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Evaluation of *Cerrena unicolor* BCC 300 and their Enzymes for Decolorization of Synthetic Dyes

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A B S T R A C T

The aim of this study was to evaluate the capability of few WRB to produce lignin-modifying enzymes in the presence of dyes and to establish parameters providing maximum and fast decolorization of selected dyes by crude laccase. In the submerged cultivation in the glycerol-based medium supplemented with 0.3 mM synthetic dyes, *Cerrena unicolor* BCC300, Coriolopsis gallica BCC1184, and *Trametes versicolor* BCC13 produced 69.3-88.4 U/ml, 54.3-59.0 U/ml, and 1.7-14.3 U/ml laccase, respectively, and demonstrated a high decolorization potential of Amaranth, Remazol Brilliant Blue R, and Indigo carmine. Involvement of laccase in these dyes decolorization was assessed and proved using the crude laccase obtained from the *C. unicolor* BCC300 culture liquid. Against Amaranth, the *C. unicolor* BCC300 laccase was most effective at pH 6-7, toward Indigo carmine at pH 6, whereas the decolorization of RBBR occurred at a maximum rate at pH 5. The decolorization activity by the enzyme decreased with increasing dye concentration. Nevertheless, it completely decolorized 0.08% RBBR and Indigo carmine after 24 h incubation. Amaranth was more resistant to the laccase action and only 55% of dye was decolorized after 24 h incubation. Concentration of laccase of 1 U/ml was sufficient to completely decolorize RBBR and Indigo carmine. However, increasing the enzyme concentration to 3 and 10 U/ml enhanced the rate of decolorization. Overall, the isolated from *C. unicolor* BCC300 crude laccase showed catalytic properties required for their biotechnological applications.

Keywords: Basidiomycetes, Laccases, Synthetic Dyes, Decolorization, Enzyme, C.unicolor.

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Introduction

In recent years, comprehensive and intensive studies have been carried out to detect easily available laccase sources with high catalytic efficiency, broad substrate specificity, and resistance to various environmental parameters for their biotechnological application, in particular, in bioremediation processes.

Due to low cost and color stability, synthetic dyes are used in many industrial process. However, during processing in textile industry, 10-15% of the used dyestuffs are lost in the industrial effluents leading to considerable environmental pollution [1]. Most of these dyes are health-risk factors with mutagenic and carcinogenic effect and cannot be completely removed by conventional wastewater treatment systems. Even low concentrations of dyes in aqueous ecosystems are highly visible and undesirable, reduce sunlight penetration, causing inhibitory effect on photosynthesis, and toxic aromatic amines can be generated, if dyes are broken anaerobically [2-5]. Application of different available physical or chemical methods for dye removal from wastewaters is limited due to expensive operational costs, formation of hazardous by-products, and intensive energy requirement [6]. As an alternative, biological processes have received increasing interest since they can offer a low-cost and environmentally friendly solution [6-8]. Various eukaryotic and prokaryotic microorganisms have been used for decolonization and degradation of synthetic dyes. Among them, most efficient in breaking down synthetic dyes are the white-rot basidiomycetes (WRB) which are capable of mineralizing a diverse range of persistent organic pollutants [6, 9]. This ability of

WRB to oxidize a wide variety of organic pollutants, including synthetic dyes, is due to an extracellular enzymatic system consisting of lignin peroxidases, manganese peroxidases, and laccases [1, 6, 10, 11] However, the cost of enzymes is still too high and there is need in new and potent enzyme sources and cheap production technologies. Moreover, fungal strain capable of growing in wide range of pH and temperature conditions and resisting the toxicity of the dyes at high concentrations should be chosen.

The objectives of this study were to evaluate the capability of few WRB to produce lignin-modifying enzymes (LME) in the presence of dyes and to establish parameters providing maximum and fast decolorization of selected dyes by crude laccase.

