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Effect of Fungal Laccase and Low Molecular Weight Mediators on Decolorization of Direct Blue Dye

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This study was performed to examine the role of laccase from white–rot fungi, *Cerrena unicolor* and *Trametes versicolor*, with low molecular weight mediators, acetovanillone (AV) and acetosyringone (AS), in decolorizing of the textile dye, Chicago sky blue 6B (Direct Blue 1, DB1). The tested fungi were shown the ability to remove colour from DB1. The decolorizing activity of the dye was closely related to the laccase from fungi. In the presence of AV or AS as co–substrate, decolorization was more extensive than that of the control. Moreover, at the presence of AS, the decolorization process was more effective than AV. The highly purified laccase of *C. unicolor* and *T. versicolor* also decolorized the dye. The addition of AV or AS enhanced this process.

INTRODUCTION

Fungal laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) are multicopper oxidases, which are involved in reduction of oxygen to water and simultaneously performing one–electron oxidation of aromatic hydrogen donors. Laccases and other blue copper oxidases not only oxidize the phenolic and methoxyphenolic acids, but also decarboxylate them (Agematu *et al*., 1993) and modify their methoxyl groups by demethylation (Leonowicz *et al*., 1984) or demethoxylation (Leonowicz *et al*., 2000; Potthast *et al*., 1995). These reactions constitute an important step in the initial transformation of lignin polymer (Leonowicz *et al*., 1999a; Leonowicz *et al*., 1999b; Leonowicz *et al*, 2001).

Laccases and similar polyphenol oxidases can be used as a free or immobilized preparation both in water and water miscible organic solvents to improve several biotechnological processes (Burton *et al*., 1995; Luterek *et al*., 1998; Milstein *et al*., 1993). The possible applications include that the enzymes can be used as a stabilizer during the must and wine processing (Lante *et al*., 1992) or as a dechlorinating factor (Cho *et al*., 1999b; Roy–Arcand and Archibald, 1991). Laccases can be used as a bleaching agent the color of mill effluents of pulp mills (Bourbonnais *et al*., 1997), or aromatic derivatives containing phenolic compounds or chlorine compounds from other industries (Bollag and Leonowicz, 1984; Brenna and Bianchi, 1994). The enzyme renders phenolic compounds less toxic via degradation or polymerization reactions and/or cross–coupling of pollutant phenols with phenols in nature (Bollag, 1983; Huttermann *et al*., 1980; Jonsson *et al*., 1998; Leonowicz *et al*., 1999b; Ullah *et al*., 2000). Several processes using laccases (also immobilized) have been developed for the treatment of phenolic effluents (Davis and Burns, 1992), oxidation of polycyclic aromatic hydrocarbons (Pickard *et al*., 1999), transformation and degradation of diazo dyes (Shliephake *et al*., 2000) and decolourization (bleaching) of aromatic textile dyes (Reys *et al*., 1999; Schneider *et al*., 1999, Abadulla *et al*., 2000). Decolorization of industrial dyes is currently performed by adsorption, precipitation, chemical degradation or photodegradation, but these methods are highly expensive, which limits their application. In contrast, white–rot fungi could be used in this regard, which degrade lignin biopolymers by producing a range of extracellular enzymes including laccase.

The bleaching activity of *Trametes versicolor* was studied by Livernoche *et al*. (1981). Nevertheless the application of living organism which is non reusable and needs sterile condition is very strenuous in industry. Immobilization on cheap matrices certifies their reusability and economy of application (Leonowicz *et al*., 1988; Sarkar *et al*., 1989). On the other hand, it is well known that the enzyme alone has only a limited effect on decolorizing of dyes due to its oxidative specificity (Cho *et al*., 1999a; Leonowicz *et al*., 1997). Fortuitously it is now well known that laccases can cooperate with low molecular compounds as mediators, which greatly improve enzyme activity. Such phenomenon showed by pioneer works of Call (1994) and Bourbonnais *et al*. (1995) for bleaching process of wood pulp. The low molecular mediators of internal or external origin migrate from the enzymes and oxidize lignin or wood pulp. Some of these are veratryl alcohol, oxalate, malate, fumarate, and 3–hydroxyanthranilic acid. Those compounds are enable the fungi to colonize and degrade the wood structure more effectively than

