International Biodeterioration & Biodegradation 85 (2013) 173–181

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

The enzymatic decolorization and detoxification of synthetic dyes by the laccase from a soil-isolated ascomycete, *Paraconiothyrium variabile*

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ARTICLE INFO

Article history:
Received 24 May 2013
Received in revised form
14 July 2013
Accepted 15 July 2013
Available online 3 August 2013

Keywords:
Decolorization
Detoxification
Industrial dye
Laccase
1-Hydroxybenzotriazole
Paraconiothyrium variabile

ABSTRACT

The enzymatic decolorization of 13 synthetic dyes with azo- and anthraquinone-based chemical structures using the purified laccase from *Paraconiothyrium variabile* was assessed in the presence and absence of 1-hydroxybenzotriazole (HBT) as a laccase mediator. The highest decolorization percentage was found for Acid Red 18 (97%) followed by Direct Red 81 (68.3%), Reactive Yellow 15 (60.2%), and Disperse Blue 56 (58%) after 15 min of incubation in the presence of 0.1 U mL^{-1} of the enzyme. The use of the laccase-HBT system increased decolorization percentage of Reactive Orange 16, Reactive Black 5, Direct Blue 71, Disperse Red 177, and Acid Yellow 36, while other chemical dyes were not affected by an increase of the HBT concentration. The kinetic parameters of the purified laccase for each textile dye were also calculated. A microtoxicity study with respect to the inhibition of bacterial growth showed a decrease in toxicity of the laccase-treated dye solution.

1. Introduction

The extensive application of synthetic dyes in various industries such as pulp and paper, textiles, printing, cosmetics, and pharmaceuticals has led to the production of more than 100,000 hazardous dyes of different types such as heterocyclic, anthraquinone, phthalocyanine, triphenylmethane, and azo-based chemical structures (Cristovao et al., 2008, 2009; Ayed et al., 2011; Tang et al., 2011). Almost all synthetic dyes, especially the azo dyes (one of the most applied dye groups in textile factories), are found to be toxic, carcinogenic, and/or mutagenic (Kunamneni et al., 2008; Zeng et al., 2011; Saratale et al., 2011). In textile industries and during the dyeing processes (classified as direct, disperse, reactive, acid, basic, and vat dye processes), about 10–15% of applied dyes pass into the effluents (Kunamneni et al., 2008; Saratale et al., 2011). The discharge of industrial wastes rich in such toxic dyes, together with the resistance of many of them to physicochemical removal techniques such as coagulation, flocculation, adsorption, ion-exchange, oxidation, and electrochemical methods (Khlifi et al., 2010), prompts researchers to study biological or enzymatic methods for the degradation of hazardous dyes (Murugesan et al., 2007; Saratale et al., 2011). The enzymatic removal of such pollutants is an economic and environmentally friendly procedure because of the low energy required and the minimal impact on ecosystems (Telke et al., 2011; Forootanfar et al., 2011; Sadighi and Faramarzi, 2013). Furthermore, the immobilization of effective enzymes on different matrices allows the biocatalysts to be reused many times (Cristovao et al., 2011).

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) are copper-containing oxidases mainly produced by fungal strains, specifically white-rot fungi (Aghaie-Khouzani et al., 2012; Forootanfar et al., 2012a). In contrast to the most substrate-specific oxidases, laccases are able to oxidize a wide range of compounds, from inorganic substrates like iodine to organic pollutants such as polyaromatic hydrocarbons (PAHs) (Forootanfar et al., 2011, 2012a). During recent decades, many studies have been done on laccase-producing strains and purified laccases in

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http://dx.doi.org/10.1016/j.ibiod.2013.07.006

both free and immobilized forms for the removal of environmental pollutants, especially synthetic dyes (Saratale et al., 2011; Cristovao et al., 2011). The application of the laccase-mediated system — for example, the laccase-HBT system — has been reported as efficient in the degradation of resistant pollutants (Couto et al., 2005; Khlifi et al., 2010).

The aim of the present work was to study the decolorization and detoxification ability of the purified laccase obtained from *Paraconiothyrium variabile*, a newly soil-isolated ascomycete (Forootanfar et al., 2011; Faramarzi and Sadighi, 2013), on 13 synthetic dyes in the presence and absence of HBT as the enzyme mediator. Furthermore, the influences of conditional parameters such as the enzyme activity, temperature, and pH on the decolorization were investigated. The enzyme kinetic parameters (K_m and V_{max}) on the applied dyes were also calculated.

2. Materials and methods

2.1. Chemicals, dyes, and the enzyme

2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and 1-hydroxybenzotriazole (HBT) were purchased from Sigma–Aldrich (St. Louis, MO, USA) and Merck (Darmstadt, Germany), respectively. Thirteen synthetic dyes (Fig. 1 and Table 1) were obtained from Alvan Sabet Co. (Tehran, Iran). All other reagents and chemicals were of the highest purity available. The extracellular laccase from *P. variabile*, a soil-isolated ascomycete, was purified (Forootanfar et al., 2011) and applied in decolorization experiments.

2.2. Purification of extracellular laccase

The extracellular laccase from *P. variabile* was purified using the method described by Forootanfar et al. (2011). Briefly, the fungal strain was cultivated in sabouraud-2%-dextrose broth (SDB) for 10 days and the broth was then harvested after removing of fungal mycelia by filtration. The laccase from *P. variabile* was then purified at four consequent steps of ammonium sulfate precipitation, anion exchange chromatography on Q-Sepharose XL and gel filtration chromatography by Sephadex G-100 after ammonium sulfate precipitation of active fraction obtained from anion exchange column. All purification steps were carried out at 4 °C. The purified enzyme was then applied decolorization experiments.