Materials and Methods
Organisms and inoculum preparation

The following WRB from the basidiomycete's culture collection of the Agricultural University of Georgia have been used in this study: *Cerrena unicolor* BCC300, *Coriolopsis gallica* BCC1184, *Trametes versicolor* BCC13. The fungal inocula were prepared by growing their mycelia taken from agar slants on a rotary shaker at 27 °C and 150 rpm in 250-ml flasks containing 100 ml of the medium (g/l): glucose, 10; NH_4NO_3, 2; KH_2PO_4, 1; $MgSO_4{\cdot}7H_2O$, 0.5; yeast extract, 2. After 7 days of cultivation the fungal biomass was homogenized in a Waring laboratory blender.

Cultivation conditions

The submerged cultivation of fungus was conducted in the Innova 44 shaker (New Brunswick Scientific, USA) at 27 °C and 150 rpm. The homogenized mycelium (5 ml) was used to inoculate the 250-ml flasks containing 50 ml of the medium (g/l): glycerol, 15; ammonium tartrate, 2; KH_2PO_4, 1; yeast extract, 3; $CuSO_4{\cdot}5H_2O$, 0.02; $MnSO_4$, 0.01; pH 5.8. The nutrient medium was supplemented with synthetic dyes (Amaranth, Indigo carmine, Remazol Brilliant Blue R) at the concentration of 0.3 mM. At predetermined time intervals, 1 mL of culture was sampled and solids were separated by centrifugation (Eppendorf 5417R, Hamburg, Germany) at 10,000 *g* for 5 min at 4 °C. The supernatants were analyzed for pH, dyes decolorization, and enzyme activities.

All experiments were performed twice using three replicates. All results were expressed as the mean ± SD with only $p \leq 0.05$ considered as statistically significant.

Analytical methods

The fungal biomasses were measured gravimetrically after recovering mycelium with centrifugation of whole cultures at 8000 *g* for 20 min and oven-drying at 60 °C for 24 h. Microbial decolorization of the synthetic dyes was evaluated in each taken samples by measuring the decrease in absorbance of culture liquids containing Amaranth (523 nm), RBBR (595 nm), and Indigo carmine (610 nm).

The laccase activity was determined spectrophotometrically (Camspec M501, UK) at 420 nm as the rate of 0.25 mM ABTS (2,2′-azino-bis-[3-ethyltiazoline-6-sulfonate]) oxidation in 50 mM Na-acetate buffer (pH 3.8) at room temperature [12]. MnP activity was measured at 610 nm by following by oxidation of Phenol Red [13] in the presence of 0.1 mM H2O2. One unit of laccase or MnP activity was defined as the amount of enzyme that oxidized 1 μmol of substrate per minute.

Enzymatic dyes decolorization

The decolorization experiments were performed using concentrated laccase preparation (400 U/ml) obtained by precipitation with ammonium sulfate from *C. unicolor* culture liquid. The enzymatic dyes decolorization was initiated by adding of 0.5 ml of the adequately diluted enzyme to 9.5 ml dye solution prepared by dissolving of each synthetic dye at a required concentration in 0.05 M citrate-phosphate buffer. The reaction mixture was incubated in 50 ml flasks at 27 °C and 150 rpm for 24 h. During incubation, 1 ml samples were taken after 0 h, 1 h, 2 h, 4 h, and 24 h and the solutions absorbance was measured at the λ_{max} of each dye. All experiments were performed twice using two replicates, the results were expressed as the mean and decolorization percent was calculated.

Results and discussion
Microbial decolorization of synthetic dyes

To elucidate the capability of the selected basidiomycetes strains to grow and produce lignin-modifying enzymes in the presence of three synthetic dyes their cultivation was performed in the submerged conditions in the glycerol containing medium. The fungi grew well in all media in the form of pellets. Slight delay in the fungal biomass accumulation was visually observed during the first three days' cultivation in the presence of 0.3 mM dyes and the maximum values of dry biomass yields in the fungi cultivation in the presence of several dyes appeared to be rather lower as compared with those in the control media (Table 1). It is interesting that the *C. gallica* 1184 growth accompanied with increase of the media pH while in the cultivation of T. versicolor 13 the media pH decreased.

