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Purification and Characterization of the Laccase Involved in Dye Decolorization by the White-Rot Fungus *Marasmius scorodonius*

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Marasmius scorodonius secretes an extracellular laccase in potato dextrose broth, and this enzyme was purified up to 206-fold using $(NH_4)_2SO_4$ precipitation and a Hi-trap Q Sepharose column. The molecular mass of the purified laccase was estimated to be ~67 kDa by SDS-PAGE. The UV/vis spectrum of the enzyme was nontypical for laccases, and metal content analysis revealed that the enzyme contains 1 mole of Fe and Zn and 2 moles of Cu per mole of protein. The optimal pH for the enzymatic activity was 3.4, 4.0, and 4.6 with 2,2′-azino-*bis*(3-ethylbenzothazoline-6-sulfonate) (ABTS), guaiacol, and 2,6-dimethoxy phenol as the substrate, respectively. The optimal temperature of the enzyme was 75°C with ABTS as the substrate. The enzyme was stable in the presence of some metal ions such as Ca^{2+}, Cu^{2+}, Ni^{2+}, Mg^{2+}, Mn^{2+}, Ba^{2+}, Co^{2+}, and Zn^{2+} at a low concentration (1 mM), whereas Fe^{2+} completely inhibited the enzymatic activity. The enzymatic reaction was strongly inhibited by metal chelators and thiol compounds except for EDTA. This enzyme directly decolorized Congo red, Malachite green, Crystal violet, and Methylene green dyes at various decolorization rates of 63–90%. In the presence of 1-hydroxybenzotriazole as a redox mediator, the decolorization of Reactive orange 16 and Remazol brilliant blue R was also achieved.

Keywords: Laccase, *Marasmius scorodonius*, synthetic dyes, decolorization

Introduction

Laccases (benzenediol: oxygen oxidoreductase; E.C. 1.10.3.2) belong to the family of multicopper oxidases that catalyze the oxidation of a great variety of phenolic compounds and aromatic amines with the concomitant reduction of dioxygen to water [1]. In the presence of appropriate mediators such as 2,2′-azino-*bis*(3-ethylbenzothazoline-6-sulfonate) (ABTS) or 1-hydroxybenzotriazole (HBT), the substrate range of laccases can be extended to nonphenolic compounds, polycyclic aromatic hydrocarbons, and dye pollutants [2]. Owing to their broad substrate specificity, laccases have attracted considerable interest in terms of applications to many fields, such as the pulp and paper industry [3] and food production [4]. Moreover, laccase is a useful enzyme because it has a high potential for environmental detoxification and because some fungal laccases have been reported to perform dye decolorization [5, 6].

Synthetic dyes are widely used in several industries, including textiles, food processing, paper printing, cosmetics, and pharmaceuticals. It is estimated that 10–15% of the dyes are lost in the effluent during the dyeing process [7]. Dye-containing effluents cause serious environmental problems, and they are poorly decolorized by the conventional wastewater treatments such as activated sludge and trickling filter [8]. To overcome this problem, white-rot fungi and their ligninolytic enzymes, especially laccases that can degrade synthetic dyes, are being studied for their potential application in textile effluent treatments [9]. In recent years, white-rot fungi such as *Pleurotus ostreatus* [9], *Rigidoporus lignosus* Wl [10], *Trametes trogii*, *T. villosa*, and *Coriolus versicolor* [7] were shown to have ability to decolorize different synthetic dyes. Nevertheless, there is a demand to find new strains of white-rot fungi that can degrade recalcitrant aromatic compounds, including dyes present in industrial effluents that cause environmental problems.

The basidiomycetous fungus *Marasmius scorodonius* ("garlic mushroom") is a small edible species that grows on wood and further lignified plant materials [11]. In a previous study, we reported optimal conditions for the production of a laccase, a polyphenol oxidase involved in lignin degradation, from *M. scorodonius* [12]. In the present study, we investigated the purification and characterization of the laccase from the white-rot fungus *M. scorodonius* (MsLAC) as well as its properties related to the decolorization of some synthetic dyes.

