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The Ligninolytic System of the White Rot Fungus *Pycnoporus cinnabarinus*: Purification and Characterization of the Laccase

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The white rot fungus *Pycnoporus cinnabarinus* was characterized with respect to its set of extracellular phenoloxidases. Laccase was produced as the predominant extracellular phenoloxidase in conjunction with low amounts of an unusual peroxidase. Neither lignin peroxidase nor manganese peroxidase was detected. Laccase was produced constitutively during primary metabolism. Addition of the most effective inducer, 2,5-xylidine, enhanced laccase production ninefold without altering the isoenzyme pattern of the enzyme. Laccase purified to apparent homogeneity was a single polypeptide having a molecular mass of approximately 81,000 Da, as determined by calibrated gel filtration chromatography, and a carbohydrate content of 9%. The enzyme displayed an unusual behavior on isoelectric focusing gels; the activity was split into one major band (pI, 3.7) and several minor bands of decreasing intensity which appeared at regular, closely spaced intervals toward the alkaline end of the gel. Repeated electrophoresis of the major band under identical conditions produced the same pattern, suggesting that the laccase was secreted as a single acidic isoform with a pI of about 3.7 and that the multiband pattern was an artifact produced by electrophoresis. This appeared to be confirmed by N-terminal amino acid sequencing of the purified enzyme, which yielded a single sequence for the first 21 residues. Spectroscopic analysis indicated a typical laccase active site in the *P. cinnabarinus* enzyme since all three typical Cu(II)-type centers were identified. Substrate specificity and inhibitor studies also indicated the enzyme to be a typical fungal laccase. The N-terminal amino acid sequence of the *P. cinnabarinus* laccase showed close homology to the N-terminal sequences determined for laccases from *Trametes versicolor*, *Coriolus hirsutus*, and an unidentified basidiomycete, PM1. The principal features of the *P. cinnabarinus* enzyme system, a single predominant laccase and a lack of lignin- or manganese-type peroxidase, make this organism an interesting model for further studies of possible alternative pathways of lignin degradation by white rot fungi.

Lignins constitute the second most abundant group of biopolymers in the biosphere; thus, their biodegradation occupies an important position in the global carbon cycle. Studies of lignin biodegradation are also of great importance for possible biotechnological applications, since lignin polymers are a major obstacle to the efficient utilization of lignocellulosic materials in a wide range of industrial processes (15). The degradation of lignins occurs primarily through the action of white rot fungi; consequently, this ecological group has received a considerable amount of research attention.

Most of our understanding of the enzymology of lignin biodegradation stems from studies of a single species of white rot fungi, *Phanerochaete chrysosporium*. Ligninolytic activity by *P. chrysosporium* is closely correlated with secretion of two specific peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP) (20, 28, 48). Since their discovery, *P. chrysosporium* LiP and MnP have been extensively characterized with respect to their biochemistry. These studies have, no doubt, led to a better understanding of the regulation and structure of the ligninolytic system produced by *P. chrysosporium*. Yet, with all of these advances, it has proven surprisingly difficult to demonstrate extensive lignin depolymerization using isolated LiP or MnP, and to date, no successful in vitro biotechnological application of these enzymes has been reported.

Until very recently, it was generally believed that *P. chrysosporium* does not produce laccase, the third phenoloxidase implicated in lignin degradation by many white rot fungi. Consequently, laccases have received much less attention, and their role in lignin biodegradation remains unclear, although an increasing number of studies indicate that these enzymes may be more important than was initially thought. Interestingly, this trend has recently been supported by Srinivasan et al. (43), who demonstrated that even *P. chrysosporium* produces laccase. Laccase production by *P. chrysosporium* seems to be repressed by glucose, commonly used as the carbon source in ligninolytic studies, but laccase activity was detectable when the fungus was grown on cellulose. Clearly, a great number of white rot fungi seem to efficiently degrade wood without the production of LiP: *Dichomitus squalens* (35) and *Cerioporiopsis subvermispora* (38) are two examples. The mechanism by which lignin is broken down in the absence of LiP, an enzyme with an exceptionally high oxidation potential, remains a mystery.

Bourbonnais and Paice (3) first described a mechanism by which laccases might assume some of the duties of LiP. In the presence of a suitable redox mediator, 2,2′-azino-bis(3-ethylthiazoline-6-sulfonate) (ABTS), for instance, laccase from *Trametes* (*Coriolus*) *versicolor* oxidized nonphenolic lignin model compounds, which are not substrates for laccase alone. This strategy recently led to the development of a laccase-based treatment for pulp bleaching which represents the first promising biotechnological application of a process based on a single ligninolytic enzyme (8). Furthermore, the production of a

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physiological laccase-redox mediator system by *Pycnoporus cinnabarinus* has recently been demonstrated in our laboratory.

