccase enzymes in denim finishing: DeniLite®. Laccases could bleach indigo-dyed denim fabrics to lighter shades with the help of a mediator molecule [10].

Nevertheless, LMS has not yet been applied at large scale due to the cost of mediators and their toxicity. The present work is an attempt to evaluate the natural compounds (phenols, anilines, and amino acids) and synthetic VIO to mediate the oxidative reactions catalyzed by laccase from *Fome lignosus* with the aim of identifying cheaper, more efficient, and eco-friendly mediators for textile and environmental applications. Furthermore, to improve the decolorization, the effects of mediator concentration, pH, and copper ion on decolorization were also studied.

Materials and methods

Chemicals

The natural compounds mentioned in Table 1, as well as the synthetic compounds violuric acid monohydrate and ABTS, were purchased from Sigma-Aldrich. All chemicals were of analytic grade or higher. The decolorizations of different dyes were monitored at their absorption maxima and were described in Table 1.

Enzymes

Laccase was produced by the laccase-secreting engineered strain *Pichia pastoris* GS115 (*Lse*), and the laccase gene was cloned from *F. lignosus* [15]. The *Lse* was cultivated in YPD (1% yeast extract, 2% peptone, 2% glucose) containing 0.6 mM copper ions. After 72 h at 30°C, 270 rpm, the optical density reached 30 and the laccase activity was maximal. The cells were separated by centrifugation at 1,000×*g*, 4°C, and the supernatant was filtered (0.45-μm pore size) and concentrated to 10 ml in an ultrafiltration cell (Amicon; Millipore, Bedford, Mass) equipped with a 30-kDa cutoff membrane. Laccase preparations were used for dye decolorization without purification. Laccase activity was determined spectrophotometrically as ΔA at 420 nm for 0.5 mM ABTS in 100 mM pH 4.5 sodium acetate buffer, ($\varepsilon_{420} = 36{,}000\ M^{-1}\ cm^{-1}$), reaction time was 5 min.

Mediator screening

Screening for mediators was based on the decolorization of indigo carmine by laccase in the presence of each compound. It was estimated by using 0.6 U ml^{-1} laccase, 20 μM indigo carmine, and 200 μM mediators in 100 mM citric acid buffer, pH 4.5 for 1 h at 24°C in 1.5-ml cuvettes. Decolorization of indigo carmine was by laccase alone as control.

Mediator concentration

The effects of the mediator concentration on dyes decolorization were studied by the use of 20 μM indigo carmine in the presence of 60, 120, 180, 240, 300, and 360 μM concentrations of mediator (thymol, phloroglucinol, and VIO, for 2 h with 0.6 U ml^{-1} laccase in the citric acid at pH 4.5 and 24°C in 1.5-ml cuvettes.

Decolorization of different types of dye

Assays of decolorization of 20 μM Coomassie brilliant blue G-250 (CBB), Acid red, Azure A, and 40 μM RBBR were performed with 0.6 U ml^{-1} laccase in the presence of 200 μM VIO, thymol, and phloroglucinol, in the pH 4.5 citric acid buffer for 0.5, 6 h in 1.5 ml cuvettes at 24°C. Decreases in the absorbance maxima were measured after different incubation time.

The effect of pH on the decolorization of dye in LMS

The effect of pH on dye decolorization was studied by treating 20 μM indigo carmine with 0.6 U ml^{-1} laccase in the presence of 200 μM mediators, at different pH citrate buffer

Table 1 Structures, concentrations, and maximum absorbance wavelengths of dyes

Dye type	Dyes	Concentrations of dyes (μM)	Maximum absorbance wavelength (nm)
Azo dyes	Acid red B	20	518
Anthraquinonic dyes	Remazol brilliant blue R	40	590
Triphenylmethane dyes	Coomassie brilliant blue G-250	20	585
Indigo carmine	Indigoid dye	20	610
Azure A	Heterocyclic dye	20	620

(pH 3, 4, 5, 6) and phosphate buffers (pH 7 and 8) for 1 h at 24°C in 1.5 ml cuvettes.

In 1.5-ml cuvettes, 40 μM RBBR, 20 μM CBB, and Acid red were oxidized by 0.6 U ml^{-1} laccase in the presence of 200 μM VIO in a range of pH from 3 to 8 by using suitable buffer for 1 h at 24°C. Controls were dyes decolorized by laccase alone.