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the other organisms (Eggert *et al*., 1996; Lundquist and Kirk, 1978; Hofrichter *et al*., 1998; Leonowicz *et al*., 1999a; Traquair, 1987). It was also found that delignification of kraft pulp by laccase with support of some external (*i.e*. non–produced by fungi and absent in pulp) low molecular dyes or other aromatic hydrogen donors as acting mediators such as 2,2'azinobis–(3–ethylbenzenthiazoline–6–sulfonic acid) (ABTS) (Bourbonnais *et al*., 1995) or 1–hydroxybenzotriazole (HBT) (Call, 1994). The mediators were also used for the bleaching of some industrial dyes by fungal laccase. For example Chicago sky blue 6B (Direct Blue 1 dye, DB1; Fig. 1) decolorization by the enzyme containing culture extract with a mediation of two phenolic compounds–acetosyringone (AS) and methyl syringate (Schneider *et al*., 1999).

The textile dyes, which are an essential for dyestuff of industries, may cause environmental pollution. They are usually aromatic and heterocyclic compounds, often recalcitrant for elimination, toxic and even carcinogenics. DB1, an anthracene derivative, represents a group of often toxic organopollutants. Laccase–rich fungal extract being added to alkaline textile detergents is able to oxidize various dyes to bleach and consequently to clean cloths (Schneider *et al*., 1999).

In our earlier reports we have shown that purified inducible forms of laccase isolated from *Kuechneromyces mutabilis* and *Pleurotus ostreatus* decolorized carminic acid (CA) (Cho *et al*., 1999a). The present work was to test whether the cultures of some white rot fungi and the highly purified laccases of *Cerrena unicolor* and *Trametes versicolor* can operate on decolorizing of the textile dye, Direct Blue 1 (DB1; Fig. 1) with acetovanillone (AV) and acetosyringone (AS) as mediators.

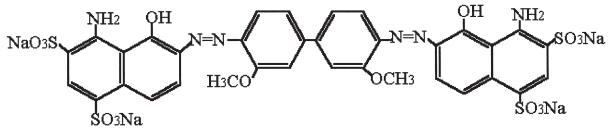

Fig. 1. The structure of Chicago sky blue 6B (DB1).

MATERIALS AND METHODS

Reagents

All chemicals were reagent grade quality. Chicago Sky Blue 6B (Direct Blue 1 DB1; $C_{34}H_{24}N_6O_{16}S_4Na_4$), acetovanillone (AV; 4–hydroxy–3–methoxyacetophenone HOC_6H_3 (OCH_3) $COCH_3$), acetosyringone (AS; 3,5–dimethoxy–4–hydroxyacetophenone HOC_6H_2 $(OCH_3)_2$ $COCH_3$), syringaldazine (4–hydroxy–3,5–dimethoxybenzaldehyde azine $[HOC_6H_2\,(OCH_3)_2\,CH{=}N–]_2$), and DEAE Toyopearl 650 S were obtained from Tosoh Corporation (Tokyo, Japan). 3–aminopropyltriethoxysilane (APTES) and glutaraldehyde were from Sigma Chemical Co (St Louis, Missouri, USA), and bovine liver catalase was from Aldrich Chemie (Steinheim, Germany). Sephadex G–25, Sephadex G–50, DEAE–Sephadex A–50, ConA–Sepharose and AH–Sepharose were from Pharmacia (Uppsala, Sweden), and agar, malt extract and yeast extract from Junsei Chemical Co. (Tokyo, Japan). Britton and Robinson (B&R) buffers were prepared by mixing 0.1 M boric acid, 0.1 M acetic acid, and 0.1 M phosphoric acid with 0.5 M NaOH (Schneider *et al*., 1999).