2.3. Laccase assay

The laccase activity of the purified enzyme was measured by oxidizing the ABTS as a substrate according to the method previously described (Faramarzi and Forootanfar, 2011). Briefly, 0.5 mL of

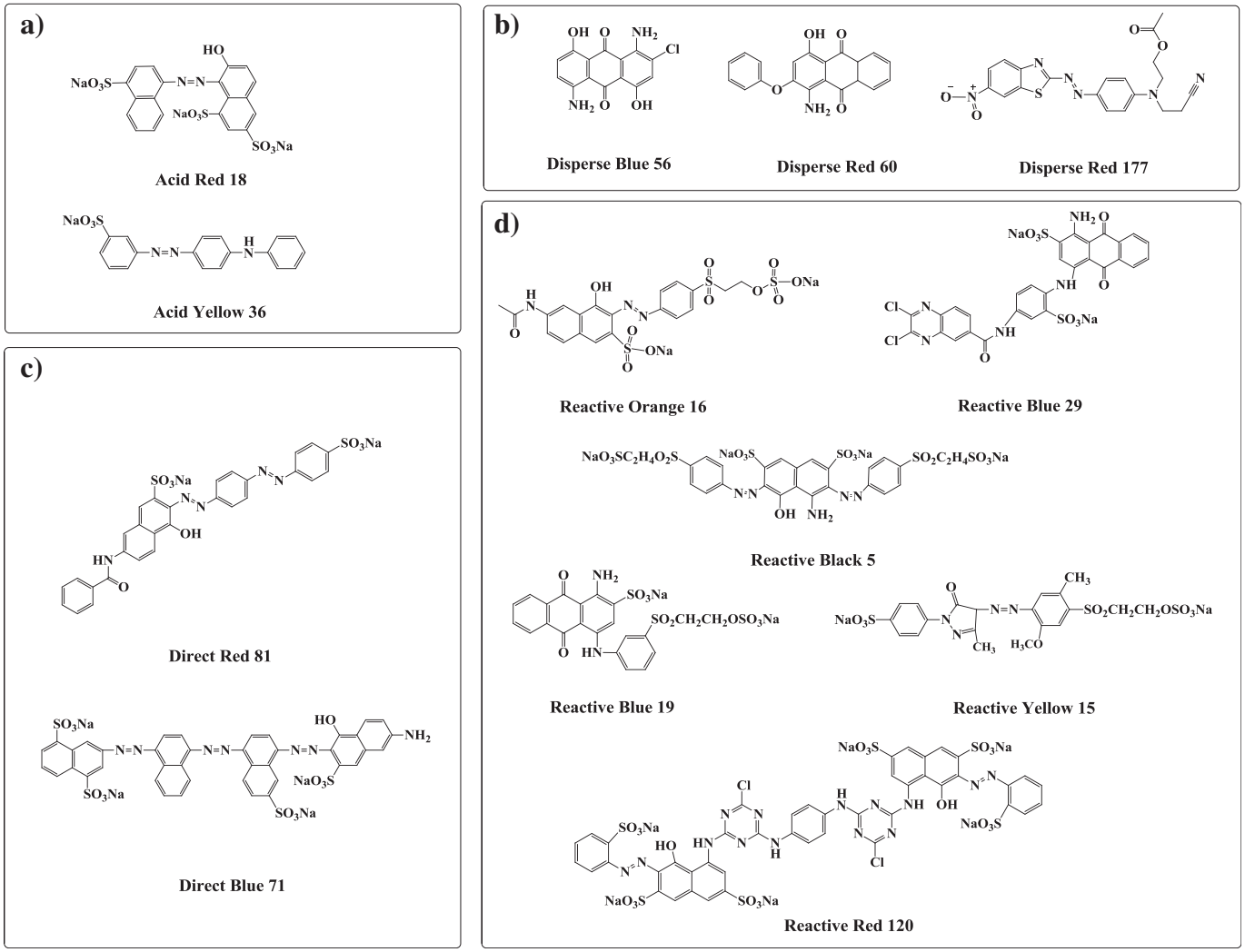

Fig. 1. Chemical structures of a) acid, b) disperse, c) direct, and d) reactive dyes applied in the present study.

Table 1
Kinetic parameters of the purified laccase of *P. variabile* on studied synthetic dyes.

Dye name	Classification	λ_{max} (nm)	K_m (μM)	V_{max} (mmol min^{-1} mg^{-1})
Acid Red 18	Monoazo	506	14.8	250
Acid Yellow 36	Monoazo	428	155	14.3
Direct Blue 71	Triazo	584	800	2.5
Direct Red 81	Diazo	509	27.5	100
Disperse Blue 56	Anthraquinone	316	25	125
Disperse Red 177	Monoazo	523	300	10
Disperse Red 60	Anthraquinone	592	48.6	50
Reactive Black 5	Diazo	607	130	16.7
Reactive Blue 19	Anthraquinone	592	38.3	50
Reactive Blue 29	Anthraquinone	589	83.8	20
Reactive Red 120	Diazo	508	362	9
Reactive Orange 16	Monoazo	490	190	12.5
Reactive Yellow 15	Monoazo	416	44.6	50

the enzyme sample was added to 0.5 mL of the ABTS solution (5 mM in citrate buffer 0.1 M pH 5) and incubated at 40 °C and 120 rpm for 10 min. The change in the absorbance at 420 nm was then determined using a UV–visible spectrophotometer (UVD 2950, Labomed, Culver City, USA), and the enzymatic activity was calculated using the molar extinction coefficient of ABTS ($\varepsilon_{420} = 36{,}000\ M^{-1}\ cm^{-1}$). One unit of enzyme activity was defined as the amount of the enzyme that can oxidize 1 μmol of ABTS per minute under the assay condition (Alberts et al., 2009).

