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Effect of Fungal Laccase and New Mediators, Acetovanillone and Acetosyringone, on Decolourization of Dyes

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Several wood rotting fungi decolourized Remazol brilliant blue R (RBBR) and carminic acid (CA). Parallel activity of laccase in these fungi was studied. The addition of acetovanillone (AV) or acetosyringone (AS) intensified these processes: decolourization was more extensive than in the experiment omitting these compounds. At the presence of AS the decourization was more extensive than AV. However the level of destaining was relatively low in comparison to laccase activity on syringaldazine. The highly purified constitutive form of *Cerrena unicolor* and inducible form of *Trametes versicolor* laccases also decolourized both dyes. The fungus, *Lentinus lepideus*, which has no laccase activity did not decolourize at all. Nitrogen starvation induced the laccase and decolourization activity in both organisms, irrespective of nitrogen availability. This fact indicates laccase not solely responsible for decolourization, and probably decolourization of dyes involves more than one mechanism.

INTRODUCTION

Fungal laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) are multicopper oxidases, capable of reducing oxygen to water and simultaneously involved in oxidation of aromatic hydrogen donors. These enzymes and very similar polyphenol oxidases can be used as free or immobilized type both in water or in some organic solvents for improving several biotechnological processes (Burton *et al*., 1995; Luterek *et al*., 1998; Milstein *et al*., 1993). Of possible applications, the enzyme is considered as a bleaching agent in the pulp and paper industry (Bourbonnais *et al*., 1997), as a stabilizer during the must and wine processing (Lante *et al*., 1992) or as a dechlorinating agent in the effluent (Cho *et al*., 1999b; Roy–Arcand and Archibald, 1991). These blue copper enzymes not only oxidize phenolic compounds, but also decarboxylates 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). Those reactions play an important role in the initial transformation of lignin polymer (Leonowicz *et al*., 1999a). Laccase is also considered as bleaching agent of some industrial dyes (Schneider *et al*., 1999), but according to our recent findings the enzyme has a limited effect on decolorizing of dyes, mainly due to their oxidative specificity (Cho *et al*., 1999a; Leonowicz *et al*., 1997).

The bleaching activity of *Trametes versicolor* was studied by Livernoche *et al*. (1981). In our earlier reports we have shown that the inducible form of laccase isolated from *Kuechneromyces mutabilis* and *Pleurotus ostreatus* decolourized carminic acid (CA, Δ_{max}=465 nm, E_{456}=919 M^{-1} cm^{-1}) (Cho *et al*., 1999a). On the other hand there have been studied that laccase could be bleachable hardwood pulp by depolymerizing and solubilizing lignin in the presence of so–called mediator compounds (Bourbonnais *et al*., 1995; Call, 1994). Therefore, in recent years, many studies have been focused on such potential low–molecular mediators, which posses high enough redox potentials (> 900 mV) to attack lignin by its migration into the lignified cell wall matrix. Examples of such substances include veratryl alcohol, oxalate, malate, fumarate, and 3–hydroxyanthranilic acid. Those compounds are produced as a result of fungal metabolism and their secretion enables the fungi to colonize and degrade the wood more effectively than the other organisms (Eggert *et al*., 1996; Hofrichter *et al*., 1998; Leonowicz *et al*., 1999a; Lundquist and Kirk, 1978; Traquair, 1987).

It was also found that delignification of kraft pulp by laccase can be supported by some low molecular compounds 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 in bleaching of some industrial dyes with fungal laccase. For example direct blue 1 dye was decolourized by the enzyme containing culture filtrates through so–called mediation of two phenolic compounds – acetosyringone (AS) and methyl syringate (MS) (Schneider *et al*., 1999).

This study was carried out to examine discolourization of industrial dyes, such as Remazol brilliant blue R (RBBR, Δ_{max}=592 nm, E_{592}=1,916 $M^{-1}cm^{-1}$) and carminic

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acid, by laccase enzyme from the some wood rotting fungi, with low molecular weight mediators, acetovanillone (AV) and acetosyringone (AS).

MATERIALS AND METHODS

Organisms

All fungal strains, *Cerrena unicolor* (Bull.ex Fr.) Murr., *Gleophyllum odoratum* (Wulf.ex Fr.) Imaz., *Lentinus lepideus* Fr., 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, registrated as FCL in the Information Center for European Culture Collections, Braunschweig, Germany, and the Fungal Culture Collections, School of Forest Resources, Chungbuk National University, Cheongju, Korea. The fungal cultures were maintained on 2% (w/v) malt agar slants.

