Applied and Environmental Microbiology, Dec. 1997, p. 4627–4632 Vol. 63, No. 12
0099-2240/97/$04.00+0
Copyright © 1997, American Society for Microbiology

Reactivities of Various Mediators and Laccases with Kraft Pulp and Lignin Model Compounds

R. BOURBONNAIS,[1]* M. G. PAICE,[1] B. FREIERMUTH,[2]† E. BODIE,[3] and S. BORNEMAN[3]

Pulp and Paper Research Institute of Canada, Pointe Claire, Quebec H9R 3J9, Canada[1]; Ciba-Geigy Ltd., D-79630 Grenzach-Wyhlen, Germany[2]; and Genencor International Inc., Palo Alto, California 94304-1013[3]

Received 24 March 1997/Accepted 6 September 1997

Laccase-catalyzed oxygen delignification of kraft pulp offers some potential as a replacement for conventional chemical bleaching and has the advantage of requiring much lower pressure and temperature. However, chemical mediators are required for effective delignification by laccase, and their price is currently too high at the dosages required. To date, most studies have employed laccase from *Trametes versicolor*. We have found significant differences in reactivity between laccases from different fungi when they are tested for pulp delignification in the presence of the mediators 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) and 1-hydroxybenzotriazole (HBT). A more detailed study of *T. versicolor* laccase with ABTS and HBT showed that HBT gave the most extensive delignification over 2 h but deactivated the enzyme, and therefore a higher enzyme dosage was required. Other mediators, including 1-nitroso-2-naphthol-3,6-disulfonic acid, 4-hydroxy-3-nitroso-1-naphthalenesulfonic acid, promazine, chlorpromazine, and Remazol brilliant blue, were also tested for their ability to delignify kraft pulp. Studies with dimeric model compounds indicated that the mechanisms of oxidation by ABTS and HBT are different. In addition, oxygen uptake by laccase is much slower with HBT than with ABTS. It is proposed that the dication of ABTS and the 1-oxide radical of HBT, with redox potentials in the 0.8- to 0.9-V range, are required for pulp delignification.

The move away from the use of molecular chlorine for kraft pulp bleaching has opened up new opportunities for biotechnology in this field. Xylanase was the first enzyme reported to facilitate pulp bleaching (23) and is now used in several mills. However, the effect of xylanase on bleaching is limited to about 25% chemical savings. Bleaching of kraft pulps with the white-rot fungus *Trametes* (*Coriolus*) *versicolor* has been shown to produce larger chemical savings, but the rate of fungal pulp delignification is too slow for commercial application (15, 18). The fungal bleaching effect is accompanied by the secretion of at least two lignin-oxidizing enzymes, laccase and manganese peroxidase (3, 16, 19). Recently, these two enzymes were shown to significantly increase pulp brightness when they were combined with subsequent alkaline peroxide treatment (17).

Laccase is a multicopper oxidase which reduces oxygen to water and simultaneously performs one-electron oxidation of many aromatic substrates (20). Laccase alone has a limited effect on pulp bleaching due to its specificity for phenolic subunits in lignin (11). We found that the substrate range of laccase can be extended to nonphenolic subunits of lignin by inclusion of a mediator, such as 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) (Fig. 1) (2). Furthermore, the laccase-ABTS couple has also been shown to effectively demethylate and delignify kraft pulp (3). It seems likely that the oxidized ABTS must function as a diffusible lignin-oxidizing agent, because laccase is a large molecule (molecular weight, around 70,000) and therefore cannot enter the fiber secondary wall to contact the lignin substrate directly. Recently, a natural laccase mediator, 3-hydroxyanthranilic acid, was found to be produced by the white-rot fungus *Pycnoporus cinnabarinus* (8). In the presence of this mediator, laccase oxidizes nonphenolic lignin model compounds, although no evidence of pulp delignification has been found to date.

Over 55% delignification of kraft pulp has been reported with laccase and another mediator, 1-hydroxybenzotriazole (HBT) (6), or after repeated treatment with laccase-ABTS followed by alkaline extraction (4). These observations have provoked considerable interest in enzyme-catalyzed oxidative bleaching of kraft pulps. Also, several phenothiazines have been reported to mediate laccase-catalyzed bleaching of textile dyes (22). In the present paper, we compare the reactivities of ABTS, HBT, phenothiazines, and several new mediators on kraft pulp and lignin model compounds in combination with various fungal laccases.

MATERIALS AND METHODS

Chemicals. Veratryl alcohol, Remazol brilliant blue (RBB), HBT, promazine (PZ), chlorpromazine (CPZ), 1-nitroso-2-naphthol-3,6-disulfonic acid (NNDS), 4-hydroxy-3-nitroso-1-naphthalenesulfonic acid (HNNS), and benzotriazole (BT) were obtained from Aldrich Chemical Co., and ABTS was obtained from Boehringer Mannheim. The lignin model dimers 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)-propane-1,3-diol (dimer I) and 1-(3,4-dimethoxyphenyl)-2-phenylethanediol (dimer II) were synthesized by N. G. Lewis (Washington State University), and 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)-3-hydroxy-1-propanone (dimer III) was kindly provided by T. Fukuzumi (Tokyo University of Agriculture). The structures of the mediators and lignin model compounds are shown in Fig. 1.