2.4. Dye decolorization experiments

Decolorization experiments were performed by adding purified laccase (with the activity of 0.1 U mL^{-1}) to each dye solution (a concentration of 100 mg L^{-1}), which was previously prepared by dissolving each dye in a citrate-phosphate buffer (0.1 M, pH 5.0), followed by the incubation of the reaction mixture (final volume of 2.5 mL) at 40 °C and 50 rpm for 3 h. The decolorization percentage was then determined by monitoring the absorbance of the samples taken (each 30 min to 3 h), using a UV–visible spectrophotometer at the maximum absorbance of each dye. Incubation of the reaction mixture was then continued overnight to confirm any significant change in decolorization percentage. The following equation was used to estimate the percentage of decolorization: Decolorization (%) = $[A_i - A_t/A_i] \times 100$: where A_i is the initial absorbance of the reaction mixture and A_t is the absorbance after incubation time (Couto, 2007; Khlifi et al., 2010; Zhuo et al., 2011). A negative control was also prepared by adding the heat-inactivated enzyme to the dye solution and by incubating as described above. All experiments were performed in triplicate and the means of decolorization percentages were reported. Decolorization studies were then investigated at different temperatures (30–60 °C) while pH was 5 and also at various pH levels (3–8) while initial temperature was 40 °C. In addition, the effect of laccase activity on decolorization was examined by adding the purified laccase to the reaction mixture to reach the enzyme

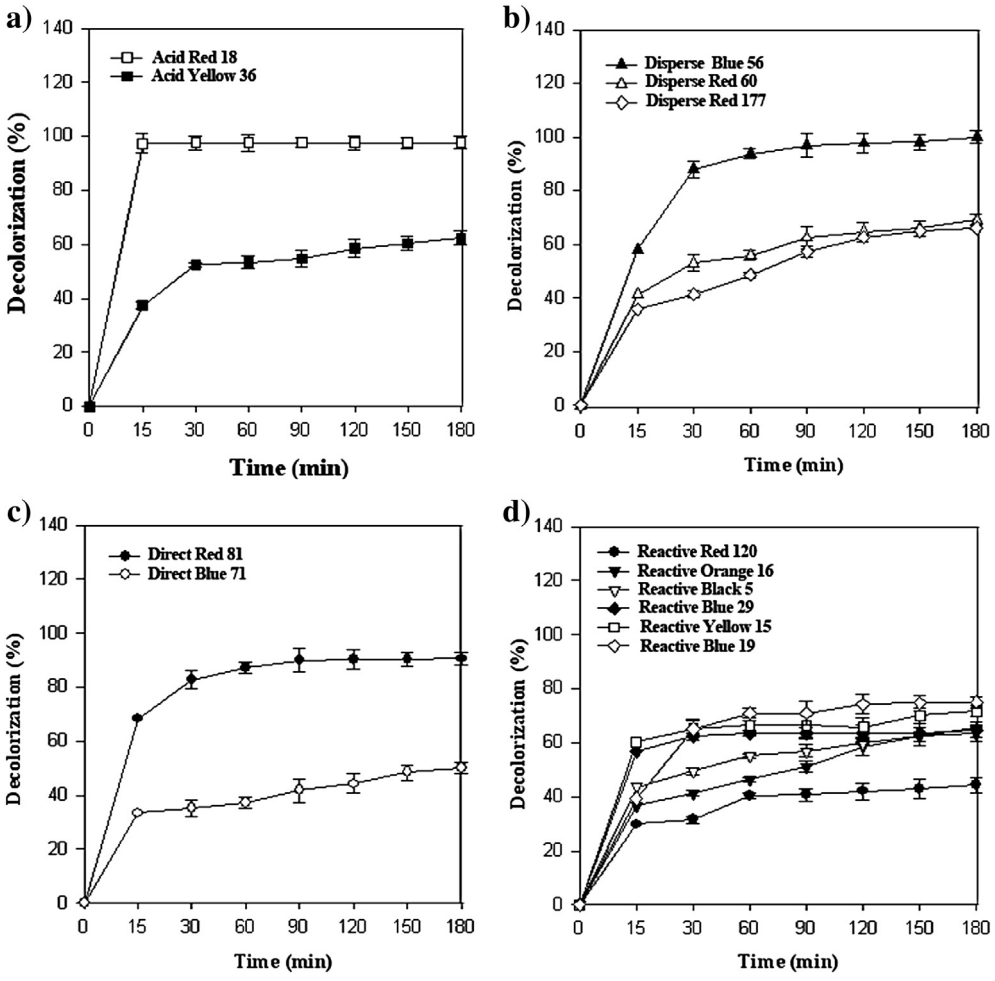

Fig. 2. Decolorization profiles of a) acid, b) disperse, c) direct, and d) reactive dyes using the purified laccase of *P. variabile* (0.1 U mL^{-1}) during 180 min of incubation at 40 °C.

activity of 0.025–0.3 U mL^{-1} followed by incubation at 40 °C and 50 rpm for 3 h and measuring of decolorization percentage.

2.5. The laccase-HBT mediated system for dye decolorization

The laccase-mediated system was made by introducing HBT as a non-phenolic laccase mediator to the reaction mixture (dye, 100 mg L^{-1} and laccase, 0.1 U mL^{-1} in citrate-phosphate buffer 0.1 M pH 5) to reach the final concentrations of 0.1, 1, and 5 mM. Decolorization was determined as described in the previous section.

2.6. Kinetic studies

The kinetic parameters of the purified laccase for each dye were obtained after the determination of velocity for different concentrations of each dye. Next, a Michaelis–Menten curve was drawn by plotting the obtained velocity (V) against the dye concentrations (S). K_m (Michaelis constant) and V_{max} (maximal velocity) were then calculated using the Lineweaver–Burk transformation of the Michaelis–Menten equation.

2.7. Dye toxicity

A susceptibility toxicity assay was performed in order to evaluate the toxic effects of both the untreated and treated dye solutions based on the inhibitory growth of three gram-negative bacterial strains (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, and *Salmonella typhi* ATCC 19430) and three gram-positive bacterial strains (*Micrococcus luteus* ATCC 10240, *Staphylococcus aureus* ATCC 6538, and *Bacillus subtilis* ATCC 6633). Each tested bacterial strain was firstly cultivated in nutrient broth to reach the OD_{600} of 0.1. Thereafter, the samples obtained from enzymatic treatment of each dye and the untreated dye solution (final concentration of 100 mg L^{-1}) were separately added to the prepared bacterial broth and incubated at 37 °C. Changes in the OD_{600} of each bacterial strain were then recorded every 2 h for 10 h. A negative control (cultivated bacterial strain in the absence of dye) was also designed for each experiment. The percentage of growth inhibition (GI%) was defined as $[(1 - D_{600S}/OD_{600C}) \times 100]$, where OD_{600S} is the OD_{600} of sample and OD_{600C} is the OD_{600} of control. All experiments were performed in triplicate.

