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ORIGINAL PAPER

H. Claus · G. Faber · H. König

Redox-mediated decolorization of synthetic dyes by fungal laccases

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Abstract Laccases from the lignin-degrading basidiomycetes *Trametes versicolor*, *Polyporus pinisitus* and the ascomycete *Myceliophthora thermophila* were found to decolorize synthetic dyes to different extents. Differences were attributed to the specific catalytic properties of the individual enzymes and to the structure of the dyes. Due to their higher oxidative capacities, the laccases from the two basidiomycetes decolorized dyes more efficiently than that of the ascomycete. The azo dye Direct Red 28, the indigoid Acid Blue 74 and anthraquinonic dyes were directly enzymatically decolorized within 16 h. The addition of 2 mM of the redox-mediator 1-hydroxybenzotriazole further improved and facilitated the decolorization of all nine dyes investigated. Laccases decolorized dyes both individually and in complex mixtures in the presence of bentonite or immobilized in alginate beads. Our data suggest that laccase/mediator systems are effective biocatalysts for the treatment of effluents from textile, dye or printing industries.

Introduction

Synthetic dyes are extensively used in industrial dying and printing processes. Over 7×10^5 tons and approximately 10,000 different dyes and pigments are produced annually worldwide, of which about 10% are released by industrial effluents (Young and Yu 1997). Containing various substituents such as nitro and sulfonic groups, synthetic dyes are not uniformly susceptible to biodecolorization in conventional aerobic processes. Attempts to develop aerobic strains for dye decolorization often resulted in very specific organisms showing restricted decolorization capability on individual dyes (Kulla 1981; Young and Yu 1997). Under anaerobic conditions the industrially important azo dyes are cleaved by azo-reductases to the corresponding amines, many of which are mutagenic and/or carcinogenic (Chung and Cerniglia 1992; Abadulla et al. 2000). In order to enhance microbial degradation of industrial dyes, a combination of anaerobic and aerobic treatments has been suggested (O'Neill et al. 2000).

Laccases (benzendiol:oxygen oxidoreductases, EC 1.10.3.2) are Cu-containing glycoproteins which require O_2 to oxidize phenols, polyphenols, and aromatic amines as well as non-phenolic organic substrates by one-electron abstractions resulting in the formation of H_2O and reactive radicals undergoing further depolymerization, repolymerization, demethylation, dehalogenation, or quinone formation. Until recently, laccases have been found mainly in lignin-degrading white-rot fungi and a few actinomycetes (Sjoblad and Bollag 1981; Thurston 1994) but there is increasing evidence for their occurrence in different species of bacteria (Alexandre and Zhulin 2000). The biotechnological usefulness of these oxidative enzymes for the treatment of xenobiotic pollutants has been discussed in reviews by Filip and Claus (1995) and Durán and Esposito (2000). The rather broad substrate specificity of laccases can be further extended by addition of small molecular redox mediators (Bourbonnais et al. 1997; Li et al. 1999).

Recent studies have shown that fungal laccases are able to decolorize and detoxify industrial dyes in vitro (Chivukula and Renganathan 1995; Rodriguez et al. 1999; Abadulla et al. 2000; Schliephake et al. 2000). The aims of our study were (1) to compare the capacity of fungal laccases from *Trametes versicolor*, *Polyporus pinisitus* and *Myceliophthora thermophila* to decolorize a set of selected synthetic dyes both individually and in mixtures, (2) to test the effect of a redox mediator on this process and (3) to investigate the possibility of using immobilized laccases.