The effect of copper ions on laccase activities and dye decolorization

The effect of copper ions on laccase activities was investigated. In presence of 0–0.6 mM copper ion concentration, the laccase activity was determined as above described method. Assay system was 0.5 mM ABTS in 100 mM pH 4.5 sodium acetate buffer, absence of mediator and dye.

Five dyes were decolorized by laccase alone or in the presence of a mediator at different copper ion concentrations for 6 h with 0.6 U ml^{-1} laccase in 100 mM pH 4.5 sodium acetate buffers. The concentration of mediators was at 200 μM, the concentration of RBBR was 40 μM, and that of the other dyes were 20 μM.

The effect of dye and mediator on laccase activities

The effect of dyes and mediators on laccase activities was studied by using 120 μM indigo carmine, RBBR, CBB, Acid red, Azure A, or 200 μM thymol and VIO in the culture supernatant (30 U ml^{-1} laccase activity), at pH 5.0 and 24°C. After 12 h, laccase activities were determined. Control was in the culture supernatant without dye or mediator.

As for the study of mediator screening, the effect of mediator concentration, pH on decolorization different types of dyes, the dyes were decolorized by laccase alone as control. As for study of the effect of copper ions on laccase activities, copper ion was absent as control. For the study of the effect of dye and mediator on laccase activities, control was in the culture supernatant without dye or mediator. All experiments were performed in triplicate. The report results were average values of triplicates.

Results

Mediators screening

Among the 14 compounds screened, 10 compounds promoted the decolorization of indigo carmine by laccase (Fig. 1). Methionine, cysteine, catechol, and 2,6-methoxyphenol had no effect on decolorization due to the low redox potential or the formation of a colored product. Reduced glutathione, antiscorbic acid, and tyrosine had little capability to mediate the oxidation (less than 40%); four kinds of phenols and synthetic compound VIO produced decolorizations higher than 70% (Fig. 1). Among them, thymol, phloroglucinol and VIO provided the highest decolorizations and were selected for additional study.

The effects of the mediator concentration on decolorization

Mediator concentration has an impressive effect on decolorization (Fig. 2). In the presence of thymol or phloroglucinol, the laccase produced the strongest decolorization at the mediator/dye ratio of 6:1 and 3:1, respectively. However, for VIO, the strongest decolorization was attained at the mediator/dye ratio of 2:1. Thus, VIO is more efficient than the natural mediators.

Decolorization of different types of dyes

Phloroglucinol, thymol, and VIO were evaluated for the oxidation of four types of dye with different chemical

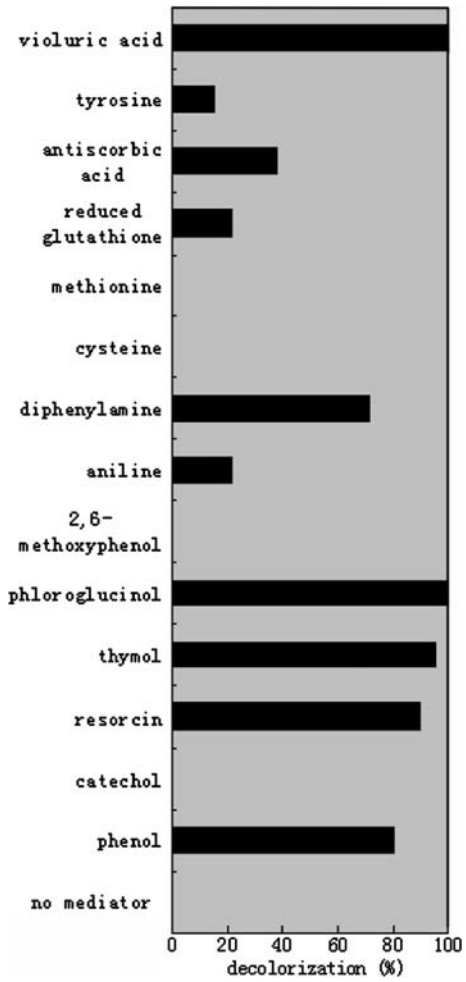

Fig. 1 Screening for mediators based on decolorization of indigo carmine (20 μM) in presence of mediator (200 μM) for 1 h monitored at 610 nm)