Table 1. *Effect of dyes on the basidiomycetes growth and enzyme activity in the submerged cultivation in the glycerol containing medium*

Dyes	Final pH	Biomass (mg/ml)	Laccase (U/ml)	MnP (U/ml)	Decolorization %
			C. unicolor BCC300		
Control	6.0 ± 0.2	6.2 ± 0.1	54.0 ± 4.8^{12}	0.26 ± 0.04^{10}	
Amaranth	5.5 ± 0.1	6.0 ± 0.2	69.3 ± 7.8^{10}	0.68 ± 0.09^{12}	100^{5}
RBBR	5.9 ± 0.2	7.0 ± 0.1	88.4 ± 14.2^{12}	1.18 ± 0.15^{7}	100^{5}
Indigo	5.9 ± 0.2	6.3 ± 0.1	74.2 ± 10.7^{7}	0.36 ± 0.05^{7}	100^{3}
			C. gallica BCC1184		
Control	6.5 ± 0.1	6.3 ± 0.3	40.2 ± 6.9^{10}	0.17 ± 0.02^{10}	
Amaranth	7.1 ± 0.1	5.6 ± 0.2	54.3 ± 8.7^{10}	0.24 ± 0.04^{7}	100^{5}
RBBR	7.4 ± 0.2	5.9 ± 0.2	59.0 ± 10.2^{10}	0.41 ± 0.07^{10}	100^{5}
Indigo	7.2 ± 0.2	5.6 ± 0.2	54.9 ± 7.7^{7}	0.33 ± 0.05^{7}	100^{3}
			T. versicolor BCC13		
Control	5.5 ± 0.1	5.1 ± 0.1	3.2 ± 0.4^{10}	0.07 ± 0.01^{10}	
Amaranth	5.5 ± 0.3	5.3 ± 0.2	9.0 ± 0.5^{15}	0.30 ± 0.01^{15}	100^{5}
RBBR	5.8 ± 0.1	4.7 ± 0.2	14.3 ± 0.3^{5}	0.39 ± 0.03^{15}	100^{5}
Indigo	5.7 ± 0.1	4.4 ± 0.1	1.7 ± 0.2^{5}	0.08 ± 0.01^{15}	100^{3}

The measurement of the laccase activity revealed that all dyes promoted this enzyme secretion by *C. unicolor* 300. Among them, RBBR followed by Indigo increased laccase activity by 64% and 34%, respectively, as compared with the control (Table 1). Analogically, 1.5-fold increase in laccase activity was detected when *C. gallica* 1184 was grown in the presence of RBBR and more than 30% increase of laccase activity was observed in the presence of other dyes. Another picture was revealed in the cultivation of *T. versicolor* 13. Specifically, RBBR caused almost 5-fold increase of laccase activity of this fungus while Amaranth promoted 3-fold increase of this enzyme activity as compared with the control medium. Taken into account that the specific laccase activity *T. versicolor* 13 in the control medium and media with RBBR and Amaranth are equal 0.63 U/mg biomass, 3.04 U/mg biomass, and 1.70 U/mg biomass, respectively, one may conclude that these dyes induced laccase production with the induction ratio of 4.8 and 2.7, respectively. By contrast, Indigo carmine not only delayed the biomass accumulation of *T. versicolor* 13 but also decreased laccase activity of this strain.

RBBR and Amaranth are very appropriate elicitors of MnP synthesis. Especially, RBBR caused 4.5-fold and 5-fold increase of this enzyme activity of *C. unicolor* 300 and *T. versicolor* 13, respectively, as compared with their control media (Table 1). Other dyes, with the exclusion of Indigo in the cultivation of *T. versicolor* 13, also favored MnP secretion by the tested fungi. Undoubtedly, high enzyme activity accumulated during the submerged cultivation of the fungi provided efficient and complete decolorization of all dyes during maximum 5 days. It means that even at the dyes concentration as high as 0.3 mM, *C. unicolor* 300, *C. gallica* 1184, and *T. versicolor* 13 are capable to well grow and produce high activity of LME and they are promising strains for their application in bioremediation of textile industry effluents.