Materials and Methods

Fungal Strain, Media, and Culture Conditions

The *M. scorodonius* strain (NO. 42740) was obtained from the Korean Agricultural Culture Collection (KACC). The fungus was grown on a potato dextrose agar plate at 25ºC for 7 days, and maintained at 4ºC. In order to produce laccase, the fungus was inoculated into a medium supplemented with 1% galactose, 0.4% yeast extract, and 0.05% Tween 80, and cultured at 25ºC for 15 days on a rotary shaker, as described previously [12]. The resulting culture supernatant was then used as the source of the enzyme to carry out the purification and characterization in this study.

Protein Purification

The culture fluid was filtered through a Whatman No. 1 filter paper and centrifuged at 10,000 ×*g* for 10 min. Ammonium sulfate was added to the supernatant to give 80% saturation, and the precipitated proteins were collected by centrifugation at 10,000 ×*g* for 20 min. The precipitate was then dissolved in an appropriate volume of a 50 mM sodium acetate buffer (pH 6.0), dialyzed against the same buffer for 24 h, and concentrated by ultrafiltration using an YM-10 membrane (Amicon, USA). The concentrated proteins were applied to a HiTrap Q HP column (GE Healthcare, USA) and washed with at least 10 column volumes of a 50 mM sodium acetate buffer (pH 6.0), and the bound proteins were eluted at a flow rate of 2 ml/min with a linear gradient of 0 to 0.5 M NaCl in the same buffer using ÄKTAprime (GE Healthcare). The fractions containing laccase activity were pooled and concentrated by ultrafiltration using an YM-10 membrane. All processes of protein collection and purification were performed at 25ºC. Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on 10% (w/v) gels and visualized using Coomassie brilliant blue staining. PAGE under non-denaturing conditions at pH 7.5 was performed in 10% (w/v) gels, and the gels were stained for laccase activity by using ABTS in sodium acetate buffer at pH 4.0 [13].

UV-Visible Spectra and ICP-MS Assay

The purified protein (0.63 mg/ml) was dialyzed against metal ion absence buffer (50 mM sodium acetate buffer, pH 6.0). The UV-visible absorption spectra of the dialyzed protein were obtained at room temperature using a UV-Vis spectrophotometer (Optizen 2120UV; Mecasys, Korea). The metal ion content of the enzyme was determined by dilution of 50 μg of enzyme with 5 ml of 0.5% (v/v) HNO_3 to digest the protein and release the metal ions, and the solutions were analyzed by inductively coupled plasma mass spectrometry (ICP-MS) (model 7700; Agilent, Japan) by the Korea Basic Science Institute (KBSI, Korea).

N-Terminal Amino-Acid Sequence Analysis

After SDS-PAGE, the purified protein on the gel was transferred to a polyvinylidene difluoride (Bio-Rad, USA) membrane by electroblotting and stained with Coomassie brilliant blue R-250. The stained band was excised and analyzed by the automatic Edman degradation method using a Procise 491 capillary protein sequencer (Applied Biosystems, USA).

Enzyme Assay

Laccase activity was assayed by measuring the oxidation of ABTS. Formation of the cation radical was monitored at 420 nm (ε_{max} = 36,000 $M^{-1} \cdot cm^{-1}$). An aliquot of enzyme solution was incubated in 1 ml of 50 mM sodium acetate buffer (pH 3.4) containing 1 mM ABTS at 70ºC. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 mM of substrate per minute under described conditions.

Temperature and pH Optimum

The temperature dependence of the activity was determined in 50 mM sodium acetate buffer (pH 3.4) at temperatures ranging from 40ºC to 95ºC using ABTS as a substrate. The optimum pH for its reaction was estimated by monitoring activity with ABTS, guaiacol, or 2,6-dimethoxy phenol (2,6-DMP) as a substrate at pHs of 1.0–9.0 using the following buffers: 50 mM HCl-KCl buffer for pH 1.0–1.8; 50 mM glycine-HCl buffer for 2.2–3.0; 50 mM sodium acetate buffer for pH 3.4–5.4; 50 mM sodium phosphate for 6.0–7.0; and 50 mM Tris-HCl buffer for pH 7.5–9.0.