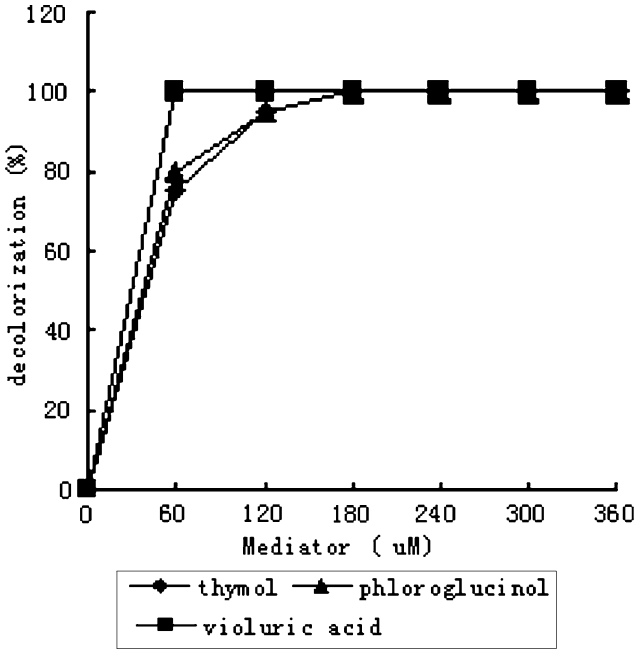

Fig. 2 Effect of mediator concentrations on the oxidation of indigo carmine by laccase (2-h treatment of 20 μM indigo carmine monitored at 610 nm)

Table 2 Decolorization of four dyes after different treatment times by laccases in the presence of a different mediator

Dye and treatment time	Decolorization (%)				
	Phloroglucinol	Thymol	VIO	Control[a]	Mediator[b]
RBBR					
0.5 h	0	77	80	0	25
6 h	10	100	100	30	59
Acid red					
0.5 h	7	9	75	0	30
6 h	10	15	100	0	80
CBB					
0.5 h	10	8	72	15	0
6 h	17	10	76	32	0
Azure A					
0.5 h	12	40	0	0	0
6 h	21	47	0	0	0

[a] Control: oxidation of dye by laccase alone

[b] Mediator: oxidation of dye was performed in the presence of a mediator without laccase. A different mediator was used for different dyes (i.e., for RBBR, CBB, and acid red it was VIO; for azure A, thymol was chosen as its mediator). The concentration of mediator was 200 μM

structures (Table 2). The results indicated that VIO was effective mediator in decolorization of RBBR, Acid red, and CBB. Thymol was able to mediate decolorization of RBBR and Azure A. Phloroglucinol had slight effect for RBBR, Azure A, and no effect on CBB. Our results also showed that indigo carmine (indigoid) and RBBR (anthraquinoid) were relatively easier to decolorize. They can be completely decolorized in the presence of the thymol or VIO. However, slight decolorization of Azure B was obtained for phloroglucinol and thymol as mediators. These results were in agreement with the previous results. For example, Camarero [4] also indicated that anthraquinoid (Reactive Blue 19) and indigoid (Acid Blue 74) were completely oxidized, but heterocyclic Azure B was just partly decolorized in LMS. Liu [16] also showed that Anthraquinoid dye was decolorized more easily than diazo dye and Triphenylmethane dyes. Therefore, dye decolorization in LMS was related to the type of mediator and the chemical structures of dye.

Our results also indicated that a mediator have capability to decolorize the dye in the absence of laccase (Table 2).

The effect of pH on dye decolorization in LMS

The optimal pH for the decolorization of indigo carmine in the presence of different mediator

The effect of pH on the decolorization of indigo carmine was examined. In the presence of phloroglucinol, thymol, the optimal pH were approximately 7, whereas for antiscorbic acid and VIO, the optimal pH were approximately 4 (as shown in Fig. 3), respectively. These results showed that the optimal pH of laccase for a specific substrate was significantly related to the type of the mediator.