Culture conditions and decolourization

SMY–agar medium contained 10 g sucrose, 10 g 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% (w/v) CA or 0.05% (w/v) RBBR and/or 10 μm AV and AS in 90 mm Petri dishes, and incubated at 27 °C. Fungal growth and decolourizing activities were measured as colony diameter and diameter of decolourized zone in mm. The decolourization was evaluated three replications an each treatment.

Determination of laccase and decolourizing activities

At the end of plate experiments, the culture fluids were separated from the mycelia by filtration. Laccase activity was measured with syringaldazine according to Leonowicz and Grzywnowicz (1981) at pH optimum for particular fungi, but with morpholineethane sulfonic acid buffer replaced by 0.1 M (McIlvaine, 1921) citrate – phosphate buffer as described Bollag and Leonowicz (1984). To exclude endogenous peroxide, the 10 min. preincubation (stirring) of the enzyme sample with catalase (10 mg/ml) was performed. The laccase activity was calculated in nkat/litre.

Decolourizing activity was assayed with a standard reaction mixture containing of either 50 $\mu\ell$ of 1 mM RBBR or CA purified by gel filtration according to Ulmer *et al.* (1984), 250 $\mu\ell$ of culture fluid or laccase in distilled water, and 200 $\mu\ell$ of 0.2 M Na–succinate buffer (pH 7.0). The concentration of each mediator, AV and AS, in the standard reaction mixture was 10 $\mu\ell$, respectively. The decrease in absorbency was assayed at 592 nm for RBBR and 456 nm for CA. The activity was calculated in nkat per litre with using molar absorption coefficients 1916 $M^{-1}\,cm^{-1}$ for RBBR and 919 $M^{-1}\,cm^{-1}$ for CA, respectively, according to Eguchi *et al.* (1994).

Nitrogen starvation of fungal cultures

For the nitrogen starvation of the fungal cultures the method of Staszczak *et al.* (1996) based on mineral medium (Lindeberg and Holm, 1952) was employed. The mycelia were transferred to media deprived *L*–asparagine after 10 day incubation. Enzymatic activities were assayed 24 h after exchange of the media.

Isolation and purification of constitutive laccase

For the constitutive *C. unicolor* laccase purification, the method of Leonowicz *et al.* (1997) with some our and Gianfreda *et al.* (1998) modifications was applied. The modifications were as followed, the aerated cultures at the tops of 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 preparations 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 20% sucrose (or, even better, 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 inducible laccase

The fermenter culture of *T. versicolor* based on Lindeberg and Holm (1952) medium was run at 28 °C (Rogalski *et al.*, 1990). To stimulate the production of inducible forms of laccase, ferulic acid was added as an inducer to the concentration of 0.2 mM (Leonowicz *et al.*, 1978). Isolation and preliminary purification of inducible laccase form by chromatography on Sephadex G–50 and DEAE–Sephadex A–50 columns were also done according to the method of Leonowicz *et al.* (1978). The further purification processes were same as in the case of constitutive laccase.

RESULTS AND DISCUSSION

Decolourizing activities

Aromatic dyes and phenols as well as mediators which are present in yeast extract caused in cultures inducible conditions. The results of decolourizing of fungi and its laccase activity are summerized in Table 1. The majority of used fungi showed the ability to remove colour from both RBBR and CA. The high decolouriza-

Table 1. Decolourizing activity of fungi on RBBR and CA during 24 h plate test

Strain No.	Decolourizing activity of RBBR, mm			Decolourizing activity of CA, mm			Laccase activity nkat/l		
	Control	AV	AS	Control	AV	AS	Control	AV	AS
C. unicolor	8.7	9.2	9.6	9.5	10.7	11.6	1,324	1,377	1,387
G. odoratum	10.9	12.3	13.1	15.3	17.5	18.2	3,874	3,998	4,024
L. lepideus	0.0	0.0	0.0	0.0	0.0	0.0	0	0	0
T. versicolor	9.8	10.1	10.9	12.3	14.2	16.1	1,212	1,253	1,249

tion of RBBR and CA occured exclusively by fungi producing laccase. Further the addition of AV or AS to the cultures caused a quantitative improving the decolourizing. The decoloured zones were larger in the presence of AC than those without mediators (Table 1).