Organisms

Two fungal strains tested, *Cerrena unicolor* (Bull.ex Fr.) Murr. and *Trametes versicolor* (L.ex Fr.) Pil., were obtained from several fungal culture collections, the Fungal Culture Collection of the Department of Biochemistry, University of Lublin, Poland. The fungal cultures were maintained on 2% (w/v) malt agar slants.

Culture conditions and decolorization experiments

SMY–agar medium contained 10 g sucrose, 10g malt extract, 4 g yeast extract and 20 g agar in 1000 ml deionized water. Fungal strains were inoculated onto 20 ml of SMY–agar medium containing 0.05% DB1 or 0.05% DB1 and 10 μm AV or 10 μM AS in 90 mm Petri dishes and incubated at 27 °C. Fungal growth and decolorizing activities were measured as colony diameter and diameter of decolorized zone in mm.

Determination of laccase and decolorizing activities

For determination of laccase, the shallow stationary cultures after inoculation with small pieces of mycelium taken from the malt agar slants were incubated at 27 °C in 250 ml conical flasks containing 50 ml SMY medium. The culture fluids were separated from the mycelia by filtration. Laccase activity was measured with syringaldazine as a substrate (absorption coefficient E = 65,000 M^{-1} cm^{-1}, Leonowicz and Grzywnowicz , 1981) at pH optimum for particular fungi, but with morpholineethanesulfonic acid buffer replaced by 0.1 M boric–acetic–phosphoric acids buffers as described Schneider *et al*. (1999). To exclude endogenous peroxide, 10 min. preincubation (stirring) of the enzyme sample with catalase (10 mg ml^{-1}) was performed.

Decolorizing activity was assayed in a standard reaction mixture containing of 50 μl of 1 mM DB1 purified by gel filtration (Ulmer *et al*., 1984), 250 μl of culture fluid or laccase in distilled water, and 200 μl of 0.2 M Na–succinate buffer (pH 7.0). In the experiments with using mediators the concentration of each in the standard reaction mixture was 10 μM. The decrease in absorbency was assayed according to Schneider et al. (1999) at the concentration corresponding to an absorbance 0.6 at 610 nm. The decolorizing activity was calculated in nkat l^{-1} with using molar absorption coefficient 1,312 M^{-1} cm^{-1} measured (Eguchi *et al*., 1994). All data represent means of triplicate measurements.

Nitrogen starvation of fungal cultures

For the nitrogen starvation of the fungal cultures, the method of Staszczak *et al*. (1996) based on

Lindeberg and Holm (1952) mineral medium was used, but the mycelia were transferred to media deprived L–asparagine after 10 day incubation period. Enzymatic activities were assayed 24 h after exchange of the media.

Isolation and purification of laccase from C. unicolor

For the laccase purification the method of Leonowicz *et al*. (1997) with some our and Gianfreda *et al*. (1998) modifications was applied as follows. The aerated cultures at the top laccase activity were filtered through Miracloth (Calbiochem, Lucerne, Switzerland). Each filtrate was desalted on the Sephadex G–25 column. The enzyme solutions were concentrated to *ca*. one tenth of the volume at 4˚C with an Amicon ultrafiltration system equipped with a filter type PTGC (pore size 10,000 NMWL) and dialyzed 7 times to the deionized water and (before the end of dialysis) to 0.1 M phosphate buffer, pH 6. Then 25 ml portions of enzyme were applied onto the ConA–Sepharose columns (7× 1.5 cm), which were then washed with 0.1 M phosphate buffer, until all unbound proteins were removed. The specifically bound proteins, including laccase, were eluted from the column with 10% α–methyl mannoside in 0.1 M phosphate buffer, pH 6. The fractions showing the highest laccase activity were concentrated by ultrafiltration, poured onto DEAE–Toyopearl column (25× 1.5 cm) and eluted by a linear gradient of 0–0.35 M NaCl in 5 mM Tris/HCl buffer, pH 6.0. The fractions around the top of laccase activity, eluted by NaCl were collected, dialyzed to 0.5 mM Tris/HCl buffer, pH 6 and applied onto a column of AH–sepharose 4B coupled to syringaldehyde. The enzyme was eluted by 0.5 M ammonium sulfate dissolved in the same buffer, pH 6.0, and stored as freeze–dried.