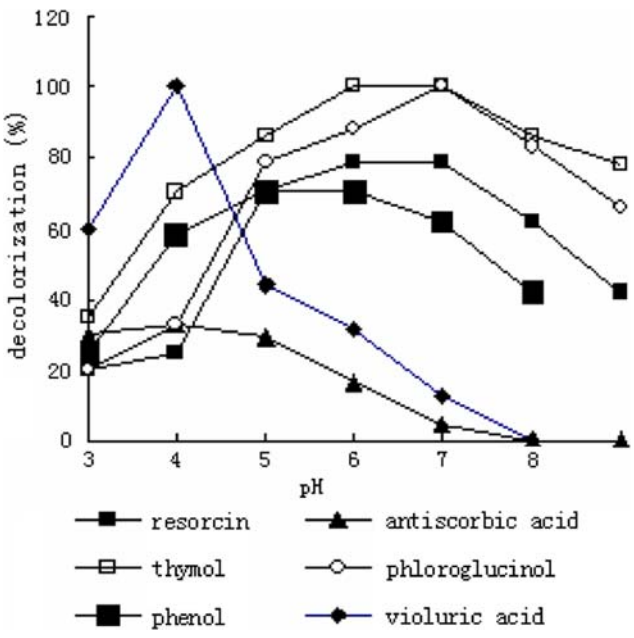

Fig. 3 Influence of pH on decolorization of 20 μM indigo carmine by laccase at 200 μM mediators different mediators (1-h treatment of 20 μM indigo carmine monitored at 610 nm)

Optimal pH for the decolorization of RBBR, CBB, and acid red in the presence of VIO

The RBBR, CBB, and acid red were oxidized in the presence of VIO at different pH (Fig. 4). The optimal pH of the three dyes ranged from 4 to 5, and with the increasing of pH, the decolorization of the three dyes obviously decreased. In combination with the results of optimal pH on the oxidation of indigo carmine (Fig. 3.), it is likely that the optimal pH in the LMS was related to the mediator rather than the dye type.

The effect of copper ion on laccase activity and dye decolorization

Copper ion has a positive effect on laccase activity (Fig. 5). Until the copper concentration reached at 0.6 mM, the laccase activity was improved as the increasing of copper ions (shown in Fig. 5. However, copper ions have different effects on dye decolorization by laccase alone and by LMS (Table 3). The decolorization of RBBR by laccase alone was improved from 30 to 100% with the addition of copper ion. However, in LMS, in the presence of copper ion, the oxidation rate of RBBR, CBB, Acid red and Azure A decreased obviously. As for indigo carmine, increasing copper concentration did not increase activity in absence of mediator, and caused no decrease of activity in the presence of the mediator.

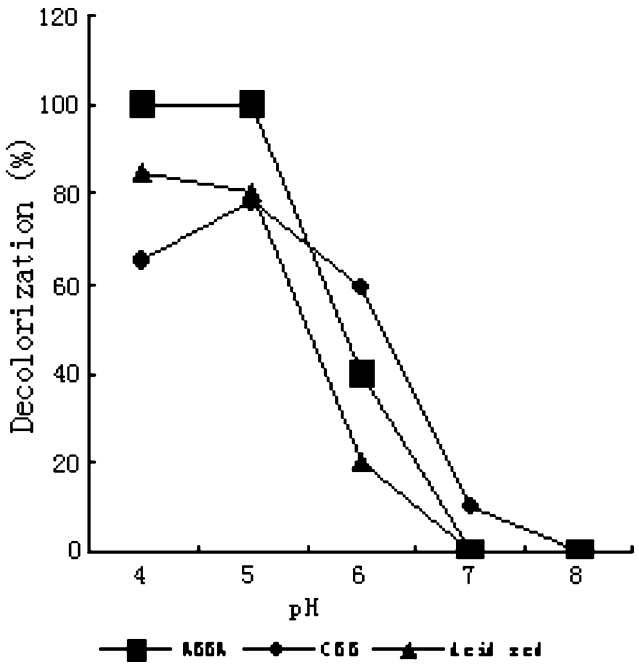

Fig. 4 Effect of pH on the decolorization of 40 μM RBBR, 20 μM CBB, and acid red by laccase in the presence of 200 μM VIO (1-h treatment of 40 μM RBBR, 20 μM CBB, and acid red, monitored at 590, 585, and 518 nm, respectively)