2.8. Statistical analysis

All mentioned experiments performed in triplicate and the reported data are presented as mean ± standard deviation. The statistical significance between mean values was calculated using the independent sample *t*-test and one-way analysis of variance (ANOVA) with Dunnett's T3 post hoc test (SPSS 15.0, SPSS Inc). Probability values <0.05 were considered significant.

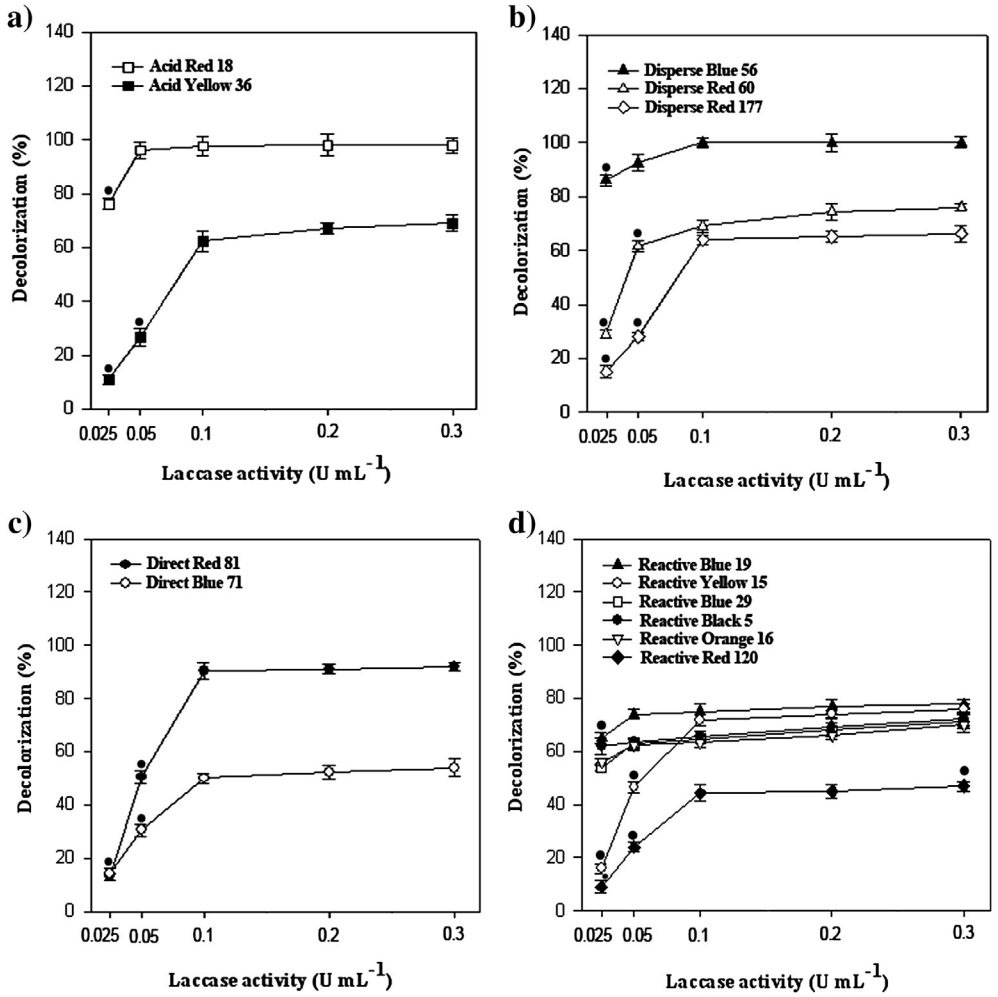

Fig. 3. Influence of laccase activity on decolorization of a) acid, b) disperse, c) direct, and d) reactive dyes after 180 min of incubation at 40 °C. Significant values (●, *p*-value < 0.05) were achieved after ANOVA analysis with Dunnett's T3 post hoc test.

3. Results and discussion

3.1. Dye decolorization by the purified laccase

Preliminary studies revealed that all the synthetic dyes in the present work (Fig. 1) were decolorized over 3 h by the purified laccase of *P. variabile* (Fig. 2). Further incubation of the reaction mixtures for 24 h did not show any increase in decolorization percentages (data not shown). No color removal was detected in the negative controls. The highest decolorization percentages were found for Acid Red 18 (97%) followed by Direct Red 81 (68.3%), Reactive Yellow 15 (60.2%), and Disperse Blue 56 (58%) after 15 min of enzymatic incubation (Fig. 2).

The removal of mutagenic and carcinogenic synthetic dyes discharged from industrial effluents into the environment using laccases as a valuable oxidizing enzyme, has received more attention during recent decades (Couto and Toca-Herrera, 2006). As is evident, depending on the sources of laccases (bacteria, fungi, etc.), the chemical structure of applied dyes, and other physical factors such as pH and temperature, the decolorization rates of diverse groups of synthetic dyes are different (Mechichi et al., 2006; Pereira et al., 2009). Some very recent studies have revealed that laccase from the isolate *P. variabile* is able to remove a wide range of chemicals such as chlorophenols, synthetic dyes, and benzodiazepines (Forootanfar et al., 2011, 2012a, b; Aghaie-Khouzani et al., 2012; Mogharabi et al., 2012; Ostadhadi-Dehkordi et al., 2012). The present study showed that almost all the azoic- and anthraquinone-based colorants selected from different groups of reactive, acid, disperse, and direct industrial dyes (Table 1) were successfully decolorized, although not to the same extent, using the purified laccase of *P. variabile* after a 3-h period of incubation. Grassi et al. (2011) reported a 65% removal of Fast Blue RR as well as 30% of Azure B and Methylene Blue using the purified laccase of *Trametes trogii* after a 24-h period of incubation. After the decolorization parameters were optimized, 60% of Reactive Black 5 (a diazo dye) was removed by the crude laccase from *Trametes pubescens* following 20 min of incubation (Roriz et al., 2009). In general, azo dyes that are classified as monoazo, diazo, and triazo, depending on the number of N=N bonds, have been identified as recalcitrant dyes, while anthraquinones are recognized as easy substrates for laccase oxidation (Saratale et al., 2011; Zeng et al., 2011).