H. Claus (✉) · H. König
Johannes Gutenberg-Universität Mainz,
Institut für Mikrobiologie und Weinforschung, Becherweg 15,
55099 Mainz, Germany
e-mail: hclaus@mail.uni-mainz.de
Tel.: +49-6131-3923547, Fax: +49-6131-22695

G. Faber
Erbslöh Lohrheim GmbH, Schaumburger Strasse 34,
65558 Lohrheim, Germany

Materials and methods

Enzymes and dyes

Laccase preparations from the culture medium of the thermophilic ascomycete *M. thermophila* and the basidiomycete *P. pinisitus* (*Coriolus pinisitus* or *Trametes villosa*) were kindly provided by Novo Nordisk (Bagsvaerd, Denmark). A laccase preparation from a *Trametes* sp. was obtained from Erbslöh Getränketechnologie (Geisenheim, Germany). Some characteristic features of the enzymes used in this study are listed in Table 1. Immobilization of laccases by entrapment in alginate beads (∅ 1 mm) was performed by Cavis Gesellschaft für Immobilisierungssysteme (Mainz, Germany).

The influence of clay minerals on enzymatic activities was investigated with bentonite (type BII) obtained from Erbslöh Lohrheim (Germany).

Dyes representing different chemical classes were purchased from Sigma-Aldrich (Deisenhofen, Germany). Some essential characteristics are listed in Table 2. Stock solutions (0.2% in water) were stored in the dark at 4°C.

Determination of laccase activities

Laccase activities were measured spectroscopically by oxidation of ABTS [2,2′-azino-di-(-ethylbenzothiazoline-6-sulfonic acid)] according to Bourbonnais et al. (1997). The assay contained 2 mM ABTS in sodium-phosphate buffer (100 mM, pH 5.9) and an appropriate amount of enzyme. Oxidation of ABTS was monitored at 420 nm (ε_{420} =3.6×10^4 M^{-1} cm^{-1}). Enzyme activities are expressed as katals (kat). One nkat corresponds to the oxidation of 1 nmol substrate per second. Protein concentrations were determined according to Bradford (1976).

Electrophoresis

Laccase preparations were resolved by electrophoresis in 10% SDS-polyacrylamide gels (10×10 mm) with a 4% stacking gel in Laemmli buffer at 150 V for 45 min. Estimation of the isoelectric points (IEP) of laccases was performed using analytical polyacrylamide gels (Servalyte 3–10) and pI marker proteins (Serva, Heidelberg). For staining of total protein, gels were incubated in Coomassie blue R250. Laccase activities were visualized using ABTS as the substrate.

Decolorization experiments

Stock solutions of dyes were diluted in either deionized water, 50 mM MES (pH 5.5) or sodium phosphate buffer (100 mM; pH 5.9), usually to a final concentration of 20 mg/l. Fungal laccases were added at activities of 40 nkat/ml or 20 alginate beads containing immobilized enzymes. One bead corresponded to an average laccase activity of 2, 30 or 40 nkat for *T. versicolor*, *P. pinisitus* and *M. thermophila*, respectively. The experiments were conducted in a total volume of 10 ml (in 15 ml plastic screwcap tubes) in triplicate. The controls contained dye solutions without enzymes and vice versa. After incubation for 16 h at 30°C on a rotary shaker (100 rpm), color was measured spectroscopically at the absorbance maximum of the dye (Table 2). In the case of mixtures of different dyes, the absorbance spectrum between 400 and 800 nm was measured with a Shimadzu photometer UV-160A.

The effect of the redox mediator 1-hydroxybenzotriazole (HBT) on enzymatic decolorization was tested at a final concentration of 2 mM prepared from a stock solution (100 mM HBT in 50% DMSO and 50 mM sodium phosphate buffer, pH 5.9). The influence of a clay mineral on the decolorization activity of laccases was investigated with 1% bentonite BII (w/v) in a sodium-tartrate buffer (50 mM, pH 3.5). Acid reaction conditions were chosen in order to allow adsorption of laccases onto bentonite.

Results

Laccase preparations from fungal cultures were tested for purity by SDS-PAGE and isoelectric focusing. In each case, one main protein band, which correlated with activity staining (not shown), could be detected. Only in the *M. thermophila* preparation were faint additional protein bands (less than 40 kDa) detected. The apparent molecular masses, IEPs and specific activities are compiled in Table 1.