Effects of Inhibitors and Metal Ions on Enzyme Activity

Effects of potential inhibitors on laccase activity were determined with 1 mM ABTS as the substrate in 50 mM sodium acetate buffer (pH 3.4) and the presence of an inhibitor. The effects of 2-mercaptoethanol, L-cysteine, dithiothreitol, sodium azide, tropolone, *p*-coumaric acid, SDS, and ethylenediaminetetraacetic acid (EDTA) on its activity were determined after 10 min of reaction of the enzyme with the various inhibitors at 70ºC. After pre-incubating the enzyme solutions containing each metal ion in 50 mM sodium acetate buffer (pH 3.4) at 25ºC for 30 min, substrate ABTS (1 mM) was added, and the enzyme activity was measured as described above under standard conditions. The ions tested were 1 and 10 mM of $CaCl_2$, $CuCl_2$, $NiCl_2$, $MgCl_2$, $MnCl_2$, $BaCl_2$, $CoCl_2$, $ZnCl_2$, and $FeCl_2$.

Kinetic Calculations

Rates of substrate oxidation were examined by spectrophotometry using the molar extinction coefficients of various substrates, which were determined in 50 mM sodium acetate buffer (pH 4.0) at 70°C. The initial substrate concentration was 0.01 mM in all cases. For the kinetic analyses, various concentrations of ABTS (0.01–2.0 mM), 2,6-DMP (0.01–2.0 mM), and guaiacol (0.01–5.0 mM) were used. The K_m value was determined measuring the initial velocity, and the apparent k_{cat} was determined from k_{cat} $t = V_{max}$/[enzyme]. All kinetic studies were performed in triplicates and the kinetic data were calculated according to the procedure of Michaelis–Menten by the EZ-Fit program [14].

Decolorization of Synthetic Dyes

Malachite green, Crystal violet, Congo red, Methylene green, Reactive orange 16, Remazol brilliant blue R, Eriochrome black T, Indigo carmine, Methyl red, Nile blue, Methylene orange, Rhodamine B, and Trypan blue at a final concentration of 200 mg/l were solubilized in water, and then membrane-filtered through a 0.45 µm cellulose nitrate filter. The reaction mixture contained 50 mM sodium acetate buffer (pH 4.0), dye, and laccase (8 U/ml). The reaction was initiated with enzyme addition and incubated at 70°C. Reaction mixtures containing dyes, without the enzyme, were used as controls. The dyes partially or not decolorized by laccase were tested in the presence of 1 mM HBT, a common laccase mediator, to increase the oxidative effect of the enzyme. Samples were withdrawn at 0.5–1 h intervals and subsequently analyzed. Decolorization was determined spectrophotometrically by measuring the decrease in the absorbance at maximum wavelength for each dye. Decolorization was evaluated as follows: Decolorization (%) = [(initial absorbance) – (final absorbance)]/(initial absorbance) × 100.

Results and Discussion

Purification and Characterization of the Extracellular Laccase

The purification steps and enzyme yields are summarized in Table 1. The purified enzyme showed a single band after PAGE under non-denaturing conditions when the gel was stained using the activity of the enzyme with ABTS as the substrate (Fig. 1A). Under denaturing conditions, we observed a major protein band with a molecular mass of ~67 kDa, which is consistent with that of most fungal