It has been shown that several polymeric dyes were decolourized by *Phanerochaete chrysosporium* under those conditions which favour lignin degradation, suggesting that the ligninolytic activity was responsible for the decolourization of these dyes (Glen and Gold, 1983; Spadaro *et al*., 1992). Besides, the most studies on *P. chrysosporium*, *P. ostreatus* (Kim *et al*., 1996; Platt *et al*., 1985; Shin *et al*., 1997; Vyas and Molitoris, 1995), *Coriolus versicolor* (Livernoche *et al*., 1981), Streptomyces sp. (Pasti and Crawford. 1991) and *Geotrichum candidum* (Kim *et al*., 1995) have been done for decolourizing activity. The fungi which have laccase activity were shown decolourization of RBBR and CA. It indicates the relationship between laccase and decolorization activity. The fungus, *Lentinus lepideus*, which has no laccase activity did not decolourize at all.

Effect of purified laccase

Table 2 and Table 3 show the results of purification of laccase from *C. unicolor* and *T. versicolor* at each purification step. The specific activities of first filtered solutions of two laccases are 42 nkat/mg and 53 nkat/mg, which are about 300 times higher than previously reported one (Gianfreda *et al*., 1998). Desalting and ultrafiltration could remove the other proteins and specific activities increased about 4 and 25 times, respectively. It is obvious that this procedure is very effective and can remove low molecular weight proteins and the other small impurities. The main increases in specific activity were obtained after running various columns. The specific activities increased 118 and 100 times. The specific activity of 4,980 −5,300 nkat/mg is one of the most active laccase ever reported (Heinzkill, 1998; Kim *et al*., 2002). The enzyme solution looks brownish blue color implying other brown colored proteins are still remained although the activity is quite high.

The results presented in Table 4 and Table 5 show that purified constitutive and inducible laccases decolourize both dyes. Decolourization was maximum in CA than RBBR. Furthermore, decolourization also increased by the use of mediators. As high amounts of laccase activities were applied (average about 8.2 times in the case of constitutive laccase of *C. unicolor* and about 8.5 times for *T. versicolor* inducible form of laccase as calculated from Tables 4 and 5), the decolorization did not coincide with laccase activity on syringaldazine, *i.e*. it increased much slower (maximum around 3 times without mediators and not more than 5 times with using mediators).

Further the nitrogen starvation method was introduced for the induction of ligninolytic activity in fungal cultures (Glenn and Gold, 1983). The manner of nitrogen starvation proposed by Staszczak *et al.* (1996) was applied. According to them, the changes into nitrogen deprived medium from nitrogen rich ones induces the secondary metabolism of fungi. Nitrogen starvation is known to induce the laccase production (Staszczak *et al*., 1999). Similarly low molecular aromatic compounds (mediators) were also known to increase the decolour-

Table 2. Isolation and purification of constitutive laccase from *C. unicolor*

Purification	Activity		Yield (%)	Purification (fold)
	Total (nkat/vol)	specific (total/mg prot)		
Filtrate	62,634	42	100	1.0
Sephadex G–25	38,792	83	61.9	2.0
Ultrafiltration	34,627	167	55.3	4.0
ConA–Sepharose	31,323	1,893	50.0	45.1
DEAE–Toyopearl	7,982	3,067	12.7	73.0
Syringyl–AH–Sepharose 4B	3,235	4,982	5.2	118.6

Table 3. Isolation and purification of inducible laccase from *T. versicolor*

Purification	Activity		Yield (%)	Purification (fold)
	Total (nkat/vol)	specific (total/mg prot)		
Filtrate	15,276	53	100	1.0
Sephadex G–50	13,111	99	86.0	1.9
DEAE–Sephadex A–50	7,467	1,327	49.0	25.0
Ultrafiltration	7,124	1,411	46.6	26.6
ConA–Sepharose	6,074	1,899	40.0	35.8
DEAE–Toyopearl	1,334	3,221	8.7	60.8
Syringyl–AH–Sepharose 4B	522	5,311	3.4	100.2

Table 4. Decolourizing activity of purified constitutive laccase

Laccase activity nkat/l	Decolourizing activity of RBBR, nkat/l			Laccase activity nkat/l	Decolourizing activity of CA, nkat/l		
	Control	AV	AS		Control	AV	AS
737	1	15	19	815	17	46	67
2,694	0	19	25	3,072	29	99	149
6,120	1	48	51	6,412	54	142	315
2,740*	0*	0*	0*	2,790*	0*	0*	0*

* The enzyme preparations were boiled after measuring the activity with syringaldazine.