Effect of Initial Dye Concentration on *C. unicolor* BCC300 Growth and Enzyme Activity

Subsequently, the capability of *C. unicolor* BCC300 to withstand higher concentrations of two dyes and to produce the target enzyme activities was studied varying the Amaranth and RBBR concentrations from 0 to 1 mM. The data received evidence that in the submerged cultivation in the presence of Amaranth, the fungus formed the same biomass yields as in the control medium (Table 2). Lower concentration of RBBR even favored the fungal biomass accumulation. However, the highest concentration of RBBR significantly delayed the fungus growth and decreased the biomass yield by 31%.

Table 2. *Effect of dyes concentration on the C. unicolor BCC300 growth and enzyme activity*

Dyes	mM	Final pH	Biomass gain, (mg/ml)	Laccase, (U/ml)	MnP_{610}, (U/ml)	Decolorization, (%)
Control	0	6.0 ± 0.1	6.2 ± 0.1	54.0 ± 0.8^{12}	0.26 ± 0.05^{10}	
Amaranth	0.1	5.9 ± 0.1	6.0 ± 0.2	64.4 ± 0.7^{10}	0.52 ± 0.06^{12}	100^{5}
	0.3	5.5 ± 0.1	6.0 ± 0.2	69.3 ± 0.8^{10}	0.68 ± 0.12^{12}	100^{5}
	1.0	5.9 ± 0.2	6.1 ± 0.1	76.1 ± 1.1^{12}	1.24 ± 0.3^{12}	100^{8}
RBBR	0.1	5.7 ± 0.1	7.2 ± 0.1	85.9 ± 1.2^{12}	0.53 ± 0.06^{5}	100^{5}
	0.3	5.8 ± 0.2	7.0 ± 0.1	88.7 ± 1.1^{12}	1.18 ± 0.3^{7}	100^{5}
	1.0	5.6 ± 0.2	4.3 ± 0.1	94.5 ± 1.3^{12}	1.64 ± 0.2^{10}	100^{8}

C. unicolor BCC300 was found to produce both laccase and MnP activity in all the tested media (Table 2). However, the values of enzyme activity depended on the dyes concentration. In particular, the higher was dye concentration the higher was enzyme activity. However, laccase activity increase was not so remarkable as compared with that of MnP activity. Thus, the laccase activity in the presence of Amaranth increased by 19-41% while in the presence of RBBR by 59-75%. However, in the same cultivation conditions MnP activity increased 2-4.8-fold in the presence of Amaranth and 2-6.3-fold in the presence of RBBR.

The measurement of culture liquids absorbance during the submerged cultivation of *C. unicolor* BCC300 revealed an efficient decolorization of both synthetic dyes. It is worth noting that a complete decolorization of 1 mM Amaranth and RBBR needed a longer time and it was achieved after 8 days of the fungus cultivation in the synthetic glycerol-containing medium.

Enzymatic decolorization of synthetic dyes

The tested fungi, including the best enzyme producer *C. unicolor* BCC300, demonstrated a high decolorization potential of three synthetic dyes. It was necessary to prove the involvement and role in this process of fungal extracellular enzymatic system. Therefore, the dyes decolorization was assessed using the crude laccase obtained from the *C. unicolor* BCC300 culture liquid.

Taking into account that laccase catalytic activity depends on the reaction mixture pH and on affinity towards the substrate, in the first set of experiments, the effect of pH on the selected dyes decolorization was studied using laccase at a final concentration of 1 U ml^{-1}. The reaction mixtures contained 0.04% of dyes (final concentrations). Data represented in Fig. 1 show that the dyes decolorization took place at all tested pH. However, the highest rate of Amaranth decolorization was observed at pH 6-7. Dye' treatment with laccase at this pH caused absorbance decrease by 57-63% after 24 h incubation of reaction mixture.