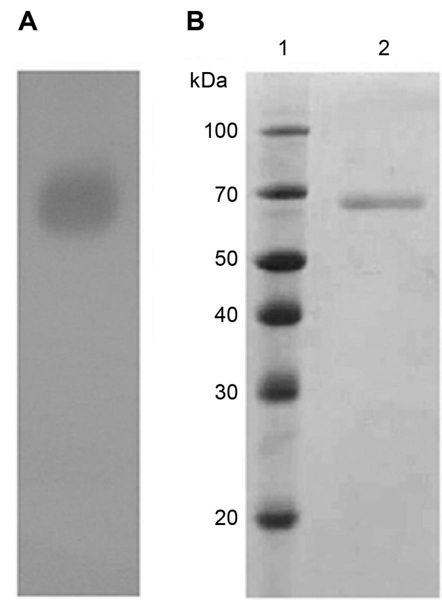

Fig. 1. SDS and native PAGE of the purified *M. scorodonius* laccase.
(**A**) Activity staining with ABTS after PAGE under non-denaturing conditions; (**B**) SDS-PAGE of the purified enzyme after Coomassie brilliant blue staining. Lane 1, molecular mass marker; lane 2, the purified laccase.

laccases (50–90 kDa) [15]. The N-terminal amino acid sequence of the purified protein was found to be AIGPVADLVI, which is the same as those of laccases from *Marasmius* sp. [12] and *Moniliophthora perniciosa* FA553 [16]. However, the purified enzyme is distinct from laccases isolated from *Marasmius* sp. (53 kDa) and *M. perniciosa* FA553 (57 kDa) with respect to molecular mass [12, 16]. Thus, this enzyme (MsLAC) was identified as a laccase and classified as a new member of the multicopper oxidase family.

To determine the state of its catalytic center, the purified enzyme was characterized spectroscopically. The 610 nm peak, which indicates the presence of a type 1 Cu(II), was absent in the UV-visible spectrum of the purified enzyme, whereas a broad peak at 400–500 nm was present (Fig. 2) [17]. In this spectrum, the presence of a shoulder at 330 nm

Table 1. Purification of extracellular laccase from *M. scorodonius.*

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification factor (fold)	Yield (%)
Culture filtrate	2,000	1,969	4,833	2.1	1	100
$(NH_4)_2SO_4$ precipitation	34	127	802	6.3	3	17
HiTrap Q	17	14	258	432.8	206	5

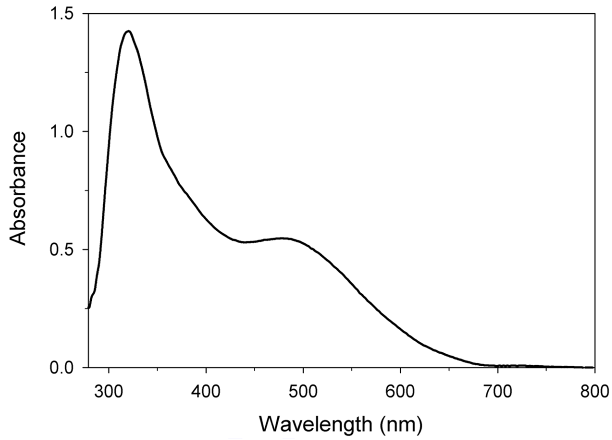

Fig. 2. UV-visible spectrum of purified *M. scorodonius* laccase (9.4 μM) in 50 mM sodium acetate buffer (pH 6.0).

could indicate a type 3 binuclear Cu(II) pair [18]. The amounts of Cu, Mn, Fe, Zn, Ni, and Co in the purified enzyme were measured by ICP-MS. The enzyme contains 1 mole of Fe and Zn each and 2 moles of Cu per mole of the protein. Mn, Co, and Ni were not detected. This result indicates the MsLAC belongs to the nonblue laccase family. Nonblue laccases containing two Cu atoms [19–22] or only one Cu atom [23] were reported earlier. In these nonblue laccases, the typical maximum of blue laccases in the UV-visible spectra at 610 nm was absent, whereas a broad band near 400 nm was detected [23]. Besides the single Cu atom, these nonblue laccases also contain two Zn atoms and one Fe or one Mn atom [23, 24] or two Fe and one Zn atom [25]. These additional non-Cu metal atoms might form a four-site reaction center comparable to that of blue laccases [26].