3.2. The effect of laccase activity on decolorization

The pattern of decolorization by increasing laccase activity is illustrated in Fig. 3. Except for Reactive Black 5 (Fig. 3d), decolorization percentages of all applied dyes in the present study were significantly increased when laccase activity increased from 0.025 U mL^{-1} to 0.1 U mL^{-1} (Fig. 3). The same was observed in Murugesan et al.'s study (Murugesan et al., 2007), which indicated that with 25 U mL^{-1} laccase activity, maximum decolorization was obtained and the decolorization percentage remained constant.

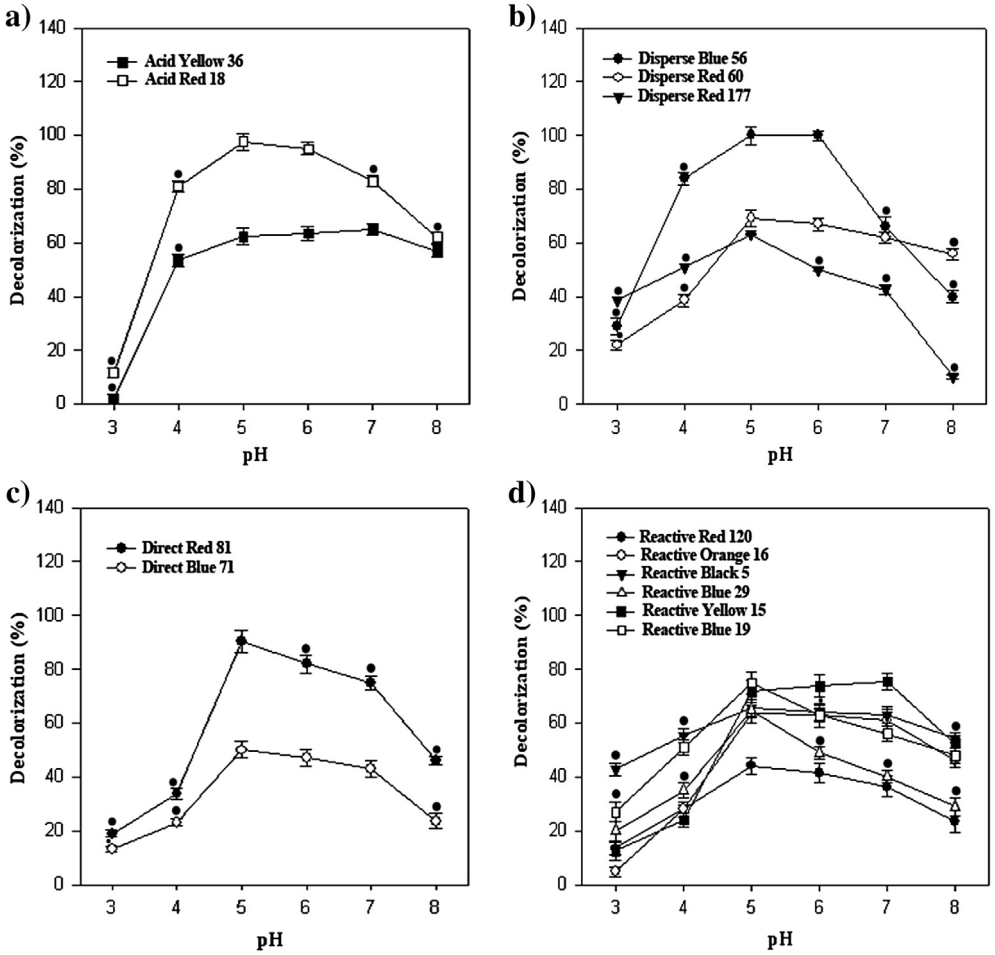

Fig. 4. Decolorization of a) acid, b) disperse, c) direct, and d) reactive dyes in the presence of the purified laccase of *P. variabile* (0.1 U mL^{-1}) after 180 min of incubation at different initial pH values. Significant means (●, p-value < 0.05) were obtained after ANOVA analysis with Dunnett's T3 post hoc test.

3.3. The effect of pH on decolorization by laccase

As shown in Fig. 4, the synthetic dyes — except for two (Acid Yellow 36 and Reactive Yellow 15, whose maximum decolorization was obtained at pH 7) — were maximally decolorized at pH 5, which was previously introduced as the optimum pH for the activity of the purified laccase from *P. variabile* (Forootanfar et al., 2011). Alteration of pH to acidic range of 3 and 4 led to significant decrease of decolorization in the case of all studied dyes (Fig. 4). In the study of Zeng et al. (2011), the pH values of 4 and 5 were optimal for the decolorization of four synthetic dyes. Murugesan et al. (2007) revealed that the laccase of *Ganoderma lucidum* could efficiently decolorize Remazol Black 5 and Remazol Brilliant Blue R at a pH of 4, and a sharp decrease in the decolorization percentage was achieved at higher and lower pH values.

3.4. The effect of temperature on enzymatic decolorization

The optimum temperature for the decolorization of six dyes, namely, Disperse Red 60, Direct Red 81, Reactive Blue 19, Reactive Blue 29, Reactive Black 5, and Reactive Red 120, was found to be 50 °C (Fig. 5). However, in the cases of Acid Red 18, Acid Yellow 36, Disperse Blue 56, Reactive Yellow 15, Disperse Red 177 and Reactive Orange 16, 40 °C was the best temperature for maximum decolorization (Fig. 5). Maximum decolorization percentages for Direct Blue 71 were achieved at 60 °C (Fig. 5). These results were in agreement with the findings of Zeng et al. (2011), who determined maximum dye decolorization between 40 °C and 60 °C; a sharp drop in decolorization activity was observed above 60 °C. Most of the fungal laccases showed their optimal activity in the temperature range of 50–70 °C, although the maximum activity of laccase from *G. lucidum* was at 25 °C (Morozova et al., 2007). A recent study (Forootanfar et al., 2011) found that purified laccase from *P. variabile* at 50 °C was optimal for maximum laccase activity.