P. cinnabarinus degrades wood as efficiently as *P. chrysosporium* (22) and has been successfully used for the decolorization of pigment plant effluents (42). In this work, we describe the purification and characterization of a laccase secreted by the white rot fungus *P. cinnabarinus*. This organism appears to be an ideal model for studies of the group of white rot fungi that lack LiP and seemingly operate with laccase as the major phenoloxidase.

MATERIALS AND METHODS

Organism. The *P. cinnabarinus* strain, PB, used in this study was isolated by one of us (C.E.) from decaying pine wood in the vicinity of Sydney, Queensland, Australia, in 1993. The culture was maintained on 2% (wt/vol) malt extract (1%) agar plates grown at 24°C and stored at 4°C. For spore production, parboiled rice was washed with deionized water, autoclaved for 25 min (60 g of rice per 1-liter Erlenmeyer flask), and inoculated with mycelium taken from malt extract agar plates after 5 days of growth at 24°C. After 10 days of growth at 30°C, the spores were harvested and aseptically filtered through six layers of cheesecloth over large Büchner funnels to remove rice and mycelium. Spores were allowed to settle by gravity overnight for repeated washing with 0.9% (wt/vol) NaCl. They were kept at 4°C in the dark and used within 2 weeks of storage. The stored spore suspensions were adjusted to a concentration of about 1.5×10^8 spores ml^{-1}.

Culture conditions. For laccase production and induction studies, 1.0 ml of spore suspension was used for inoculation of 400 ml of culture medium contained in 1,000-ml Erlenmeyer flasks. If not otherwise indicated, basal liquid medium contained the following (per liter): glucose, 3.0 g; KH_2PO_4, 1.0 g; NaH_2PO_4, 0.26 g; $(NH_4)_2SO_4$ (2.4 mM), 0.317 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CuSO_4 \cdot 7H_2O$, 0.5 mg; 2,2-dimethylsuccinic acid, 2.2 g; $CaCl_2 \cdot 2H_2O$, 74 mg; $ZnSO_4 \cdot 7H_2O$, 6 mg; $FeSO_4 \cdot 7H_2O$, 5 mg; $MnSO_4 \cdot 4H_2O$, 5 mg; $CoCl_2 \cdot 6H_2O$, 1 mg; vitamin solution, 500 μl (26). The pH of the medium was adjusted to 4.5 with 1 N NaOH. Cultures were incubated at 30°C on a rotary shaker (125 rpm; diameter, 18 mm). In flask experiments, 1 mM veratryl alcohol was added to the basal medium to stimulate production of peroxidases, and cultivations were done with and without addition of Tween 80 (0.8% [vol/vol]) (13). Because of the demonstrated role of manganese ions in the induction and activity of MnP (6), in preliminary experiments, the manganese concentration of the medium (as $MnSO_4$) was varied between 1 and 100 μM to stimulate MnP production.

To study the nitrogen regulation of laccase production, with glucose concentration fixed at either 3 or 8 g/liter, the nitrogen concentration was varied between 1.2 and 24 mM $(NH_4)_2SO_4$. For laccase induction studies, the investigated inducers, i.e., 2,5-xylidine, veratric acid, guaiacol, and lignosulfonate, were filter sterilized and added to the cultures at the time points indicated in the text.

For large-scale laccase production, *P. cinnabarinus* was cultivated in a 100-liter fermentor containing 70 liters of the basal medium lacking 2,2-dimethylsuccinate. The pH was continuously monitored and maintained at pH 4.5, using 0.1 N NaOH. After 24 h of cultivation, 2,5-xylidine (10 μM) was added to the culture medium to stimulate laccase production. The fermentation was carried out at a stirrer speed of 60 rpm (diameter, 700 mm) and an aeration rate of 30 liters min^{-1} at 30°C.

Assays for enzyme activities. After the mycelium was removed by centrifugation (10 min at 5,000 × *g*), laccase activity in the culture supernatant was routinely determined by measuring the oxidation of 500 μM ABTS buffered with 50 mM sodium tartrate buffer (pH 4.5). Formation of the cation radical was monitored at 420 nm ($\varepsilon_{max} = 3.6 \times 10^4\ M^{-1}\ cm^{-1}$). In some assays, catalase (50 μg/125 U; Sigma C-10) was added to the assay solution. All spectrophotometric measurements were carried out on a Varian UV-Vis DMS 200 spectrometer (Varian, Sugarland, Tex.).

Peroxidase activity was determined by adding to the laccase assay solution (pH either 3.5 or 4.5) H_2O_2 to 100 μM final concentration and subtracting the increase in absorbance caused by laccase activity. LiP and MnP activities were measured with veratryl alcohol and phenol red, respectively, using standard methods (19, 49).