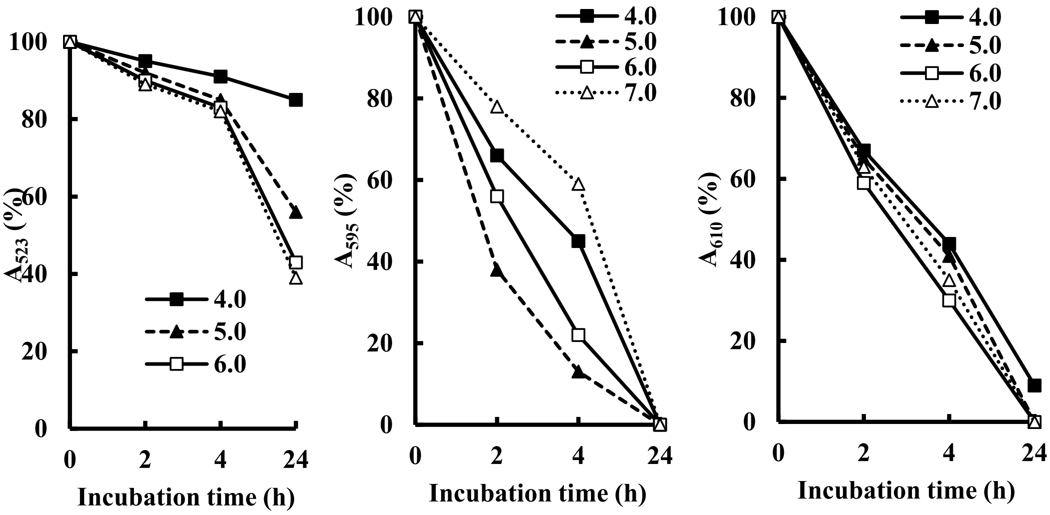

Fig. 1. *Amaranth (A), RBBR (B), and Indigo carmine (C) decolorization in dependence on the reaction mixture pH*

Unlike Amaranth, the highest rate of Remazol Brilliant Blue R decolorization was observed at pH 5.0-5.5. Dye' treatment with laccase at this pH caused 62% and 87% decolorization after 2 and 4 h incubation of reaction mixture, respectively (Fig. 1B). It is worth noting that a complete dye decolorization of RBBR was achieved at all pH after 24 h incubation. Finally, Fig. 4C show that the Indigo carmine decolorization efficiently occurred at wide range of pH with the highest rate observed at pH 6.0. Dye' treatment with laccase at this pH caused absorbance decrease by 41 and 70% after 2 and 4 h incubation of reaction mixture, respectively. Indigo carmine was completely decolorized by laccase during 24 h incubation at pH 5-7. It is worth noting that in all experiments the final laccase activity was measured after 24 h incubation of reaction mixtures. The results obtained showed that incubation at pH 4 caused a significant inactivation of enzyme and only 0.1-0.3 U/ml laccase activity was detected. At the same time, practically no enzyme activity loss was observed after 24 h incubation at pH 6-7. It means that the reduced dye discoloration at pH 4 is explained not only by the low catalytic activity of the enzyme, but also by its inactivation.

Subsequently, the ability of laccase from *C. unicolor* BCC300 to decolorize different concentrations of Amaranth, RBBR, and Indigo carmine was evaluated at the optimal for individual dyes pHs. The dyes concentration in solutions varied from 0.02 to 0.08% while the laccase concentration was 1 U/ml. Data represented in Fig. 2 show high decolorizing potential of laccase from *C. unicolor* BCC300. Enzyme is catalytically active even at dye concentration as high as 0.08% completely decolorizing RBBR and Indigo carmine after 24 h incubation. Amaranth was more resistant to the laccase action and only 55% of dye was decolorized after 24 h incubation at the initial dye concentration of 0.08%.