Enzyme production. Crude laccases from various fungal strains, obtained from the American Type Culture Collection, were prepared from 2,5-xylidine-induced liquid cultures (9) and concentrated by ultrafiltration. Laccases I and II from *T. versicolor* were produced and partially purified by ion-exchange chromatography on DEAE–Bio-Gel as described previously (5).

Enzyme assays. Laccase activity was assayed by measuring oxidation of ABTS (24). The assay mixture contained 0.5 mM ABTS, 0.1 M sodium acetate (pH 5.0), and a suitable amount of enzyme. Oxidation of ABTS was monitored by measuring the increase in A_{420} ($\varepsilon_{420} = 3.6 \times 10^4\ M^{-1} \cdot cm^{-1}$). Enzyme activity was expressed in units; 1 U was defined as 1 μmol of ABTS oxidized per min.

Oxygen consumption. Oxygen uptake during mediator oxidation by laccase was measured with a Clark oxygen electrode (Rank Brothers, Cambridge, United

* Corresponding author. Mailing address: Pulp and Paper Research Institute of Canada, 570 St. John's Boulevard, Pointe Claire, Quebec H9R 3J9, Canada. Phone: (514) 630-4100. Fax: (514) 630-4134. E-mail: Bourbonnais@paprican.ca.

† Present address: Novartis Services AG, K-127.6.70, 4002 Basel, Switzerland.

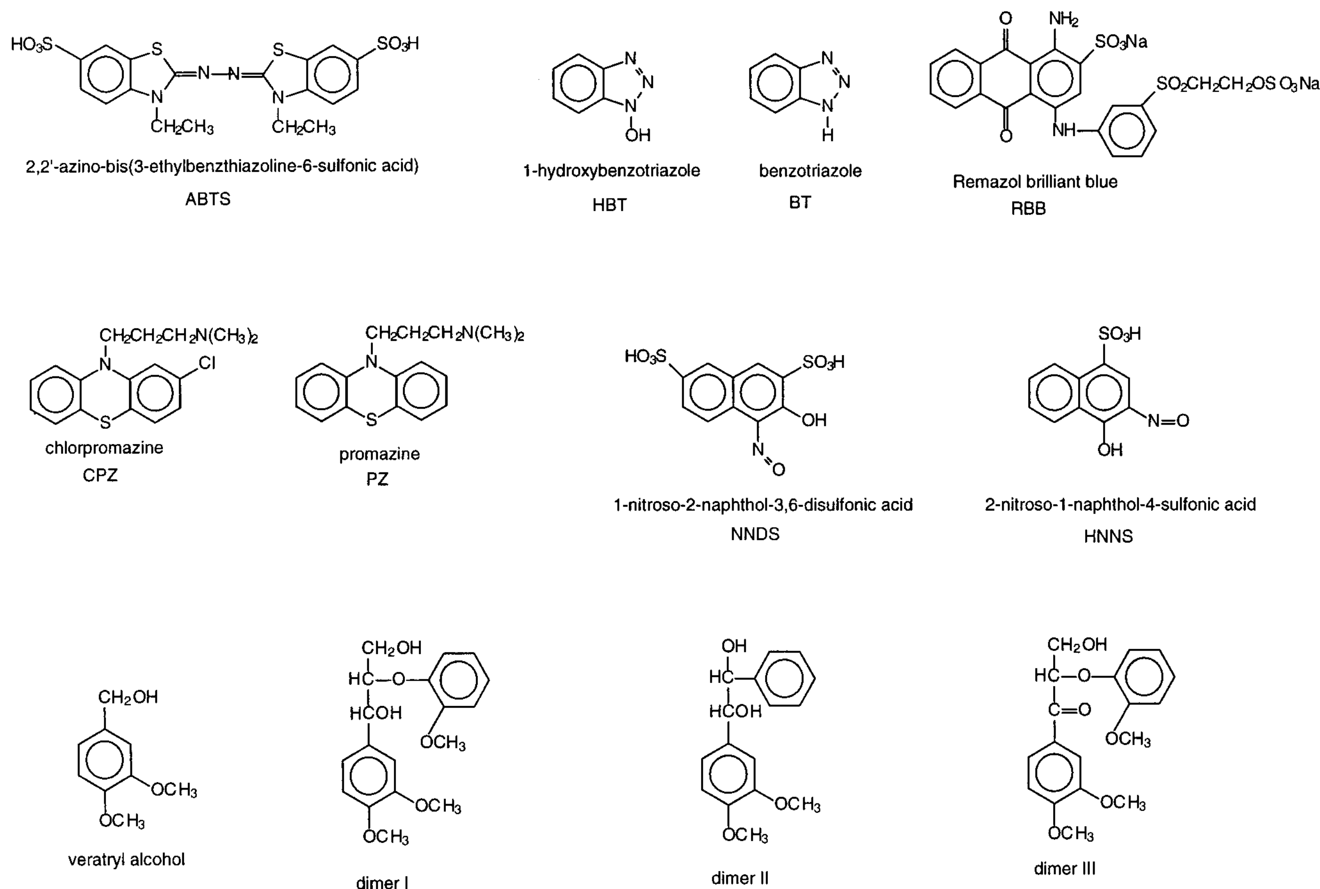

FIG. 1. Structures of laccase mediators and lignin model compounds.