Isolation and purification of laccase of T. versicolor

The fermenter culture of *T. versicolor* was run at 28˚C according to Rogalski *et al*. (1990) and filtered through Miracloth. Isolation and preliminary purification of laccase by chromatography on the Sephadex G–50 and DEAE–Sephadex A–50 columns was done according to the method of Leonowicz *et al*. (1978). The enzyme solutions were further concentrated with the Amicon ultrafiltration system, dialyzed, applied onto the ConA–Sepharose columns, eluted with sucrose, concentrated by ultrafiltration, poured onto DEAE–Toyopearl column eluted with NaCl, applied onto a column of AH–sepharose 4B coupled to syringaldehyde, eluted with ammonium sulfate, and stored as freeze–dried in 0.1 M phosphate buffer pH 6.

Laccase immobilization

The controlled porous glass beads (CPG), obtained from Cormey–Lublin, Poland, and silt loam soil (SL, pH 6.5; silt 60%; sand 14%; clay 21%; and organic matters 4,5%) were stirred in boiling concentrated nitric acid, washed thoroughly with distilled water up to pH 6.0 (Sarkar *et al*., 1989). The supports were silanized by 3–aminopropyltriethoxysilane (APTES) (Robinson *et al*., 1971) and activated by glutaraldehyde (Lappi *et al*., 1976). Laccase was coupled to silanized inorganic supports activated by glutaraldehyde according to the method of Leonowicz *et al*. (1988). The final prepa–rations were filtered, washed with 0.1 M phosphate buffer pH 7, suspended in 0.1 M phosphate buffer, pH 6 and stored at 4˚C.

RESULTS AND DISCUSSION

Decolorizing activities in the plate experiments

Although lignin is one of the most abundant polymers in nature, the mechanism of its biodegradation is poorly understood. A variety of radioactive and unlabelled lignin model compounds have been used to measure ligninolytic activity. Assays, however, are very slow. In addition the substrates are usually not commercially available. Therefore, as an alternative, the method with using aromatic dyes are receiving attention. The plate experiment was used for detection decolorizing of dyes because of convenient method for the analysis of lignin biodegradation. It has been shown that several polymeric dyes were decolorized by *Phanerochaete chrysosporium* culture under the favourable condition for lignin degradation. It suggests that the ligninolytic activity was also responsible for the decolorization of these dyes (Glen and Gold, 1983; Spadaro *et al*., 1992). Besides the most of studies on *P. chrysosporium*, *P. ostreatus* (Platt *et al*., 1985; Vyas and Molitoris, 1995, Kim *et al*., 1996; Shin *et al*., 1997), *Coriolus versicolor* (Livernoche *et al*., 1981), *Streptomyces* sp. (Pasti and Crawford, 1991) and *Geotrichum candidum* (Kim *et al*., 1995) have been investigated for decolorizing activity.

Aromatic dye and phenolic compounds as mediators which are present in culture extract caused inducible condition. The results of decolorizing activities of fungi tested and laccase production by these fungi are reported in Table 1. The two tested fungi were shown the ability to remove color from DB1. The decolorizing activity of the dye was exclusively shown by fungi producing laccase in the plate experiments. The addition of AV or AS to the growth media quantitatively improved the decolorizing activity. The decolorized zones of the plates were larger than that of without mediators. However, AS showed the larger decolorized zone compared to AV.