As shown in Table 3, five of the nine dyes tested were directly decolorized by fungal laccases after 16 h incubation at 30°C. With respect to their molecular structure, all the anthraquinone-based dyes, the azo chemical Direct Red 28 and the indigoid dye Acid Blue 74 were oxidized regardless of whether dissolved in water or buffer. The final decolorization capacity of the laccases from *P. pinisitus* and *T. versicolor* was generally significantly higher than those of *M. thermophila*.

Enzymatic decolorization was substantial after 4 h of incubation (Fig. 1). The initial decolorization rate generally increased at substrate concentrations from 10 to 100 mg/l. At higher concentrations (200 mg/l) the indigoid dye Acid Blue 24 was decolorized to a similar extent but the degradation of the two anthraquinone dyes was incomplete. The laccase from *M. thermophila* was more sensitive to high substrate concentrations than those from the two basidiomycetes (Fig. 1, Table 4). However, resistance to decolorization is mainly attributed to the molecular structure of the dyes and not to enzyme inactivation because all laccases were nearly fully active after 16 h at a dye concentration of 20 mg/l, regardless of whether dyes were oxidized or not (Table 4).

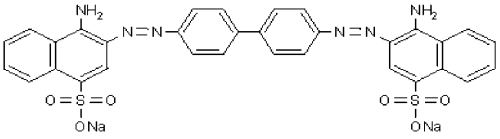
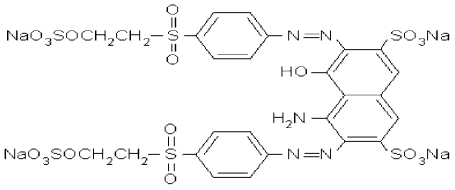
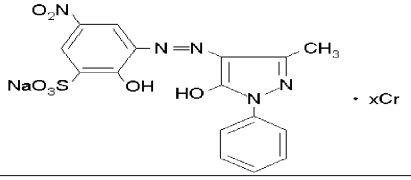
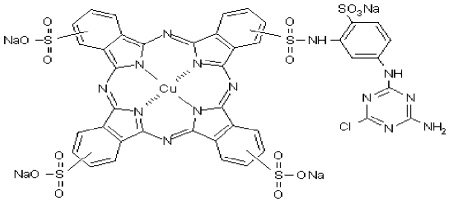
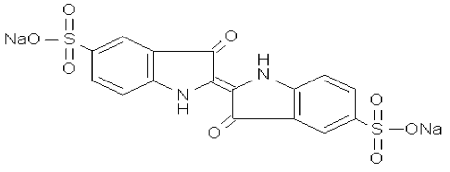
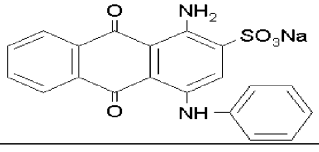
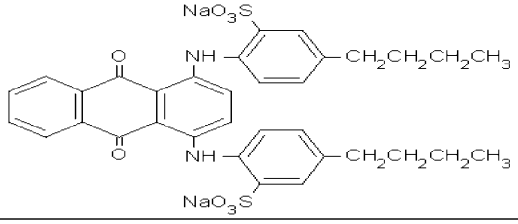
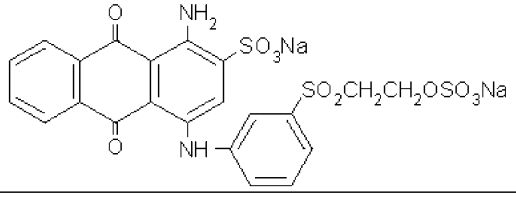
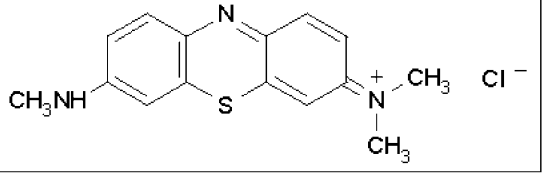
The enzyme concentration mainly determines the reaction velocity, but not the final level of dye decolorization. At a concentration of 1.6 nkat/ml, laccase from *P.*