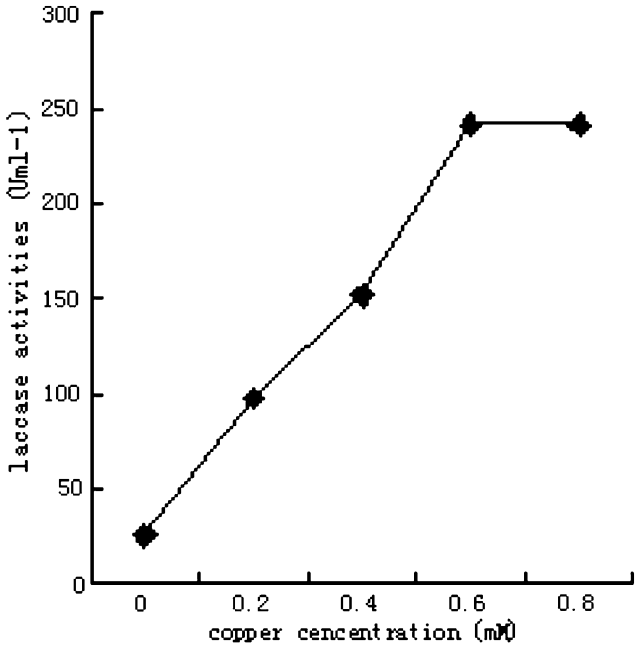

Fig. 5 Effect of copper ion on laccase activities. Laccase activities were monitored, in presence of 0–0.6 mM copper ion concentration, without mediator and dye in the assay buffer. Reaction time was 5 min

The effect of dyes and mediators on laccase activities

The effects of four types of dye and two kinds of mediators on laccase activity were studied (Fig. 6). 65% of laccase activities were remained for Indigo carmine and RBBR after 12-h treatment. In comparison, Azure A and Acid red deactivate laccase activity obviously, less than 35% of laccase activity was remained. For mediators, in presence of mediator thymol or VIO, laccase activity was lost slightly (Fig. 6.). These results indicated that different dyes and mediators have different effect on laccase activities.

Discussion

Mediator screening

Indigo carmine was not oxidized by laccase from *F. lignosus* alone, probably due to the high redox potential of this dye. However, it can be oxidized partly or completely by laccase in the presence of mediators. This reaction was used as a test to evaluate the mediating capabilities of phenols, aniline, amino acids, antiscorbic acid, and the synthetic compound VIO. Our results indicated that most of the phenols used were more efficient than aniline, amino acids, and antiscorbic acid. Mediating capabilities of some phenols were close to that of synthetic compound VIO. Furthermore,

Table 3 The effect of copper ion on the decolorization of five dyes[a]

Type of dye	Mediator	Decolorization (%) (at different copper ion concentration, mM)				
		0 (mM)	0.3 (mM)	0.4 (mM)	0.5 (mM)	0.6 (mM)
RBBR	No mediator	30	42	70	100	100
	VIO	100	90	90	85	85
Indigo	No mediator	0	0	0	0	0
	VIO	100	100	100	100	100
	Thymol	100	100	100	100	100
CBB	VIO	72	52	48	40	35
Acids red	VIO	100	20	15	10	10
Azure A	Thymol	47	15	15	15	15

[a] Five dyes were oxidized by laccase alone or in the presence of a mediator at different copper ion concentrations for 6 h. The mediators' concentrations were at 200 μM, the concentration of RBBR was 40 μM, and that of the other dyes were 20 μM

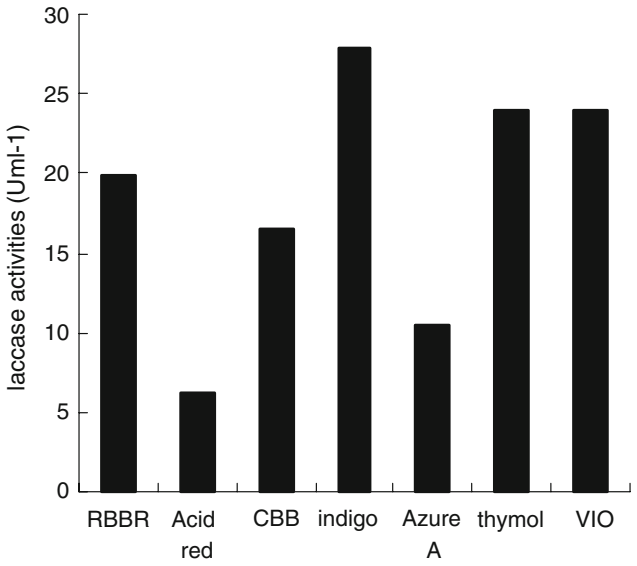

Fig. 6 Effects of dyes and mediators on laccase activities. Crude laccase activity was 30 U ml^{-1}, after the culture supernatant was incubated with dyes and mediators 12 h later, the laccase activities was determined

thymol and phloroglucinol as laccase mediators were also effective due to their low mediator/dye molar ratio of 6:1, which was much lower than those used in the studies mentioned above (ratios of 19–40) [11, 13]. Moreover, decolorization occurred more rapidly—indigo carmine can be completely oxidized in less than 1 h.