Table 5. Decolourizing activity of purified inducible laccase

Laccase activity nkat/l	Decolourizing activity of RBBR, nkat/l			Laccase activity nkat/l	Decolourizing activity of CA, nkat/l		
	Control	AV	AS		Control	AV	AS
726	3	17	23	829	27	59	82
2,763	5	22	31	3,115	45	125	197
6,400	7	54	62	6,372	67	196	384
2,900*	0*	0*	0*	3,213*	0*	0*	0*

* The enzyme preparations were boiled after measuring the activity with syringaldazine

ization activity (Schneider *et al*., 1999). These two inducing techniques were employed to understand the relationship between decolourization and laccase, using *G. odoratum* which produces laccase, and *L. lepideus* which does not produce laccase. The mediators significantly increased the decolourization in *G. odoratum*, then followed by *L. lepideus* (Tables 6 and 7). Among the mediators, the highest decolourization was recorded in AS than AV, irrespective of nitrogen availability (compare Table 6 and 7). Nitrogen starvation induced the laccase and decolourization activity in both organisms, respectively, This fact indicates laccase not solely responsible for decolourization. Probably decolourization of dyes involved by more than one mechanisms.

The results indirectly point that laccase, although shows decolourizing activity on RBBR and CA, is most probable not the only agent causing this process. The similar results were obtained earlier in the case of *P. ostreatus* and *K. mutabilis* fungi (Cho *et al*., 1999a). May be laccase supplements the activity of specific dye decolourizing enzymes similar to the enzyme reported by Vyas and Molitoris (1995). Such hydrogen peroxide dependent RBBR decolourizing enzyme was found in the solid–state culture of *P. ostreatus* in wheat straw. It seems that two decolourizing enzymes, specific and non–specific laccases, function in *P. ostreatus* (also in *K. mutabilis*, *C. unicolor*, *T. versicolor* and other fungi). The decolourizing activity of laccase on RBBR 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 CA could be decolourized by laccase

Table 6. Laccase activities and RBBR and CA decolourizing activities in nitrogen rich cultures of *G. odoratum* and *L. lepideus* in the presence of mediators

Fungus	Laccase nkat/l	Decolourizing activity of RBBR, nkat/l			Decolourizing activity of CA, nkat/l		
		Control	AV	AS	Control	AV	AS
G. odoratum	4,213	22	81	98	123	312	396
L. lepideus	0	3	3	10	29	45	79

Table 7. Laccase activities and RBBR and CA decolourizing activities in nitrogen deprived cultures of *G. odoratum* and *L. lepideus* in the presence of mediators

Fungus	Laccase nkat/l	Decolourizing activity of RBBR, nkat/l			Decolourizing activity of CA, nkat/		
		Control	AV	AS	Control	AV	AS
G. odoratum	7,340	34	121	144	378	1,197	1,372
L. lepideus	0	8	15	21	89	134	157

as shown earlier reports (Cho *et al*., 1999a; Eguchi *et al*., 1994). Our results presented here confirm that RBBR and CA can be decolourized by fungal laccases, both, constitutive and inducible. We were addictively shown that the decolourizing effect can be greatly improved by low molecular phenolics, AV and AS. Both compounds were earlier named "mediators" in the laccase context. Kim and Shin (2000) interpreting cyclic voltagrams of 23 different compounds included AV to the group of mediators which work lignin degradation with laccase. Schneider *et al*. (1999) reported that the decolourizing effect of laccase containing culture exctract on Direct Blue 1 dye was greatly improved by some phenolic compounds. Such unusual decolourizing activity of fungal laccase is not strange, as the enzyme shows very broad specificity for many hydrogen donors (Bollag & 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). This laccase probably serves as a not specific but secondary agent. Laccase enzyme probably performs the analogical role in decolourizing process.

CONCLUSIONS

This study was carried out to examine discolourization of industrial dyes, such as Remazol brilliant blue R (RBBR, Δ_{max}=592 nm, E_{592}=1,916 $M^{-1} cm^{-1}$) and carminic acid, by laccase enzyme from the some wood rotting fungi, with low molecular weight mediators, acetovanillone (AV) and acetosyringone(AS). The laccase shows decolourizing activity on RBBR and CA. It means laccase supplements the activity of specific dye, such as hydrogen peroxide dependent RBBR. The highly purified constitutive form of *Cerrena unicolor* and inducible form of *Trametes versicolor* laccases also decolourized both dyes. The addition of AV and AS improved the efficiency of dyes decolourization by wood rotting fungi and fungal laccase. Nitrogen starvation induced the laccase and decolourization activity in both organisms, irrespective of nitrogen availability.

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