Table 1 Some characteristics of fungal laccase preparations used in this study

Laccase source	Activity[a] (nkat/mg)	Protein content[a] (mg/ml)	MW[a] (kDa)	Isoelectric point (IEP)[a]	Optimum pH[b]	Thermal stability[b] (°C)
Myceliophthora thermophila	440	90	78	4.2	6.0	70–80
Polyporus pinisitus	920	34	65	3.0	4–5	50
Trametes versicolor	5,840	0.3	40	3.0	5.0	65

[a] This study
[b] Data from supplier

Table 2

Dye	Classification	λmax nm	MW	Structure
Direct Red 28	Azo dye	497	697	
Reactive Black 5	Azo dye	597	992	
Acid Orange 74	Azo dye	455	493	
Reactive Blue 15	Azo dye	675	1283	
Acid Blue 74	Indigoid dye	608	466	
Acid Blue 25	Anthraquinone	600	416	
Acid Green 27	Anthraquinone	605	707	
Reactive Blue 19	Anthraquinone	592	627	
Azure B	Heterocyclus	647	306	

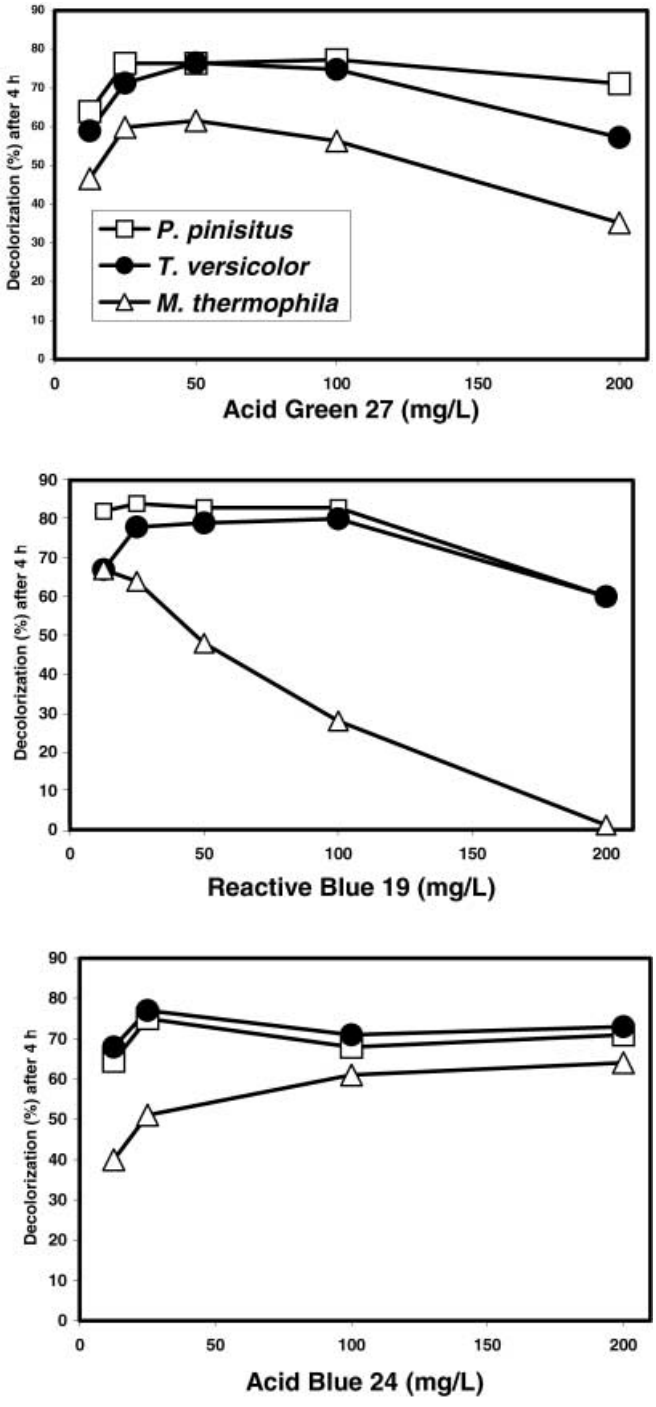


Fig. 1 Effect of dye concentration on decolorization by fungal laccases from *Polyporus pinisitus*, *Trametes versicolor* and *Myceliophthora thermophila*

pinisitus nearly completely decolorized the dye Acid Blue 74 after 16 h, whereas 17 nkat/ml required only 4 h (Fig. 2). In order to improve enzymatic decolorization, the influence of the redox mediator HBT was tested. As shown in Fig. 3, in the presence of 1–2 mM HBT, laccase from *P. pinsitus* decolorized the otherwise recalcitrant dye Reactive Black 5. At higher concentrations, the formation of colored HBT oxidation products interfered