These results are in agreement with those reported by Gianfreda and Bollag (1994). These authors demonstrated that about 2.3 mg of *T. versicolor* laccase was

Table 1. Effect of fungi and low molecular weight mediators on decolorization of Blue dye

Fungi	N–rich medium, mm			N–free medium, mm		
	Control	AV	AS	Control	AV	AS
C. unicolor	6.4	7.8	8.8	7.2	9.7	10.0
T. versicolor	5.6	7.2	7.9	7.0	8.6	9.2

immobilized on 1 g of porous glass support. Also high laccase activity was recovered on the same support earlier (Sarkar *et al*., 1989). In our further experiments we found the relationship between laccase and decolorization activity. For this reason we used the method of nitrogen starvation of fungal cultures that induce ligninolytic activity (Staszczak *et al*., 1996; Glenn and Gold, 1983). According to these authors, transformation of mycelia from nitrogen–rich to nitrogen–free medium induces the secondary metabolism of fungi. Table 2 shows that nitrogen starvation induced both laccase and dye decolorizing activity. *C. unicolor* which has higher laccase activity shows higher decolorizing activity than that of *T. versicolor*. The addition of AV or AS to the both growth media quantitatively improved the decolorizing activity. As shown in plate experiments, AS showed the higher decolorizing compared to that of AV addition.

Table 2. Laccase and DB1 decolorizing activities in the presence of mediators (deviation <10%)

Fungi	Laccase activity nkat l^{-1}	Decolorizing activity (N–rich culture medium) nkat l^{-1}			Decolorizing activity (N–free culture medium) nkat l^{-1}		
		Control	AV	AS	Control	AV	AS
C. unicolor	1,375	30	121	140	378	1,098	1,256
T. versicolor	1,187	19	82	98	112	296	317

Decolorizing activities by laccase

The extracellular laccases were isolated and purified from fermented cultures of both fungi (Table 3 and Table 4). The purification procedure was based on consecutive steps of ion–exchange and affinity chromatography. The method gives relative high purification, higher than ones reported earlier (Leonowicz *et al*., 1997, Luterek *et al*., 1998). Applying this method we achieved about 122–fold purification of *C. unicolor* extracellular laccase and 97–fold in the case of *T. versicolor* (see Table 3 and Table 4).

The purified laccases were immobilized on the silanized controlled porous glass beads (APTES–CPG) and soil (APTES–SL) after activation with glutaraldehyde. As a result of the bonding procedure, in cases of CPG and SL, about 80% and 78% protein and laccase activity were coupled to the supports, respectively. The final immobilized enzymes contained a little more than 2 mg protein per 1 g of CPG and SL as well, and their specific activities were around 60 U per mg protein, (see Table 5).

Table 3. Isolation and purification of laccase of *C. unicolor*

Purification step	Total activity (nkat/vol)	Specific activity (total/mg protein)	Yield (%)	Purification (fold)
Filtrate	59,873	40	100.0	1.0
Sephadex G–25	37,846	79	63.2	2.0
Ultrafiltration	33,208	158	55.5	3.9
ConA–Sepharose	30,976	1,799	51.7	45.0
DEAE–Toyopearl	7,620	3,125	12.7	78.1
Syringyl–AH–Sepharose 4B	3,198	4,872	5.3	121.8

Table 4. Isolation and purification of laccase of *T. versicolor*

Purification step	Total activity (nkat/vol)	Specific activity (total/mg protein)	Yield (%)	Purification (fold)
Filtrate	14,984	55	100.0	1.0
Sephadex G–50	12,986	103	86.7	1.9
DEAE–Sephadex A–50	7,341	1,347	49.0	24.5
Ultrafiltration	6,963	1,398	46.5	25.4
ConA–Sepharose	5,937	1,910	39.6	34.7
DEAE–Toyopearl	1,334	3,221	8.7	58.6
Syringyl–AH–Sepharose 4B	522	5,311	3.4	96.6

When laccase activities increase around 8.3 times for the laccase of *C. unicolor* as in Table 6 and around 8.5 times for the laccase of *T. versicolor* as in Table 7, the decolorization increases were much slower, *e.g*. about 3 times without mediators and not more than 5 times with mediators. Both free and immobilized laccases decolorize the dye, and as in laccase activity, the addition of AV or AS enhanced the decolorizing activity.