Kingdom) at room temperature (22°C) under atmospheric air saturation conditions (0.28 mM O_2). The reaction mixture contained 3 μmol of mediator and 0.6 U of laccase in 3 ml of 0.05 M sodium acetate buffer (pH 5.0).

Oxidation of nonphenolic model compounds. Each reaction mixture (10 ml) contained either 2 mM veratryl alcohol, 2 mM dimer I or 2 mM dimer II, 1 mM mediator, and 0.5 U of laccase per ml in 0.05 M sodium acetate buffer (pH 5). Samples were taken at designated times, heated in boiling water to stop the reaction, acidified to pH 2 with HCl, and filtered through a 0.45-μm-pore-size filter prior to high-performance liquid chromatography (HPLC) analysis as described previously (2).

Pulp treatments. Never dried softwood oxygen-delignified pulp from a western Canadian mill was washed with 0.05 M sodium acetate buffer (pH 5.0), filtered, and heated to 60°C. The mediator (10 mg/g of pulp) was solubilized in a sufficient amount of acetate buffer to obtain a final pulp consistency of 10%. Laccase (5 U/g of pulp, unless otherwise specified) was added to the mediator solution at 60°C and immediately mixed with the pulp in a Hobart mixer for 1 min. The pulp was then transferred to a pressurized vessel (Amicon ultrafiltration cell [capacity, 400 ml] with the membrane replaced by a plastic film to seal the chamber). The reactor was then pressurized with oxygen (140 kPa) and immersed in a water bath at 60°C. After a 2-h reaction, the pulp was filtered and washed with deionized water. The liquid extract before washing was assayed for residual laccase activity as described above and for methanol concentration by gas chromatography (3). Subsequent alkaline extraction was performed at 10% pulp consistency with 0.05 M NaOH for 90 min at 70°C.

Pulp testing. Handsheets (i.e., sheets of paper produced from pulp samples in the laboratory) were prepared with a British Standard handsheet machine (Noram) by using 4 g of pulp that had been homogenized for 30 s in a Brookfield counterrotating mixer. Each handsheet was then air dried on a blotter and tested for kappa number (standard testing method G18 of the Technical Section of the Canadian Pulp and Paper Association).

RESULTS

Oxidation of lignin model compounds by laccase and ABTS or HBT. A comparison of the rates of oxidation of veratryl alcohol (2 mM) in the presence of either 1 mM ABTS, 1 mM HBT, or a combination of the two (each at a concentration of 0.5 mM) is shown in Fig. 2. The results show that the rate of veratryl alcohol oxidation was higher with HBT than with ABTS. A combination of the two mediators gave a rate slightly higher than the rate observed with HBT alone, with almost all

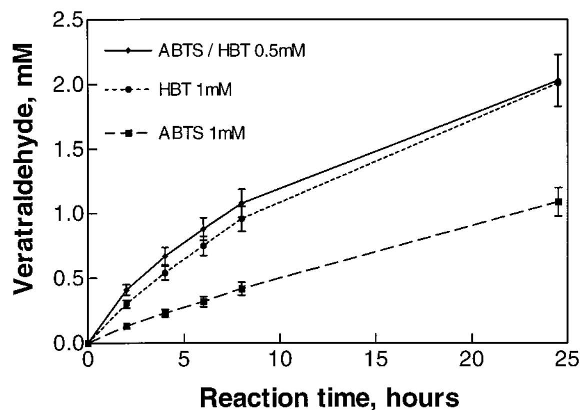

FIG. 2. Oxidation of veratryl alcohol (2 mM) to veratraldehyde by laccase II (0.5 U/ml) from *T. versicolor* in the presence of either 1 mM ABTS, 1 mM HBT, or a combination of ABTS and HBT (each at a concentration of 0.5 mM). Experiments were performed in duplicate, and the maximum deviation was less than 10%.

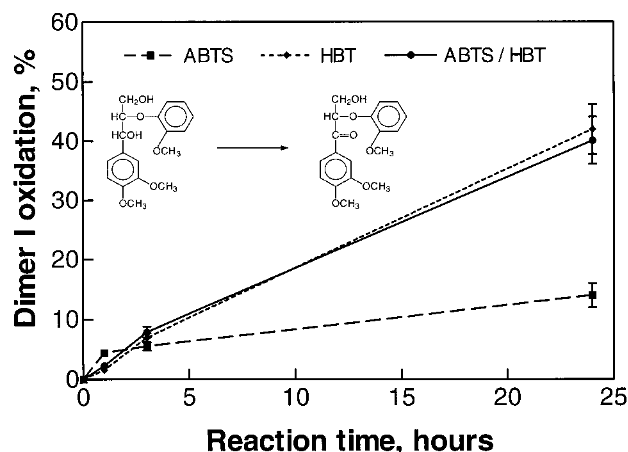

FIG. 3. Oxidation of β-O-4 dimer I (2 mM) to α-carbonyl dimer III by *T. versicolor* laccase II (0.5 U/ml) in the presence of either 1 mM ABTS, 1 mM HBT, or a combination of ABTS and HBT (each at a concentration of 0.5 mM). HPLC analyses of samples were performed in duplicate. Error bars represent maximum deviation.