Effects of pH and Temperature on Laccase Activity and Thermostability

Laccases have different pH optima, which are substrate dependent [27]. The pH dependence of the activities of laccases toward phenolic and nonphenolic substrates was evaluated next. The optimal pH for the oxidation of the phenolic substrates guaiacol and 2,6-DMP was 4.0 and 4.6, respectively (Fig. 3A). Generally, the pH dependence of the activity of the majority of fungal laccases toward phenolic substrates is narrow bell-shaped, with the optimal pH near 5 [15]. The optimal pH for the oxidation of nonphenolic substrate ABTS was found to be 3.4, similar to that of the majority of fungal laccases (Fig. 3A) [15]. The temperature profiles of a laccase usually range between 50°C and 70°C [15]. The optimal temperature for ABTS oxidation by the MsLAC was 75°C, which is similar to that of laccases from

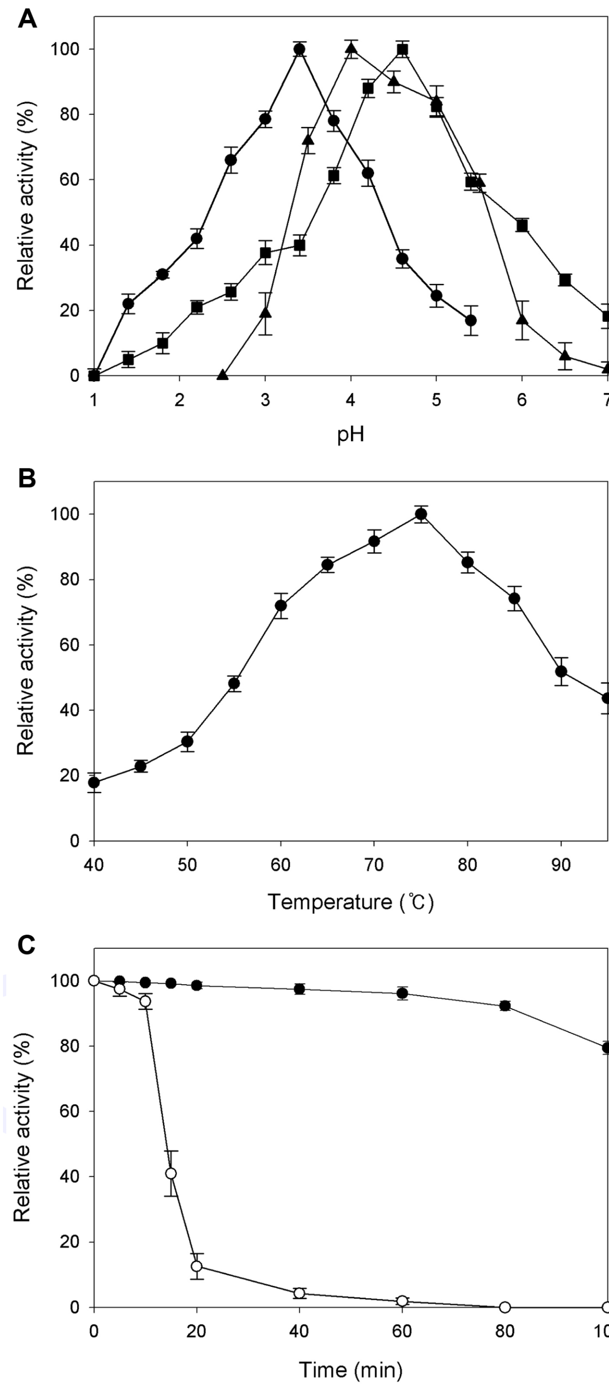

Fig. 3. Effects of pH and temperature on the activity and thermostability of *M. scorodonius* laccase.

(**A**) pH dependence of activity. ●, ABTS; ▲, guaiacol; ■, 2,6-DMP. (**B**) Temperature dependence of activity. (**C**) Thermostability of the enzyme. Laccase (0.2 μM) was incubated for various lengths of time at 70°C (●) and 75°C (○), and the residual activity of the samples was measured at 70°C and pH 3.4 with ABTS as a substrate. The data presented are the average values from triplicate repeats of measurements.