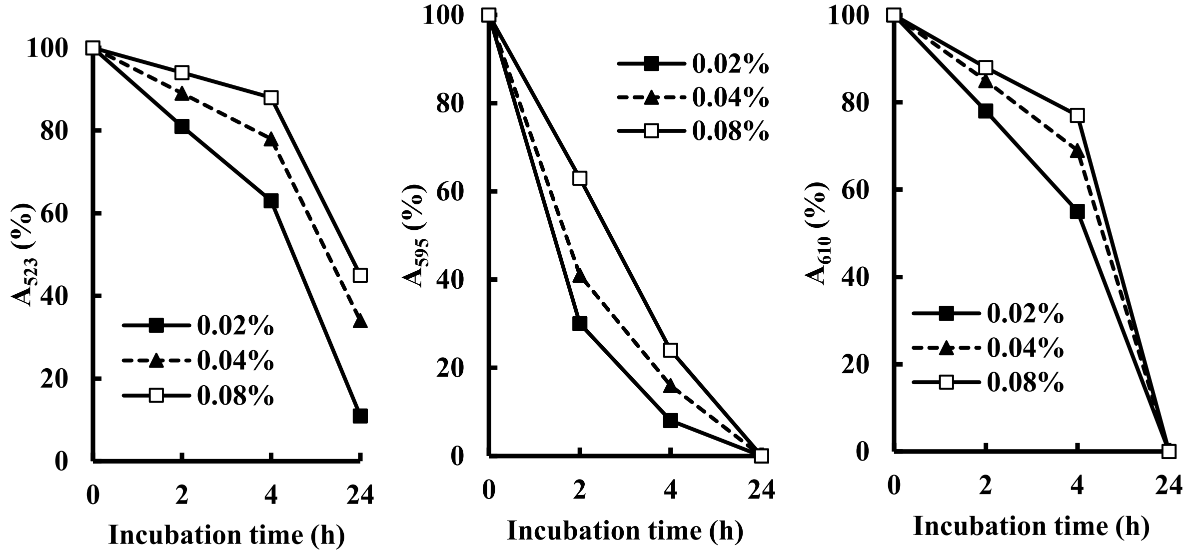

Fig. 2. *Amaranth (A), RBBR (B), and Indigo carmine (C) decolorization in dependence on the dye concentration*

Therefore, in the final experiment, the effect of the laccase concentration on Amaranth, RBBR, and Indigo carmine decolorization was studied at the dyes concentration of 0.08%. Enzyme loading is one of the reaction parameters required to achieve maximum rate of dye decolorization. Indeed, the higher was laccase concentration in the dyes solutions the higher was the rate of their decolorization. Among the dyes tested, RBBR was the most sensible to the catalytic action of *C. unicolor* BCC300 laccase (Fig. 3). Even at the laccase concentration of 1 U/ml the dye was completely decolorized after 24 h incubation. However, an increasing of laccase concentration highly accelerated the process of dye decolorization and it was practically completely decolorized after 4 h of incubation at the laccase concentration of 10 U/ml. Like in the previous experiments, Amaranth was the most resistant to the laccase catalytic activity. Nevertheless, complete decolorization of this dye was achieved when the concentration of laccase was increased to 10 U/ml.