of the veratryl alcohol oxidized to the aldehyde within 1 day. Figure 3 shows the rate of β-O-4 lignin model dimer I oxidation by laccase in the presence of ABTS and HBT. The resulting product was identified as the α-carbonyl oxidation product of dimer I based on its retention time and UV-visible light absorption spectrum relative to authentic model dimer III. No cleavage products were detected following oxidation with either mediator. The rate of oxidation was higher with HBT (1 mM) than with ABTS (1 mM); after 1 day 40% of dimer I was oxidized with HBT, compared to 12% with ABTS. The rate and extent of dimer I oxidation in the presence of both mediators (0.5 mM each) were about the same as the rate and extent of dimer I oxidation with HBT (1 mM) alone. With β-1 dimer II (Fig. 4) the reaction products were different with ABTS and HBT. We showed previously (2) that with laccase and ABTS this dimer was cleaved to give veratraldehyde and benzaldehyde, and no carbonyl dimer derivatives were detected. During the oxidation of dimer II with laccase and HBT,

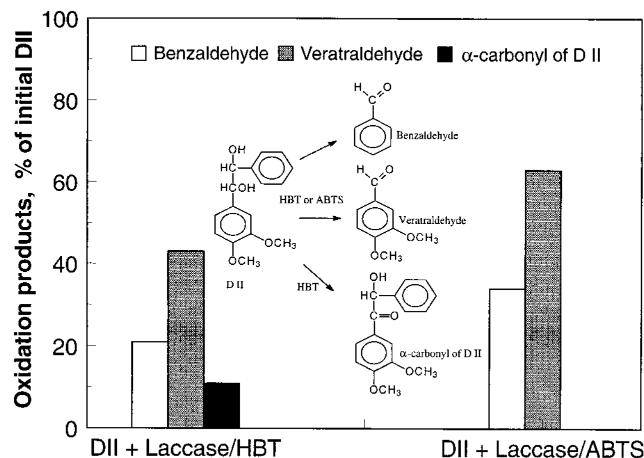

FIG. 4. Oxidation of β-1 dimer II (2 mM) by *T. versicolor* laccase II (0.5 U/ml) in the presence of either ABTS (1 mM) or HBT (1 mM) for 24 h at 23°C. The amount of the proposed α-carbonyl dimer produced was estimated from the dimer III absorption coefficient at 280 nm. HPLC analyses of samples were performed in duplicate. DII, dimer II.

TABLE 1. Oxygen uptake by laccases from *T. versicolor* with various mediators

Mediator	Rate of oxygen uptake (μmol of O_2/min, ± maximum deviation) by[a]:	
	Laccase I	Laccase II
ABTS	0.21 ± 0.01 (100)[b]	0.28 ± 0.01 (100)
HBT	0.0025 ± 0.0002 (1.2)	0.0015 ± 0.0002 (0.5)
NNDS	0.116 ± 0.009 (55)	0.129 ± 0.01 (46)
HNNS	0.122 ± 0.01 (58)	0.130 ± 0.016 (46)
RBB	0.082 ± 0.004 (39)	0.049 ± 0.001 (18)
PZ	0.106 ± 0.007 (50)	0.165 ± 0.015 (59)
CPZ	0.052 ± 0.002 (25)	0.078 ± 0.003 (28)

[a] Rates of O_2 uptake were measured in the first 1 min of reaction. Experiments were done in duplicate.
[b] The values in parentheses are percentages of the value obtained with ABTS.

veratraldehyde and benzaldehyde were still the major oxidation products, but a third compound eluting later in the chromatogram was produced with a UV-visible spectrum characteristic of carbonyl compounds. This product was purified by HPLC and analyzed by high-resolution mass spectrometry by using the fast atom bombardment technique with nitrobenzyl alcohol as the internal source of protons. A parent peak (M^+ = 273) was shown to correspond to a protonated compound with the following atomic composition: $C_{16}H_{16}O_4$. The proposed structure of this α-carbonyl dimer is shown in Fig. 4.

Oxygen uptake by laccase in the presence of mediators. The reactivity of laccases I and II from *T. versicolor* with different mediators was determined by measuring O_2 consumption (Table 1). Except for HBT, all of the mediators tested had relatively high initial rates of oxygen uptake for both laccase isozymes, ranging from 18 to 60% of the rates obtained with ABTS. However, the rate of O_2 uptake with both laccases in the presence of HBT was extremely low, only around 1% of the rate obtained with ABTS, even though HBT is as good as or even better than ABTS for lignin oxidation.

Comparison of pulp delignification with various mediators. To compare the efficiencies of various mediators for pulp delignification, we used the optimal conditions developed previously for *T. versicolor* laccase and ABTS. Table 2 shows the pulp kappa number, the amount of methanol released during demethylation, and the stability of laccase following a 2-h treatment of softwood oxygen-delignified kraft pulp under the conditions described above. Under these conditions, the maximum delignification was obtained with HBT. PZ and CPZ did not promote pulp delignification compared to controls without mediator and laccase. On a molar ratio basis (moles of lignin C_9 units released per initial mole of mediator), ABTS was by far the best mediator for pulp delignification. The mediator efficiency is the ratio of the initial molar concentration of the mediator to the amount of lignin released from the pulp and is governed by two factors: the rate of oxidation of mediator by laccase and the reactivity of the oxidized mediator with lignin. The low molecular weight of HBT makes its molar efficiency much less than that of ABTS and even less than that of RBB.