Table 3 Enzymatic decolorization of some industrial dyes. *M.t. M. thermophila*, *P.p. P. pinisitus*, *T.v.: T. versicolor*. Incubation: 40 nkat/ml laccase and 20 mg/l dye in water or 50 mM MES (pH 5.5) for 16 h at 30°C

Dye	Decolorization (%)					
	Water			MES		
	Laccase from:			Laccase from:		
	M.t.	*P.p.*	*T.v.*	*M.t.*	*P.p.*	*T.v.*
Direct Red 28	9.6	46.9	11.9	2.8	33.8	33.4
Reactive Black 5	0	0	0	0	0	0
Acid Orange 74	0	0	0	0	0	0
Reactive Blue 15	0	6.0	0	0	6.0	0
Acid Blue 74	15.2	90.4	88.4	30.9	83.1	91.7
Acid Blue 25	53.3	59.8	66.0	49.8	51.0	56.0
Acid Green 27	67.0	71.0	76.0	66.0	71.0	74.0
Reactive Blue 19	31.2	58.5	64.5	0	59.9	61.6
Azure B	0	0	0	0	0	0

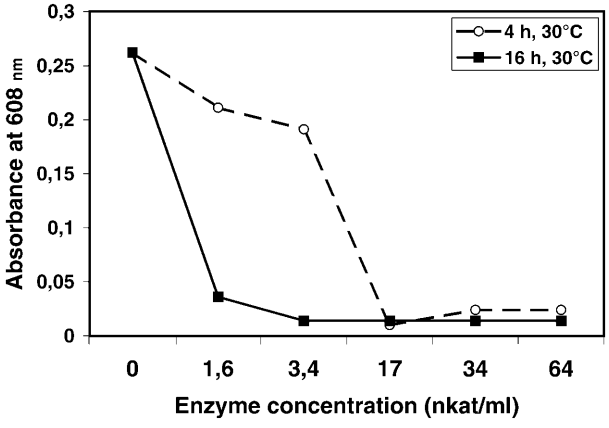


Fig. 2 Decolorization of Acid Blue 74 by different amounts of a laccase from *P. pinisitus*

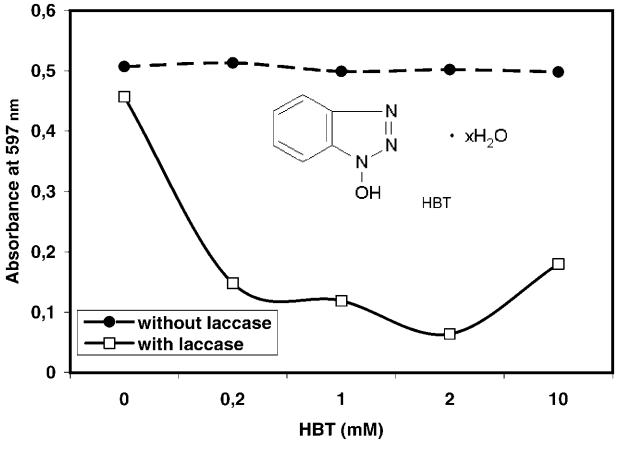


Fig. 3 Decolorization of Reactive Black 5 by a laccase from *P. pinisitus* (40 nkat/ml) in the presence of different concentrations of hydroxybenzotriazole (HBT)