Marasmius quercophilus (75°C and 80°C) and higher than that of the majority of fungal laccases (Fig. 3B) [15]. Heat inactivation of the enzyme was estimated by measuring the residual laccase activity after heat treatment at two temperatures. The enzyme still showed 80% of its activity after incubation for 100 min at 70°C, while it hardly retained any activity after heating for 40 min at 75°C (Fig. 3C). From the result, the decrease of thermal stability at 75°C was supposed to be caused by the denaturation of proteins after heating for 15 min. These results indicate that the thermostability of MsLAC gives it a high potential for industrial applications [15].

Effects of Metal Ions and Inhibitors on the Laccase Activity

Heavy metals in general are potent inhibitors of enzymatic reactions. The effects of heavy metals and other salts on laccase activity were tested with individual metal salts at two concentrations (1 and 10 mM) as shown in Table 2. It was found that for the concentration of 1 mM, the laccase activity was resistant to all the metal ions tested except for Fe^{2+}. When the concentration was increased to 10 mM, laccase activity was decreased by all the metal ions tested. The enzymatic activity was completely inhibited in the presence of 1 or 10 mM Fe^{2+}. It was reported that the purified laccases from *Ganoderma lucidum* [28] and *T. trogii* [29] are highly sensitive to Fe^{2+}. This effect may be due to an interaction of Fe^{2+} with the electron transport system of the laccase [28].

The sensitivity of the enzyme to laccase inhibitors is shown in Table 3. The metal chelators tested, NaN_3, tropolone, and *p*-coumaric acid, had strong inhibitory effects, whereas EDTA showed no inhibition of the enzymatic reaction. Likewise, it has been observed that EDTA is not an efficient inhibitor of laccases from *T. trogii* and *P. ostreatus* [30, 31]. This result was explained by reversible removal of type 2 Cu from the protein by the chelating agent EDTA [32]. In the presence of thiol compounds, L-cysteine, dithiothreitol, or 2-mercaptoethanol, the activity of the enzyme was also strongly inhibited. Inhibition by thiol compounds has been reported for several laccases [33] and is presumed to be the result of coordination of the thiol by the Cu atoms in the enzyme's active site [34].

Table 2. Effect of metal ions on *M. scorodonius* laccase activity.

Metal ions	Relative laccase activity (%)	
	1 mM	10 mM
None	100	100
Ca^{2+}	101.3	19.8
Cu^{2+}	106.7	39.2
Ni^{2+}	107.1	32.2
Mg^{2+}	100	34.2
Mn^{2+}	100	28.1
Ba^{2+}	95.6	41.3
Co^{2+}	102	44.9
Zn^{2+}	99.7	11.7
Fe^{2+}	ND	ND

Enzyme assays were performed in triplicates. The averages of triplicate activity measurements, varied <5%, were used for the calculation. ND: not detected.

Table 3. Effect of inhibitors on *M. scorodonius* laccase activity.

Compound	Concentration (mM)	Inhibition (%)
2-Mercaptoethanol	1	97
	10	100
L-Cysteine	1	100
	10	100
Dithiothreitol	1	100
	10	100
NaN_3	1	98
	10	100
Tropolone	1	78
	10	93
p-Coumaric acid	1	48
	10	87
SDS	1	43
	10	98
EDTA	1	0
	10	6

Enzyme assays were performed in triplicates. The averages of triplicate activity measurements, varied <5%, were used for the calculation.

Substrate Specificity

To study the kinetic parameters of the enzymatic activity of MsLAC, the initial reaction rates at various substrate concentrations were determined, and kinetic parameters of different substrates identified for MsLAC are summarized

Table 4. Kinetic parameters of purified *M. scorodonius* laccase.