All of the mediators, including the promazines, were able to mediate pulp demethylation (Table 2). It seems that, although demethylation of lignin occurs during pulp treatment by laccase and mediators, it is not sufficient to provide lignin removal. Laccase from *T. versicolor* is relatively stable with most of the mediators used except HBT, with which only 2% of the initial laccase activity was recovered following the 2-h pulp treatment. For this reason, we routinely added more laccase in

TABLE 2. Comparison of pulp delignification with *T. versicolor* laccase and various mediators

Mediator	Amt of demethylation (mg of CH_3OH/liter)	% Residual laccase	Kappa no., ± maximum deviation[a]	% Delignification	Mediator efficiency (no. of C_9 units/mol of mediator)[b]
Control	3.0		9.9 ± 0.1	10.8	
ABTS	58.1	32	7.3 ± 0	34.2	1.22
HBT	51.2	2	6.9 ± 0.1	37.8	0.35
NNDS	33.2	76	8.1	26.4	0.58
HNNS	44.5	50	7.6	30.1	0.64
RBB	28.0	82	9.1 ± 0.1	18.0	0.43
PZ	42.2	52	10.4 ± 0.2	6.3	−0.14
CPZ	43.3	50	9.9 ± 0	10.8	0

[a] Pulp kappa numbers were measured in duplicate.
[b] Mediator efficiency was calculated as follows: (kappa number of mediator − kappa number of control) × [(8.52×10^{-6} mol of C_9 units/g of pulp)/(moles of mediator/g of pulp)]. The molecular weight of a C_9 unit was estimated to be 196.

the presence of HBT (20 U/g of pulp versus 5 U/g for all of the other mediators).

Effect of buffer on pulp delignification with laccase and mediator. Delignifying activities of laccase with ABTS and HBT were compared in different buffers. Table 3 shows results of pulp delignification experiments following treatment in sodium acetate or citrate (pH 5 and 0.05 M) or in unbuffered preparations with the pH adjusted to between 4 and 5 with sulfuric acid. Pulp delignification by laccase was considerably greater with both mediators in unbuffered medium than in buffered medium; with HBT delignification was 22% in acetate buffer and 42% in unbuffered medium. With citrate buffer an improvement over acetate buffer was observed only when HBT was used as the mediator. In citrate buffer there was some decrease in pH in the control and with both mediators, whereas in acetate buffer the pH was more stable. The initial pH of the unbuffered reaction mixture with laccase and HBT was low (pH 4) because of the acidity of the HBT solution, and the final pH dropped to 3.3. We have shown that pulp delignification with laccase-ABTS in acetate is not changed significantly between pHs 3 and 6 (4), so the pH of the reaction mixture cannot account for the improvement in pulp delignification.

Reaction products with HBT. Following the delignification of pulp with laccase and HBT, the reaction products present in solution were determined by HPLC. The reduced species BT was identified by comparison with authentic sample and was quantified together with residual HBT (Table 3). The formation of BT, like pulp delignification, was lower in buffered solutions than in unbuffered solutions. However, BT itself did not function as a mediator. Also, BT was not detected when laccase and HBT oxidized veratryl alcohol or nonphenolic lignin model dimers.

Pulp delignification with various laccases. We found previously that laccases I and II from *T. versicolor* were both effective for demethylation and delignification of kraft pulps when the mediator ABTS was present (5). Here we compared the delignifying activities of crude laccase preparations from various white-rot fungi (Table 4). All of the laccases were tested alone and in combination with the mediators ABTS and HBT under the optimized conditions for *T. versicolor* described above. Without mediator, none of the laccases could effectively delignify kraft pulp; the kappa number reduction ranged between 7 and 17%, and a control treatment without laccase gave a kappa number reduction of 12.7%. However all of the laccases were able to delignify pulp in the presence of either ABTS or HBT, although the extents of delignification and demethylation were quite variable. In the presence of ABTS, the reduction in kappa number with all laccases was between 30 and 35%, and the pulp demethylation as measured by methanol release was between 53.3 and 69 mg/liter. With HBT, the variation was even greater; the kappa number decrease was as low as 12.7% for *Pleurotus ostreatus* laccase, and delignification was as high as 40% with *Ganoderma colossum*. In general, pulp demethylation by laccases in the presence of HBT was much lower than pulp demethylation by laccases in the presence of ABTS, except for *G. colossum* laccase, which released 75 mg of methanol per liter in the presence of HBT. Table 4 also shows that there was wide variation from one strain to another in the amount of residual enzyme activity recovered following the treatments. However, the general trend that emerged is that all

TABLE 3. Effect of buffer on pulp delignification by laccase from *T. versicolor* and mediator

Pulp treatment	Prepn	Initial pH	Final pH	Kappa no., ± maximum deviation[a]	% Delignification	% Residual HBT	% BT formed
Control	Acetate	5.0	ND[b]	9.9 ± 0.1	10.8		
	Citrate	4.7	4.4	10.4 ± 0.1	6.3	ND	ND
	H_2SO_4	4.4	4.9	9.8 ± 0.1	11.7		
Laccase and ABTS	Acetate	4.9	4.9	8.5 ± 0.1	23.4		
	Citrate	4.8	4.4	8.8 ± 0.2	20.7	ND	ND
	H_2SO_4	4.4	4.6	7.7 ± 0.1	30.6		
Laccase and HBT	Acetate	5.0	ND	8.6 ± 0.2	22.5	62.1	22.9
	Citrate	4.9	4.4	7.5 ± 0.1	32.4	40.8	39.1
	H_2SO_4	4.0	3.3	6.9 ± 0.1	42.3	48.1	44.4

[a] Kappa numbers were measured in duplicate.
[b] ND, not determined.