In recent years, some phenols, including syringaldehyde and acetosyringone, have been described as laccase mediators [5, 7]. The mediating capabilities of a phenolic compound partly depend on the stability of phenoxy radicals and the redox potential of a mediator. Groups on the aromatic ring have a great effect on the stability of phenoxy radicals and the redox of mediator [4, 7]. In the present study, Thymol and phloroglucinol were the more efficient phenolic mediators, probably due to the 5-Methyl-2-[1-methylethyl] groups or more OH groups on the aromatic ring, which therefore make its phenoxy radicals more stable. With regard to the effect of a mediators' redox potential on capability, it has been proposed that the oxidation efficiency increased proportionally with the redox potentials of the phenolic mediators up to the redox potential of the enzyme and decreased thereafter with the redox potentials exceeding this value [11]. Moreover, it was probable that the redox potential of thymol and phloroglucinol were close to that of laccase from the *F. lignosus*. In this paper, we first indicated that the simple phenols such as phenol, resorcin, phloroglucinol, and thymol can be as efficient laccase mediators for the decolorization of indigo carmine. Moreover, these compounds possess low-cost and low-toxic characteristics.

The effect of copper ion on the decolorization of different types of dyes

Laccase belongs to the multinuclear copper-containing oxidases. Thus, copper atom is very important for laccase activity. In the presence of copper ion in a culture medium, laccase activity was greatly improved [14, 17, 25]. However, the effects of copper ion on dye decolorization by laccase alone and by LMS are different. In decolorization of RBBR, copper ion improved the decolorization by laccase alone and decreased the decolorization in LMS. It was probably that RBBR was oxidized directly only by laccase. Thus, with the improvement of laccase activity, the higher decolorization was attained (Table 3). However, in a LMS, a mediator was first oxidized by laccase and then it formed an intermediate which acted with the dye. During the reaction, it was probable that copper ion increased the reaction rate between laccase and the mediator. However, it decreased the stability of the intermediate greatly, thus making the decolorization drop. Similarly, in the decolorization of CBB and Acid reds in LMS, copper ion decreased its decolorization too. Furthermore, this result was disagreed with the previous study; Mechichi [18] indicated that the decolorization of RBBR by laccase alone was slightly inhibited in the presence of copper ion. The definite mechanism was still unclear.

Decolorization of indigo carmine, RBBR

Decolorization conditions have great effects on dye decolorization. Thus, different types of dye should be decolorized by different decolorization systems. In the decolorization of indigo carmine by LMS, phloroglucinol, thymol, and VIO were highly efficient mediators. Nonetheless, in comparison with the Phloroglucinol and VIO, there were two good characteristics for thymol, one of which was the slightly blue of the buffer, which is near to the color of indigo carmine. However, in presence of phloroglucinol and VIO, it was slightly yellow or red. These colors were completely different to the blue of indigo carmine. Thus, if chosen Phloroglucinol or VIO as mediator in bleaching, it would cause trouble in the next procedure. Another one is that the optimal pH for thymol is 6; even at pH 8, the decolorization was still above 70%. This character is favorable in the process bleaching indigo by laccase, because bleaching buffer is usually biased alkalinity.

Moreover, LMS can be used in bioremediation. Textile effluent mainly containing indigo carmine can be decolorized recycling by laccase. It is because that laccase activity was kept above 80% in presence of thymol or indigo carmine (Fig. 6).

Remazol brilliant blue R was oxidized by laccase alone, and in the presence of 0.6 mM copper ion, the decolorization reached 100% after 6 h. Although thymol and VIO can improve the reactive efficiency of RBBR (100% decolorization in 2 h), in order to have less impact on the environment, it was better to oxidize RBBR by laccase without mediator.

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