3.5. Dye decolorization by laccase in the presence of HBT

The effect of HBT as a redox mediator on the decolorization of textile dyes using the purified laccase is presented in Fig. 6. The study showed that the decolorization percentages of Reactive Orange 16, Reactive Black 5, Direct Blue 71, Disperse Red 177, and Acid Yellow 36 were significantly increased in the presence of the laccase-HBT system (Fig. 6). However, in the case of other dyes (Acid Red 18, Disperse Red 60, Direct Red 81, Reactive Blue 19, Reactive Yellow 15, Reactive Blue 29, and Reactive Red 120), increasing the HBT concentration negatively affected the rate of decolorization. The negative effect was found to be significant at higher concentrations of HBT (1 and 5 mM, Fig. 6). According to the literature (Mikolasch and Schauer, 2009), laccases with a higher redox potential (one of the most important characteristics of these

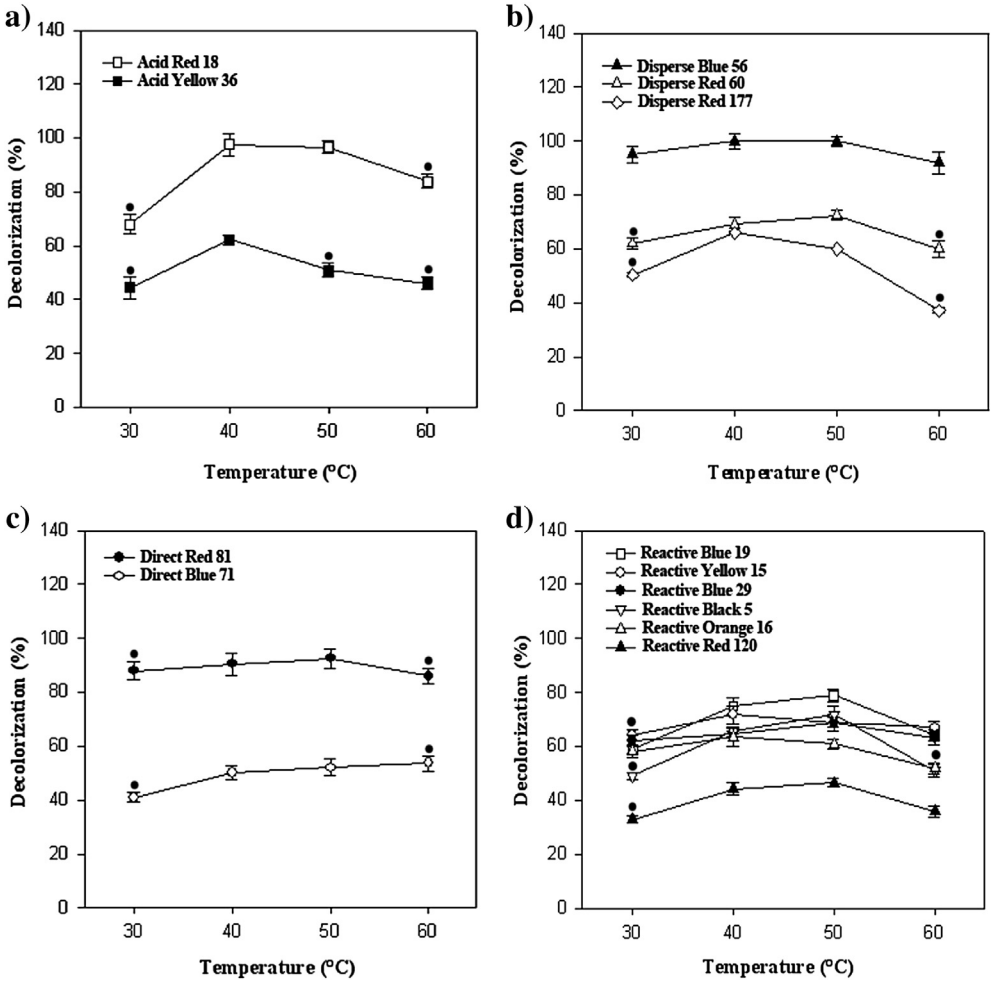

Fig. 5. Effect of various temperatures on decolorization of a) acid, b) disperse, c) direct, and d) reactive dyes using the purified laccase of *P. variabile* (0.1 U mL^{-1}) after 180 min of incubation. Obtained means were analyzed by ANOVA with Dunnett's T3 post hoc test (●, p-value < 0.05).