TABLE 4. Kraft pulp delignification with various laccases

Pulp treatment[a]	Amt of demethylation, (mg of CH_3OH/liter)	% Residual laccase	Kappa no.	% Delignification
Control pulp	2.1		9.6	12.7
Phlebia radiata (5 U/g)	3.7	30	10.2	7.3
Phlebia radiata (5 U/g) + ABTS	53.3	32	7.2	34.5
Phlebia radiata (5 U/g) + HBT	14.5	11	8.7	20.9
Lentinus edodes (5 U/g)	4.0	6	9.5	13.6
Lentinus edodes (5 U/g) + ABTS	55.6	13	7.5	31.8
Lentinus edodes (5 U/g) + HBT	12.3	8	9.1	17.3
Trametes versicolor (5 U/g)	14.0	50	9.5	13.6
Trametes versicolor (5 U/g) + ABTS	56.9	27	7.7	30
Trametes versicolor (5 U/g) + HBT	47.3	13	7.7	30
Pleurotus ostreatus (3.9 U/g)	20.5	1.1	9.5	13.6
Pleurotus ostreatus (3.9 U/g) + ABTS	ND[b]	ND	7.7	30
Pleurotus ostreatus (3.9 U/g) + HBT	12.3	1.4	9.6	12.7
Merulius tremellosus (1.2 U/g)	ND	72	9.7	11.8
Merulius tremellosus (1.2 U/g) + ABTS	58.5	86	7.4	32.7
Merulius tremellosus (1.2 U/g) + HBT	22.6	48	8.9	19.1
Fomes fomentarius (5 U/g)	10.5	57	9.4	14.5
Fomes fomentarius (5 U/g) + ABTS	60.3	15	7.3	33.6
Fomes fomentarius (5 U/g) + HBT	40.5	13	7.5	31.8
Ganoderma colossum (5 U/g)	20.8	90	9.1	17.2
Ganoderma colossum (5 U/g) + ABTS	69.0	63	7.7	30.0
Ganoderma colossum (5 U/g) + HBT	75.2	24	6.6	40.0

[a] The laccases were obtained from the organisms. The units in parentheses indicate the amount of laccase used per gram of pulp.
[b] ND, not determined.

of the fungal laccases tested are more susceptible to deactivation in the presence of HBT than in the presence of ABTS.

Veratryl alcohol oxidation with various laccases. The fungal laccase preparations were also assayed for veratryl alcohol oxidation in the presence of mediators. Table 5 shows that there is wide variation from one laccase to another in the capacity to oxidize veratryl alcohol in the presence of the mediators ABTS and HBT. Laccases I and II from *T. versicolor* differ from the other enzymes in giving almost complete oxidation of veratryl alcohol with both mediators at pH 3.5 and extensive oxidation in the presence of HBT at pH 5. Laccases from *Phlebia radiata*, *Fomes fomentarius*, and *Ganoderma colossum* were also relatively effective, with 40 to 80% of the veratryl alcohol oxidized after 1 h at pH 3.5 with both ABTS and HBT. However, laccases from *Pleurotus ostreatus*, *Merulius tremellosus*, and *Lentinus edodes* seem to be much less effective in oxidizing veratryl alcohol in the presence of both mediators, even though they are relatively effective for pulp delignification, at least with ABTS. For all enzymes, the veratryl alcohol oxidation activity was higher at pH 3.5 than pH 5 with both ABTS and HBT.

TABLE 5. Veratryl alcohol oxidation with various laccases[a]

Source of laccase	% of veratryl alcohol oxidized to veratraldehyde with:			
	ABTS (pH 3.5)	HBT (pH 3.5)	ABTS (pH 5)	HBT (pH 5)
Trametes versicolor (laccase I)	95	93	26	78
Trametes versicolor (laccase II)	80	95	16	75
Phlebia radiata	82	47	ND[b]	40
Fomes fomentarius	58	39	9	36
Ganoderma collosum	ND	48	7	31
Lentinus edodes	ND	14	8	8
Merulius tremellosus	10	10	3	20
Pleurotus ostreatus	13	2	3	3

[a] The concentrations of laccases, veratryl alcohol, and mediators were the same as those used in the experiments shown in Fig. 2. The reaction time was 1 h at 60°C in either citrate buffer (pH 3.5) or acetate buffer (pH 5).
[b] ND, not determined.