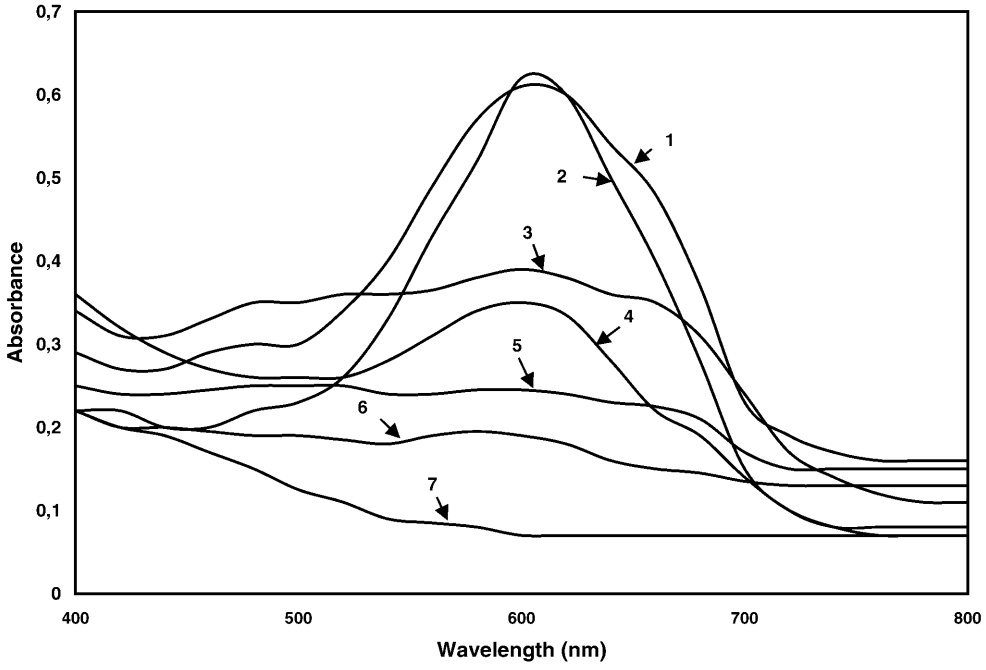


Fig. 4 Absorbance spectra of a dye mixture (eight dyes listed in Table 2 except Acid Orange 74, at a concentration of 20 mg/l for each dye in 0.1 M phosphate buffer, pH 5.9) with 2 mM HBT and/or laccases from *P. pinisitus* or *T. versicolor* (40 nkat/ml of each enzyme) after incubation for 16 at 30°C. Curves: *1* Dye mixture, *2* dye mixture +2 mM HBT, *3* dye mixture + laccase from *P. pinisitus*, *4* dye mixture + laccase from *T. versicolor*, *5* dye mixture + laccase from *P. pinisitus* + HBT, *6* dye mixture + laccase from *T. versicolor* + HBT, *7* laccases from *P. pinisitus* and *T. versicolor* + HBT

Table 4 Laccase activities in the presence of dyes. Incubation: laccase 40 nkat/ml and dye in water for 16 h at 30°C

Residual activities (%) after incubation with:								
Laccase source	Direct Red 28		Acid Blue 74		Reactive Blue 19		Azure B	
	mg/l		mg/l		mg/l		mg/l	
	20	200	20	200	20	200	20	200
M. thermophila	100	92	100	100	100	39	98	21
P. pinisitus	90	76	99	89	100	85	90	81
T. versicolor	91	89	95	95	96	83	100	60

Table 5 Enzymatic decolorization of dyes in presence of the redox mediator hydroxybenzotriazole (HBT). Incubation: 40 nkat/ml laccase from *P. pinisitus* and 200 mg/l dye in phosphate-buffer (100 mM, pH 5,9) for 16 h at 30°C

Dye	Decolorization (%)		
	2 mM HBT	Laccase	Laccase +2 mM HBT
Direct Red 28	43	47	64
Reactive Black 5	0	3	70
Reactive Blue 15	0	15	25
Acid Blue 74	4	82	86
Acid Blue 25	0	17	29
Reactive Blue 19	5	63	64
Acid Green 27	0	69	65
Azure B	0	16	73