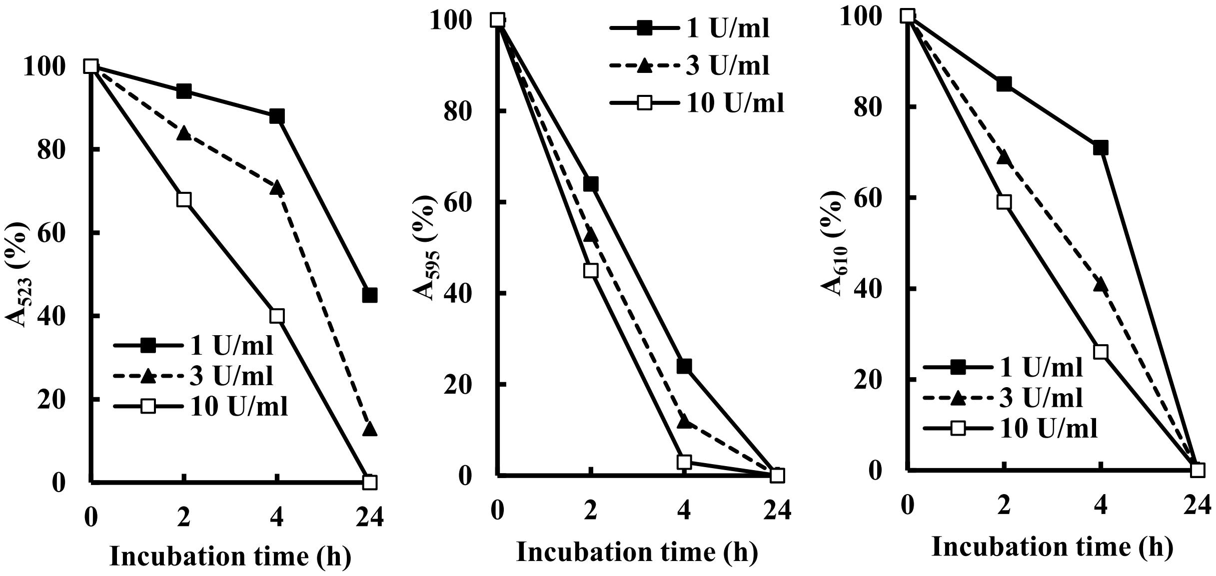

Fig. 3. *Amaranth (A), RBBR (B), and Indigo carmine (C) decolorization in dependence on the laccase concentration*

Discussion

This study showed that the selected white rot basidiomycetes had high decolorization capacity against azo dye Amaranth, anthraquinone dye RBBR, and indigoid dye Indigo carmine. *C. unicolor* BCC300, *C. gallica* BCC1184, and *T. versicolor* BCC13 were capable to withstand high concentration of dyes and to produce significant activities of laccase and MnP. Analogically, *B. adusta* CCBAS 232 was able to decolorize a number of chemically different synthetic dyes at concentrations of 2-4 g/l [14, 15]. *Polyporus* sp. S133 showed the fastest rate and efficiency (97% during 72 h) for decolorization of Amaranth (30 mg/l) under shaking conditions in glucose containing medium [16]. It is worth noting that as compared with the fungi tested in this study, *B. adusta* CCBAS 232 and *Polyporus* sp. S133 appeared to be poor enzyme producers.

Like in the study with *Trametes hirsuta* D7 enzyme [17], the obtained from liquid culture of *C. unicolor* BCC300 crude laccase was most effective at decolorizing anthraquinone dye RBBR. The crude enzyme showed good decolorization activity at acidic pH, but the optimal pH values for decolorization of the dyes were different. Against Amaranth, the *C. unicolor* BCC300 laccase was most effective at pH 6-7, toward Indigo carmine at pH 6, whereas the decolorization of RBBR occurred at a maximum rate at pH 5. At the same time, the most efficient decolorization of RBBR by crude enzyme from *Rigidoporus lignosus* W1 was obtained at pH 3.5 [18]. Enzyme extracts from *Coriolus versicolor* and *Pleurotus ostreatus* showed the maximum decolorization of RBBR at pH 4 [19].

The decolorization activity by the enzyme decreased with increasing dye concentration. In this study, the degree and rate of dyes decolorization decreased with an increase of dye concentration. Thus, 12, 22, and 37% of Amaranth decolorization was achieved during 2 h of incubation at dye concentration of 0.02, 0.04, and 0.08%, respectively. The highest rate of RBBR and Indigo decolorization was observed at the lowest dyes concentration. Similarly, maximum RBBR decolorization (95.6%) by laccase of *T. hirsuta* D7 was found at a lowest dye concentration (0.1%) [17].

In general, increasing the enzyme concentration enhanced the decolorization activity [17, 19]. In this study, concentration of laccase of 1 U/ml was sufficient to completely decolorize RBBR and Indigo carmine. Even lower concentration of laccase required to decolorize RBBR by *Trametes hirsuta* D7 [17]. By contrast, enzyme activity of 20 U/ml was optimum for decolorization of RBBR by *C. versicolor* and *P. ostreatus* [19]. However, an excessively high enzyme concentration would increase the cost of the treatment process.

Thus, three white rot basidiomycetes *C. unicolor* BCC300, *C. gallica* BCC1184, and *T. versicolor* BCC13 expressed an industrially relevant potential to decolorize structurally different synthetic dyes due to their capability to secrete extracellular laccase. Moreover, the isolated from *C. unicolor* BCC300 crude laccase showed catalytic properties required for their biotechnological applications.

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