DISCUSSION

There has been increased interest in the use of mediators with laccases since the initial discovery that nonphenolic lignin model compounds can be oxidized and pulp can be delignified by laccase and ABTS (2, 3). Since then, HBT has been found to mediate pulp delignification in the presence of laccase (6), and phenothiazines have been found to bleach and prevent redeposition of azo dyes in textile manufacturing (22). Our results show that ABTS and HBT have very similar capacities to delignify kraft pulp and to oxidize nonphenolic lignin model compounds. However, the rate of veratryl alcohol and β-O-4 dimer I oxidation was higher with HBT than with ABTS, and the reaction mechanisms with β-1 dimer II were distinct for the two mediators, as shown by the difference in the reaction products.

The oxidation of HBT by laccase is very slow, more than 85 times slower than oxidation of ABTS (as measured by O_2 uptake [Table 1]), even though HBT mediation activity is as good as or even better than ABTS mediation activity. It seems likely that the reactive form of oxidized ABTS is produced at

a rate comparable to the HBT oxidation rate. The cation radical ($ABTS^{+\cdot}$), which is produced at a high rate, was shown to be unreactive with veratryl alcohol (2, 14) and failed to delignify kraft pulp. However $ABTS^{+\cdot}$ can be oxidized to its dication form ($ABTS^{2+}$) by oxidizing agents (13). The oxidation of HBT to the benzotriazolyl-1-oxide radical (1) and the oxidation of $ABTS^{+\cdot}$ to $ABTS^{2+}$ (12), both of which occur between 1 and 1.1 V (versus normal hydrogen electrode), are likely to proceed very slowly with fungal laccases which have redox potentials between 0.7 and 0.8 V (25). It is interesting that oxidation of HBT with laccase resulted in the formation of BT, which is a reduced product of HBT, and that veratryl alcohol or nonphenolic dimers prevent BT formation. Its seems likely that in the presence of these nonphenolic compounds the reactive radical of HBT cycles back to its original state by transferring its electron to the substrate. However, in the absence of readily available nonphenolic compounds, the HBT radical seems to undergo complex redox reactions that lead to a more reduced species.

PZ and CPZ are also known to form cation radicals and dications following voltammetric oxidation (21), but neither of these compounds can mediate the laccase-catalyzed oxidation of veratryl alcohol or delignification of pulp. This may be because the dications of PZ and CPZ are unstable and decay very rapidly (7). RBB and the nitrosonaphthol compounds NNDS and HNNS were also found to mediate laccase-catalyzed veratryl alcohol oxidation and pulp delignification. However, the nature of their oxidation states which are likely to promote lignin oxidation has yet to be characterized.

We tested seven crude preparations of laccase from various white-rot fungi and found that they are all equally capable of delignifying kraft pulps when they are coupled to ABTS. However, their performances with HBT were much more variable, which can be explained in part by their rates of activity loss in the presence of this mediator. The fact that crude enzyme preparations were used in this study does not permit a clear distinction between the stability of the laccase itself and the stabilizing effect of the other proteins present in the enzyme preparations. Laccase reactivities toward oxidation of veratryl alcohol in the presence of ABTS and HBT also show wide variations. The enzymes from *P. ostreatus*, *M. tremellosus*, and *L. edodes* were much less efficient in catalyzing veratryl alcohol oxidation than the other enzyme preparations (Table 5). This same group of enzymes was also less efficient in promoting pulp delignification in the presence of HBT (Table 4). In a recent study, Xu et al. (25) compared the reactivities and redox potentials of fungal laccases and concluded that higher redox potential correlates with higher activity. They concluded that structural differences in the substrate activation site (the blue, type I copper center) control the redox potentials, as well as the substrate specificities, of the laccases. We do not know the redox potentials of the laccases used in this work, except the *T. versicolor* laccase, which has one of the highest values measured for laccases (0.77 V, as reported by Fee and Malmström [10]). The differences in the redox potentials of the laccases are likely to control their reactivities with mediators. *T. versicolor* laccase has the highest capacity to oxidize veratryl alcohol and delignify kraft pulp with both mediators.

ACKNOWLEDGMENTS

We thank Mary Ann Fitzsimmons and Francis Young for skilled technical assistance and M. Evans from the Regional Center for Mass Spectrometry of the Université de Montréal for the high-resolution mass spectrum.

This research was supported in part by Industry Canada, Genencor International, and Ciba-Geigy Ltd.