In conclusion, the results presented in Tables 2, 6 and 7 indirectly point that laccase is most probable decolorizing DB1. The similar results were obtained earlier in the case of *P. ostreatus* and *K. mutabilis* fungi (Cho *et al*., 1999a). In this case, the laccase supplements the activity of specific dye decolorizing enzymes similar to the enzyme reported by Vyas and Molitoris (1995). Such hydrogen peroxide dependent

Table 5. Properties of immobilized laccase on inorganic matrice

Immobilization	Free enzyme	Immobilized enzyme	Yield (%)	Protein /g support (mg)	Specific activity (units/mg protein)
C. unicolor					
CPG					
activity	7.21 [a]	5.19 [a]	81.1		59.7
protein	12.18 [b]	8.12 [b]	79.5	2.19	
SL					
activity	6.19 [a]	4.37 [a]	78.2		60.0
protein	11.21 [b]	9.11 [b]	78.4	2.21	
T. versicolor					
CPG					
activity	5.11 [a]	4.21 [a]	80.1		56.0
protein	12.97 [b]	10.93 [b]	84.0	2.01	
SL					
activity	4.98 [a]	4.19 [a]	81.2		57.0
protein	12.33 [b]	11.91 [b]	79.9	1.89	

[a] total activity ($U \times 10^6$)
[b] total protein (mg)

Table 6. Decolorizing activity of purified *C. unicolor* laccase with mediators (deviation <10%)

Laccase activity nkat/l	Decolorizing activity of free enzyme nkat l^{-1}			Laccase activity nkat l^{-1}	Decolorizing activity of immobilized enzyme nkat l^{-1}		
	Control	AV	AS		Control	AV	AS
694	1	11	12	736	2	13	16
2,451	2.6	33	39	2,914	4.1	45	51
5,835	3	43	59	6,124	5.8	52	78
2,432 *	0 [a]	0 [a]	0 [a]	2,390 *	0 [a]	0 [a]	0 [a]

[a] The enzymes were boiled after measuring the activity with syringaldazine.

Table 7. Decolorizing activity of purified *T. versicolor* laccase with mediators (deviation <10%)

Laccase activity nkat/l	Decolorizing activity of free enzyme nkat/l			Laccase activity nkat/l	Decolorizing activity of immobilized enzyme nkat/l		
	0	AV	AS		0	AV	AS
726	3	17	23	779	27	59	82
2763	5	22	31	3115	45	125	197
6400	7	54	62	6445	67	196	384
2900*	0*	0*	0*	3213*	0*	0*	0*

[a] The enzymes were boiled after measuring the activity with syringaldazine.

decolorizing Remazol brilliant blue enzyme was found in the solid–state culture of *P. ostreatus* in wheat straw. It seems that the enzyme in *P. ostreatus* (also in other fungi) has two functions of decolorizing activity, specific and non–specific ones. The decolorizing activity of laccase on Remazol brilliant blue was reported earlier in the case of *Pycnoporus cinnabarinus* (Schliephake and Lonergan, 1996), *P. ostreatus* (Eguchi *et al*., 1994) and *C. unicolor* (Bekker *et al*., 1990). Also carminic acid can be decolorized by laccase as in the earlier reports of Cho *et al*. (1999a) and Eguchi *et al*. (1994). Our results presented here show that DB1 can be decolorized by fungal laccase. The decolorizing effect can be greatly improved by low molecular phenolic compounds, AV and AS, which have roles of hydrogen donor similar as "mediator" in the laccase context (Kim and Shin, 2000). Schneider *et al*. (1999) reported that the decolorizing effect of laccase containing culture extract on DB1 dye was improved by some phenolic compound addition, among which AS worked the best at pH 7. Such decolorizing activity of fungal laccase is not strange, as the enzyme shows very broad specificity for many hydrogen donors (Bollag and Leonowicz, 1994), including lignin. In this case the enzyme is known as a depolymerizing agent (Leonowicz *et al*., 1985; Leonowicz *et al*., 1999b; Potthast *et al*., 1995; Szklarz and Leonowicz, 1986). The laccase probably performs as a secondary, not specific agent, in decolorizing process.

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