Table 6 Decolorization (%) of dyes in the presence of bentonite. Incubation: 40 nkat/ml laccase and 1% (w/v) bentonite in sodium-tartrate buffer (50 mM, pH 3.5) for 16 h at 30°C

Assay	Reactive blue 19	Acid Green 27
Bentonite	17.8	59.8
Laccase from *M. thermophila*	0.0	13.5
Laccase from *M. thermophila* + bentonite	30.7	82.0
Laccase from *P. pinisitus*	91.4	92.1
Laccase from *P. pinisitus* + bentonite	97.0	97.7
Laccase from *T. versicolor*	90.0	92.1
Laccase from *T. versicolor* + bentonite	61.5	97.0

with the measurements. The addition of 2 mM HBT also supported the enzymatic decolorization of Reactive Blue 15, Acid Blue 25 and Azure B (Table 5). The positive effect of HBT was also found when mixtures of different dyes were exposed to enzymatic treatment. As is evident from Fig. 4, there was a significant reduction of the absorbance between 500 and 700 nm in the mixed VIS-spectrum after incubation with fungal laccases. The decrease was enhanced by the addition of 2 mM HBT, which in the absence of laccase had no significant decolorization effect. Enzymatic oxidation of HBT led to a slight increase in the absorbance in the short wave range of the spectrum.

Clay minerals like bentonite can be used for the cleaning of different wastewaters (e.g., from dye and textile industries) as an adsorbant and to improve precipitation of insoluble materials. The laccases from *P. pinsi-*

Table 7 Enzymatic decolorization of dyes with immobilized laccases. Incubation: 20 beads with enclosed laccase and 20 mg/l dye in 100 mM phosphate buffer (pH 5.9) for 16 h at 30°C for each cycle

Laccase from	Dye	Decolorization (%)			
		1st cycle	2nd cycle	3rd cycle	4th cycle
P. pinisitus	Direct Red 28	48	26	n.d.[a]	n.d.
T. versicolor		14	21	n.d.	n.d.
P. pinisitus	Reactive Blue 19	67	49	6	0
T. versicolor		8	18	2	0
P. pinisitus	Acid Blue 25	79	77	59	0
T. versicolor		72	75	64	42
P. pinisitus	Acid Green 27	94	96	78	43
T. versicolor		90	96	93	52
P. pinisitus	Direct Blue 21	24	8	3	0
T. versicolor		4	7	4	2

[a] Not determined (beads disintegrated)

tus and *T. versicolor* had a stronger decolorization effect than bentonite alone, in contrast to that of *M. thermophila*. On the other hand, a combination of laccases and bentonite was most effective (Table 6).

Experiments with immobilized laccases showed that the enzymes enclosed in alginate beads could be reused after the first incubation for 1–3 further incubations depending on the dye under test (Table 7). The activity loss in subsequent steps may have been due to enzyme inactivation and/or mechanical destruction of the beads.

Discussion

Cultures of streptomycetes are able to decolorize anthrone-type dyes (Pasti and Crawford 1991) and those of the white-rot basidiomycete *Phanerochaete chrysosporium* polymeric and triphenylmethane dyes (Glenn and Gold 1983; Bumpus and Brock 1988). Experiments with purified enzymes show that lignin-peroxidases and Mn-peroxidases in these cultures are the main agents of dye degradation (Goszczynski et al. 1994; Spadaro and Renganathan 1994; Chivukula and Renganathan 1995; Young and Yu 1997). However, in cultures of other white-rot fungi like *Phlebia tremellosa* (Kirby et al. 2000) or *Trametes hirsuta* (Abadulla et al. 2000), laccases seem to be responsible for dye decolorization. In a study by Rodriguez et al. (1999), 16 white-rot fungi were investigated for the decolorization of 23 industrial dyes. Laccase, Mn-peroxidase, lignin-peroxidase, and aryl alcohol oxidase activities were determined in crude extracts from solid-state cultures. Only the laccase activity correlated with the decolorization capacity and purified preparations were able to perform this reaction in vitro.