REFERENCES

1. **Aurich, H. G., G. Bach, K. Hahn, G. Küttner, and W. Weiss.** 1977. Aminyloxides (nitroxides). Part XXV. Reactions of benzotriazolyl oxide radicals with aromatic compounds. J. Chem. Res. **1977:**1544–1566.
2. **Bourbonnais, R., and M. G. Paice.** 1990. Oxidation of non-phenolic substrates. An expanded role for laccase in lignin biodegradation. FEBS Lett. **267:**99–102.
3. **Bourbonnais, R., and M. G. Paice.** 1992. Demethylation and delignification of kraft pulp by *Trametes versicolor* laccase in the presence of 2,2′ azinobis-(3-ethylbenzthiazoline-6-sulphonate). Appl. Microbiol. Biotechnol. **36:**823–827.
4. **Bourbonnais, R., and M. G. Paice.** 1996. Enzymatic delignification of kraft pulp using laccase and a mediator. Tappi (Tech. Assoc. Pulp Pap. Ind.) J. **79(6):**199–204.
5. **Bourbonnais, R., M. G. Paice, I. D. Reid, P. Lanthier, and M. Yaguchi.** 1995. Lignin oxidation by laccase isozymes from *Trametes versicolor* and role of the mediator 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) in kraft lignin depolymerization. Appl. Environ. Microbiol. **61:**1876–1880.
6. **Call, H. P.** December 1994. Process for modifying, breaking down or bleaching lignin, materials containing lignin or like substances. PCT. World patent application WO 94/29510.
7. **Cheng, H.-Y., P. H. Sackett, and R. L. McCreery.** 1978. Reactions of chlorpromazine cation radical with physiologically occurring nucleophiles. J. Med. Chem. **21:**948–952.
8. **Eggert, C. B., U. Temp, and K.-E. Eriksson.** 1996. A fungal metabolite mediates degradation of non-phenolic lignin structures and synthetic lignin by laccase. FEBS Lett. **391:**144–148.
9. **Fahraeus, G., and B. Reinhammar.** 1967. Large-scale production and purification of laccase from cultures of the fungus *Polyporus versicolor* and some properties of laccase A. Acta Chem. Scand. **21:**2367–2378.
10. **Fee, J. A., and B. G. Malmström.** 1968. The redox potential of fungal laccase. Biochim. Biophys. Acta **153:**299–302.
11. **Higuchi, T.** 1989. Mechanisms of lignin degradation by lignin peroxidase and laccase of white-rot fungi. Am. Chem. Soc. Symp. Ser. **399:**482–502.
12. **Hünig, S., H. Balli, H. Conrad, and A. Schott.** 1964. Polarographie von 2,2′-azinen aromatischer Heterocyclen. Justus Liebigs Ann. Chem. **676:**52–65.
13. **Maruthamuthu, P., L. Venkatasubramanian, and P. Dharmalingam.** 1987. A fast kinetic study of formation of 2,2′-azinobis(3-ethyl-benzothiazole-6-sulfonate) radical cation in aqueous solution. Bull. Chem. Soc. Jpn. **60:**1113–1117.
14. **Muheim, A., A. Fiechter, P. J. Harvey, and H. E. Schoemaker.** 1992. On the mechanism of oxidation of non-phenolic lignin model compounds by the laccase-ABTS couple. Holzforschung **46:**121–126.
15. **Paice, M. G., L. Jurasek, C. Ho, R. Bourbonnais, and F. Archibald.** 1989. Direct biological bleaching of hardwood kraft pulp with the fungus *Coriolus versicolor*. Tappi (Tech. Assoc. Pulp Pap. Ind.) J. **72:**217–221.
16. **Paice, M. G., I. D. Reid, R. Bourbonnais, F. S. Archibald, and L. Jurasek.** 1993. Manganese peroxidase, produced by *Trametes versicolor* during pulp bleaching, demethylates and delignifies kraft pulp. Appl. Environ. Microbiol. **59:**260–265.
17. **Paice, M. G., R. Bourbonnais, and I. D. Reid.** 1995. Bleaching of kraft pulps with oxidative enzymes and alkaline hydrogen peroxide. Tappi (Tech. Assoc. Pulp Pap. Ind.) J. **78(9):**161–169.
18. **Reid, I. D., M. G. Paice, C. Ho, and L. Jurasek.** 1990. Biological bleaching of softwood kraft pulp with the fungus *Trametes* (*Coriolus*) *versicolor*. Tappi (Tech. Assoc. Pulp Pap. Ind.) J. **73(8):**149–153.
19. **Reid, I. D., and M. G. Paice.** 1994. Biological bleaching of kraft pulps by white-rot fungi and their enzymes. FEMS Microbiol. Rev. **13:**369–376.
20. **Reinhammar, B.** 1984. Laccase, p. 1–35. *In* R. Lontie (ed.), Copper proteins and copper enzymes, vol. 3. CRC Press, Boca Raton, Fla.
21. **Sackett, P. H., J. S. Mayausky, T. Smith, S. Kalus, and R. L. McCreery.** 1981. Side-chain effects on phenothiazine cation radical reactions. J. Med. Chem. **24:**1342–1347.
22. **Schneider, P., and A. H. Pedersen.** January 1995. Enhancement of laccase reactions. PCT. World patent application WO 95/01426.
23. **Viikari, L., M. Ranua, A. Kantelinen, J. Sundquist, and M. Linko.** 1986. Bleaching with enzymes, p. 67–69. *In* Proceedings of the Third International Conference on Biotechnology in the Pulp and Paper Industry, Stockholm. STFI, Stockholm, Sweden.
24. **Wolfenden, B. S., and R. L. Willson.** 1982. Radical-cations as reference chromogens in kinetic studies of one-electron transfer reactions. J. Chem. Soc. Perkin Trans. II **1982:**805–812.
25. **Xu, F., W. Shin, S. H. Brown, J. A. Wahleithner, U. A. Sundaram, and E. I. Solomon.** 1996. A study of a series of recombinant fungal laccases and bilirubin oxidase that exhibit significant differences in redox potential, substrate specificity, and stability. Biochim. Biophys. Acta **1292:**303–311.