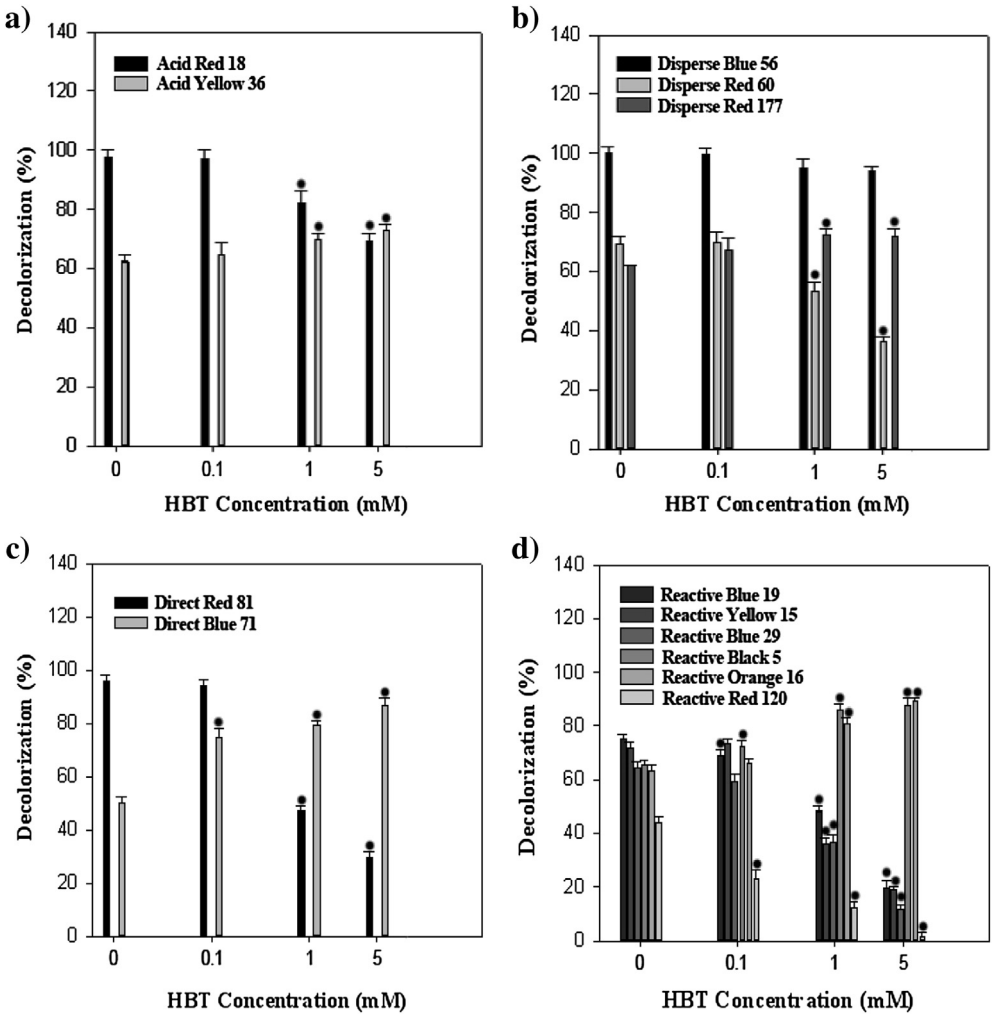

Fig. 6. Application of laccase-HBT system at a concentration of 0.1, 1, and 5 mM for the decolorization of a) acid, b) disperse, c) direct, and d) reactive dyes. Significancy (●) was checked after ANOVA analysis of obtained data (p-value < 0.05).

enzymes) are able to oxidize a wide range of substrates. For example, it was found that the purified laccase of *T. trogii* (redox potential of 0.79 V) was able to efficiently decolorize industrial dyes with various chemical structures, such as Remazol Brilliant Blue R, Reactive Blue 4, Acid Blue 129, and Acid Red 1 (Grassi et al., 2011; Zeng et al., 2011). In laccases with a lower redox potential, the addition of enzyme mediators such as HBT, vanillic acid, syringic acid, ABTS, and ferulic acid could be helpful in oxidizing recalcitrant substrates (Giardina et al., 2010; Ostadhadi-Dehkordi et al., 2012). Although the laccase-HBT system is one of the most successful laccase-mediated systems used for removal of pollutants, the elimination of some synthetic dyes (Khlifi et al., 2010), and the degradation of some pharmaceutical agents (Ostadhadi-Dehkordi et al., 2012), the mediating activity of HBT (redox potential of 1.1 V) was found to be proportional due to the destructive effect of the N–O• group on the laccase activity, especially at a high concentration (Papinutti et al., 2008). In the present study, both the positive and negative effects of HBT on the decolorization of synthetic dyes were observed. As an example, in the case of Direct Blue 71 the use of the laccase-HBT system increased the decolorization percentage from 50% (unaided laccase) to 86% (in the presence of 5 mM of HBT, p-value < 0.05), while the decolorization percentage of Acid Red 18 significantly decreased from 97% (laccase alone) to 69.4% (laccase-HBT at a concentration of 5 mM) after a 3-h enzymatic treatment (Fig. 6). In the study of Zeng et al. (2011), it was shown that increasing the concentration of HBT from a critical concentration (1 mM) reduced the decolorization of azo dyes.

3.6. Kinetic studies

The results of the kinetic study are summarized in Table 1. The lowest and the highest K_m were determined for the monoazoic dye of Acid Red 18 (14.8 μM) and triazoic dye of Direct Blue 71 (800 μM), respectively, in the presence of the purified laccase. The high K_m value of Direct Blue 71 suggests that it is not a suitable substrate for laccase oxidation and needs a mediator for being decolorized. In the study of Roriz et al. (2009), the K_m value of laccase from *T. pubescens* on Reactive Black 5 was determined to be 260 mg L^{-1}. The Michaelis–Menten constants for Reactive Black 5, Reactive Blue 114, Reactive Yellow 15, Reactive Red 239, and Reactive Red 180 in the presence of the commercial laccase Denilite® IIS were also found to be 3.14, 21.28, 99, 165, and 650 mg L^{-1}, respectively (Cristovao et al., 2008). In addition, the V_{max} value in the case of Direct Blue 71 (2.5 mmol min^{-1} mg^{-1}) confirmed the unsuitability of this dye to be removed by the purified laccase of *P. variabile*. The study of Younes et al. (2012) showed that the produced laccase of *Scytalidium thermophilum* represented the lowest V_{max} (191 μM min^{-1}) for triphenylmethane dye of Phenol Red.

Table 2
Growth inhibition percentage (GI%) of untreated and laccase-treated dyes against six bacterial strains. Values are averages of three replicates ±standard deviation.