In our study, fungal laccase preparations from *P. pinisitus*, *T. versicolor* and *M. thermophila* decolorized synthetic dyes of diverse structures to a varying extent. Anthraquinone and indigoid-based dyes and, to a lesser degree, the azo dye Direct Red 28 (Congo Red) were directly decolorized by the laccases tested. Similarly, for a laccase from *T. hirsuta*, anthraquinoic dyes were found to be better substrates than azo dyes (Abadulla et al. 2000). The differences in azo dye oxidation may be explained by the different electron-donating properties of the substituents and their location on the phenolic ring. As pointed out by Chivukula and Renganathan (1995), an azo dye has to be electron-rich to be oxidized by a laccase from *Pyricularia oryzae*. This situation allows the enzymatic generation of a phenoxy radical and a breakdown mechanism that results in the cleavage of azo linkages and release of molecular nitrogen. The result is not only decolorization of the dye but also the exclusion of toxic aromatic amine formation. In a bacterial test system, Abadulla et al. (2000) found that the toxicity of several dyes, including azo compounds, was reduced by laccase treatment, although there was no strict correlation between decolorization and detoxification.

The inability to oxidize some dyes under test is not due to enzyme inactivation, but attributed to dye structure, because at dye concentrations of 20 mg/l, all laccases were essentially active after 16 h at 30°C. Only the enzyme from *M. thermophila* showed a significant loss in activity at high concentrations (200 mg/l) of Reactive Blue 19 and Azure B.

The enzymes proved to be active in the presence of 1% (w/v) bentonite BII. Commercial preparations of this clay mineral are used at recommended concentrations of 0.5% (w/v) for the treatment of various acid or alkaline industrial wastewaters. Laccases are adsorbed on the negatively charged surface of bentonites at pH values below the IEPs of the proteins (Claus and Filip 1988). Although the reaction conditions used (pH 3.5) favored adsorption of laccases onto bentonite, enzymatic decolorization was enhanced by the clay mineral.

Laccases immobilized in alginate beads decolorized phenolic effluents more efficiently than soluble enzymes in the experiments of Davis and Burns (1990), although beads were unsuitable for continuous use because the enzymes were rapidly released into solution. Similar results were obtained in our experiments with entrapped laccases for dye decolorization. Co-polymerization of laccase with tyrosine (Davis and Burns 1990) and/or additional stabilization of the alginate matrix may further extend the potential of these enzymes for biotechnological applications.

The laccases of *P. pinisitus* and *T. versicolor* showed a high decolorization capacity, whereas that of *M. thermophila* laccase was low. This seems to be attributed mainly to the different redox potentials (E_o) of the laccases, i.e., high (790±10 mV) for *P. pinsitus* compared to 450±10 mV for *M. thermophila* (Li et al. 1999). The latter was also more sensitive to higher substrate concentrations. Michaelis-Menten constants were not determined in this study, because decolorization kinetics were too slow.

Phenolic and nonphenolic compounds with high E_o values can be oxidized by laccases through the mediation of small, redox-active substrates like ABTS and HBT

(Bourbonnais et al. 1997; Li et al. 1999; Xu et al. 2000). Redox-mediated laccase catalysis has been used in a wide range of applications, such as pulp delignification, polycyclic aromatic hydrogen degradation, pesticide or insecticide degradation, and organic synthesis (Li et al. 1999). The degradation of the textile dye indigo with purified laccases from *T. hirsuta* and *Sclerotium rolfsii* was enhanced to about 30% in the presence of the redox-mediator acetosyringone (Campos et al. 2001). In experiments performed by Soares et al. (2001), the anthraquinone dye Remazol Brilliant Blue R was only decolorized when small molecular weight redox mediators like violuric acid or HBT were added with a laccase. As shown in our study, the addition of HBT significantly improved or facilitated decolorization of nine industrial dyes by fungal laccases. This observation holds also when mixtures of different dyes were used. These results suggest that laccase/mediator systems are useful biocatalysts for the treatment of effluents from textile, dye or printing industries. Mechanisms and products of the enzymatic breakdown of dyes need to be elucidated further.

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