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\cdot s^{-1}$)
ABTS	27 ± 6	72 ± 2	2,609 ± 333
2,6-DMP	602 ± 165	68 ± 7	113 ± 42
Guaiacol	2,772 ± 706	226 ± 30	81 ± 42

Data represent the mean (±SE) of duplicate measurements.
ABTS: 2,2′-azino-*bis*(3-ethylbenzothazoline-6-sulfonate); 2,6-DMP: 2,6-dimethoxy phenol.

in Table 4. MsLAC oxidized phenolic substrates such as 2,6-DMP and guaiacol as well as nonphenolic substances such as ABTS (Table 4). Laccases generally have high affinity for ABTS with a high catalytic constant, whereas the oxidation of 2,6-DMP and guaiacol was considerably slower and the respective K_m constants were higher [15]. The MsLAC showed the highest affinity (27 µM) and catalytic efficiency (2,609 $mM^{-1}{\cdot}s^{-1}$) for ABTS. In contrast, the K_m values for guaiacol (2,772 µM) and 2,6-DMP (602 µM) were noticeably higher than that of ABTS. The catalytic efficiency (k_{cat}/K_m) toward 2,6-DMP was lower than that of the thermostable fungal laccase from *Trametes hirsuta* (250 $mM^{-1}{\cdot}s^{-1}$) [35] and was higher than that of the thermostable bacterial laccase from *Bacillus pumilus* (16.17 $mM^{-1}{\cdot}s^{1}$) [36]. As in the case of typical laccases, the MsLAC did not oxidize L-tyrosine and veratryl alcohol, which are standard substrates for tyrosinase and arylalcohol oxidase, respectively.

Enzymatic Decolorization of Dyes

The enzymatic decolorization of synthetic organic dyes representative of the most widely used industrial dyes (for detail see "Materials and Methods") was evaluated by using the purified enzyme alone or in the presence of redox mediators during incubation for 0 to 8 h at 70°C. Table 5 shows that this laccase has different effects on different dyes. Indeed, it can decolorize Congo red (90%), Malachite green (82%), Crystal violet (69%), and Methylene green (63%) without the addition of an expensive mediator. MsLAC may be a suitable enzyme for Congo red and Malachite green decolorization. In comparison with other studies, the laccase isolated from *Trametes* sp. SQ01 was found to decolorize 45% of Congo red (100 mg/l) and 100% of Malachite green (100 mg/l) after incubation for 24 h [37]. The potential of a laccase for decolorization of a synthetic dye is determined by the structure of the enzyme in question and its specificity and activity [38].

A recent study showed decolorization of dyes Neolane yellow and Maxillon blue with a laccase-mediator system by the laccase from *T. trogii* [39]. In the present report, HBT was chosen as a typical redox mediator for studying decolorization of various dyes by the purified MsLAC. The addition of HBT clearly increased the decolorization of Remazol brilliant blue R and Reactive orange 16, which were decolorized up to 61% and 48%, respectively (Table 5). The use of HBT as a laccase mediator increased the range of dyes degraded by MsLAC, and this finding is similar to the results of other reports [39–41]. In contrast to the above-mentioned dyes, the decolorization of Eriochrome black T, Indigo carmine, Methyl red, Nile blue, Methylene orange, Rhodamine B, and Trypan blue was not improved by the laccase and HBT (data not shown). This result could be caused by various factors, such as reaction conditions, the type of dye, and the structure and redox potential of the mediator. Thus, we continue our studies to improve the decolorization of dyes by MsLAC using various mediators.

Table 5. Decolorization of different synthetic dyes by *M. scorodonius* laccase in the presence and absence of 1-hydroxybenzotriazole (HBT).

Dyes	Decolorization without HBT (%)	Decolorization with HBT (%)
Malachite green	82	ND
Crystal violet	69	ND
Congo red	90	ND
Methylene green	63	ND
Reactive orange 16[a]	0	48
Remazol brilliant blue R[a]	0	61

ND: not determined.

[a]Dyes that were not decolorized were treated with enzyme in the presence of HBT.

This is the first report of the white-rot fungus *M. scorodonius* as a producer of a thermostable and acidophilic laccase that can efficiently decolorize several synthetic dyes without a redox mediator. This study is useful for understanding the precise use of *M. scorodonius* laccase in industrial bleaching processes and for decolorization of effluents containing textile dyes without the addition of an expensive mediator.

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