Bacterial strains												
Dye names	*E. coli*		*P. aeruginosa*		*S. typhi*		*B. subtilis*		*S. aureus*		*M. luteus*	
	Un-treated	Treated	Un-treated	Treated	Un-treated	Treated	Un-treated	Treated	Un-treated	Treated	Un-treated	Treated
Acid Red 18	39 ± 1.4	14 ± 0.4*	28 ± 1.0	9 ± 0.3*	37 ± 0.8	11 ± 0.8*	44 ± 0.6	14 ± 0.6*	39 ± 0.9	11 ± 0.9*	48 ± 0.7	18 ± 0.7*
Acid Yellow 36	34 ± 1.1	11 ± 0.4*	26 ± 1.2	8 ± 0.3*	33 ± 1.2	9 ± 0.7*	41 ± 1.5	11 ± 0.5*	36 ± 1.1	11 ± 0.6*	42 ± 0.8	13 ± 0.7*
Direct Blue 71	52 ± 1.8	27 ± 0.7*	38 ± 1.2	18 ± 0.6*	48 ± 1.9	21 ± 0.8*	57 ± 2.2	29 ± 0.9*	53 ± 2.1	25 ± 0.8*	62 ± 2.9	34 ± 1.0*
Direct Red 81	42 ± 1.1	19 ± 0.5*	31 ± 0.4	13 ± 0.6*	40 ± 0.8	17 ± 0.4*	47 ± 1.1	20 ± 1.0*	43 ± 1.8	20 ± 0.8*	50 ± 2.3	24 ± 0.6*
Disperse Blue 56	23 ± 0.9	5 ± 0.4*	14 ± 0.7	6 ± 0.3*	19 ± 0.3	4 ± 0.4*	27 ± 0.4	6 ± 0.2*	24 ± 0.9	5 ± 0.3*	28 ± 1.1	6 ± 0.5*
Disperse Red 177	30 ± 1.8	10 ± 0.4*	26 ± 1.4	6 ± 0.3*	29 ± 1.3	7 ± 0.6*	38 ± 1.9	9 ± 0.7*	33 ± 0.7	7 ± 0.6*	39 ± 1.2	10 ± 1.1*
Disperse Red 60	28 ± 2.1	5 ± 0.4*	20 ± 1.6	6 ± 0.7*	21 ± 0.7	4 ± 0.8*	35 ± 0.8	6 ± 0.7*	28 ± 1.1	5 ± 0.4*	33 ± 0.9	6 ± 0.3*
Reactive Black 5	45 ± 2.6	17 ± 0.8*	33 ± 2.0	12 ± 0.7*	42 ± 1.8	15 ± 0.6*	49 ± 1.7	22 ± 1.3*	45 ± 2.4	18 ± 1.1*	52 ± 3.4	23 ± 1.4*
Reactive Blue 19	23 ± 2.4	5 ± 1.2*	14 ± 1.2	6 ± 1.3*	19 ± 0.8	4 ± 0.5*	27 ± 0.6	6 ± 0.3*	24 ± 1.1	5 ± 0.6*	28 ± 0.9	6 ± 0.3*
Reactive Blue 29	23 ± 1.1	5 ± 0.4*	18 ± 1.0	6 ± 0.8*	21 ± 1.6	4 ± 0.3*	29 ± 1.3	6 ± 0.5*	27 ± 1.1	5 ± 0.3*	32 ± 1.4	6 ± 0.2*
Reactive Red 120	45 ± 2.3	23 ± 1.8*	33 ± 2.1	15 ± 0.4	42 ± 0.9	18 ± 0.7*	54 ± 3.2	26 ± 0.6*	49 ± 2.7	22 ± 1.1*	57 ± 2.9	26 ± 1.2*
Reactive Orange 16	30 ± 0.7	7 ± 0.4*	24 ± 0.8	5 ± 0.4*	27 ± 1.2	4 ± 0.4*	35 ± 1.4	7 ± 0.4*	30 ± 0.9	5 ± 0.4*	37 ± 0.8	8 ± 0.4*
Reactive Yellow 15	27 ± 1.2	7 ± 0.4*	22 ± 1.1	6 ± 0.8*	25 ± 1.1	4 ± 0.3*	34 ± 2.1	6 ± 0.1*	27 ± 1.7	5 ± 0.5*	35 ± 1.8	8 ± 0.7*

*Significancy was determined using independent sample *t*-test (p-value < 0.05).

3.7. Microtoxicity study

Different biological assays, such as phytotoxicity studies on plant seeds (*Oryza sativa* or *Triticum aestivum*) conducted by measuring root and shoot lengths after exposure to dye and its related enzymatic treated solutions (Zhuo et al., 2011), the growth inhibition of microbial strains (for example, *Bacillus megaterium*, *E. coli*, and *Saccaromyces cerevisiae* (Mendes et al., 2011; Younes et al., 2012)), and cytotoxicity studies using mammalian cell lines (for example, human cervix cells [HeLa] (Younes et al., 2012)), have been developed in order to examine the toxicity of synthetic dyes and their metabolites. In the present study, the toxicity of the applied dyes and their related laccase-treated solutions was evaluated by measuring the growth inhibition of three gram-positive and three gram-negative bacterial strains. In general, the growth inhibition percent (GI%) in the presence of all treated dyes was significantly decreased compared to the dye solution (Table 2). The triazo dye of Direct Blue 71 was the most toxic dye, inhibiting *M. luteus*, *S. aureus*, *B. subtilis*, *E. coli*, *P. aeruginosa*, and *S. typhi* by 62%, 53%, 57%, 52%, 38%, and 48%, respectively, after a 10-h exposure to each bacterial strain. However, growth of the mentioned bacterial strains was significantly improved in the presence of laccase-treated Direct Blue 71 (Table 2). On the other hand, the anthraquinone dyes Disperse Blue 56 and Disperse Red 60 showed the lowest inhibition against tested bacterial species (Table 2). After a treatment of purified laccase from *Pleurotus* ostreatus, only 5% Remazol Brilliant Blue R toxicity toward *Bacillus cereus* remained (Palmieri et al., 2005). In the study of Mendes et al. (2011), in which the decolorization of 18 dyes using recombinant CotA-laccase from *B. subtilis* was evaluated, it was found that except for Acid Red 266, Acid Black 194, Acid Black 210, Direct Black 38, Reactive Black 5, Reactive Yellow 145, and Direct Red 80, toxicity (estimated by the rate of growth inhibition in *S. cerevisiae*) was reduced after treating of dyes by the purified laccase. The growth of *B. megaterium* and *E. coli* were enhanced from 2% to 99% and 11% to 94%, respectively, after the incubation of malachite green in the presence of purified laccase originated from *S. thermophilum* (Younes et al., 2012).

4. Conclusion

The purified laccase of *P. variabile*, recently studied for its capacity in the removal of aromatic compounds (e.g., chlorophenol pollutants, hazardous synthetic dyes, and pharmaceutical agents), was applied during the decolorization process of 13 azo- and anthraquinone-based dyes in the presence and absence of HBT as the laccase mediator. All the synthetic dyes were decolorized using non-assisted laccase. The application of the laccase-HBT system improved the decolorization of some synthetic dyes, but HBT (especially at high concentration) showed a negative effect on the decolorization percentages of other dyes. The obtained results of toxicity study using standard bacterial strain showed a significant decrease in the growth inhibition percentage of applied microbes (GI%) in presence of untreated and laccase-treated dyes.

Acknowledgment

This work was financially supported by a grant (No. 92-01-90-21957) from the Biotechnology Research Center, Tehran University of Medical Sciences, Tehran, Iran.

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