Enzyme activities were expressed in units defined as 1 μmol of product formed per min. All values represent the means from duplicates of two independent experiments, with a maximal sample mean deviation of ±7% from the reported values.

Purification of extracellular laccase from *P. cinnabarinus* PB. On day 5 of cultivation, when laccase activity reached its maximum, the fermentor was harvested and mycelia were removed by centrifugation. All of the following purification steps were carried out at 4°C. The culture fluid, 70 liters, was concentrated to 2 liters in a Pellicon ultrafiltration system (Millipore, Bedford, Mass.), using a 10-kDa filter cassette. This concentrate was passed through a 300-kDa ultrafiltration cassette (Ultrasette; Filtron, Northborough, Mass.) to remove insoluble material. Further concentration and buffer exchange against 25 mM sodium acetate buffer (pH 5.0) was carried out by ultrafiltration in stirred cells (Amicon Corp., Lexington, Mass.), using 10-kDa PM membranes. The enzyme concentrate was then applied to a DEAE-M column (300 by 30 mm; Toyopearl, Montgomeryville, Pa.) and eluted with a linear gradient of 0 to 1 M NaCl in 25 mM sodium acetate buffer (pH 5.0) at a flow rate of 1.5 ml min^{-1}, using a fast protein liquid chromatography system (Pharmacia). Fractions containing laccase activity were pooled, reconcentrated, dialyzed against 1.7 M ammonium sulfate in 25 mM sodium acetate buffer (pH 5.0), and loaded onto a Toyopearl butyl-hydrophobic interaction column (200 by 20 mm). Laccase was eluted with a linear gradient of 1.7 to 0.0 M ammonium sulfate at a flow rate of 1.5 ml min^{-1}. Fractions containing laccase were pooled, concentrated, and purified to apparent homogeneity by passage over a final S-400 gel filtration (Sephacryl S-400; Pharmacia) column (980 by 25 mm). The gel filtration column was run with 25 mM sodium acetate buffer (pH 5.0) at a flow rate of 0.3 ml min^{-1}.

Homogeneity of the enzyme was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determined by the method of Bradford (5), using the Bio-Rad protein assay kit with bovine serum albumin as a standard.

Gel electrophoresis and staining. Isoelectric point and isozyme pattern of the laccase in crude (cell-free) enzyme mixtures and pure enzyme preparations were determined on isoelectric focusing (IEF) gels (IEF-PAGE) with gradients of pH 3 to 10 (100 by 100 mm, 1 mm thick; Servalyt-Precotes; Serva Biochemicals, Heidelberg, Germany), as described previously (45). At the completion of the run, duplicate lanes were stained either for protein with Serva Blue W (Serva) or for laccase activity with diaminobenzidine (0.6 mg ml^{-1}) in 50 mM sodium tartrate buffer (pH 4.0) (21). The isoelectric point of the laccase was determined by comparison with a protein standard mixture (Test Mix 9; Serva) containing amyloglycosidase (pI, 3.5), ferritin (pI, 4.4), bovine serum albumin (pI, 4.7), β-lactoglobulin (pI, 4.4), conalbumin (pI, 5.37), horse myoglobin (pI, 7.3), RNase (pI, 9.45), and cytochrome *c* (pI, 10.65). To determine purity of the protein, SDS-PAGE was performed according to Schägger and von Jagow (41). Protein bands were visualized by staining with Coomassie brilliant blue (G-250; Bio-Rad, Richmond, Calif.) and compared with molecular weight markers (broad range; Bio-Rad). For activity staining of laccase after SDS-PAGE, electrophoresis was carried out without prior boiling of the samples and using diaminobenzidine as described above. The molecular weight of the laccase was estimated by using a calibrated (molecular weight markers, broad range; Sigma) gel filtration column (Superose 12; Pharmacia) with 25 mM sodium acetate (pH 5.0) as the eluant.

Copper characterization. For the spectroscopic characterization of the Cu(II) centers of the *P. cinnabarinus* laccase, 0.2 mg of the enzyme, in 25 mM sodium acetate buffer (pH 5.0), was used. Spectrophotometric measurements were carried out on a Varian UV-Vis DMS 200 spectrometer, while electroparamagnetic resonance (EPR) spectra were recorded at 1 GHz on a Bruker ESP 300 E apparatus. A Cryofab helium transfer refrigerator unit and an Oxford ITC 4 controller maintained the samples for EPR spectroscopy at 20 K.

N-terminal amino acid sequence and degree of glycosylation. Purified laccase was electroblotted directly from an SDS-PAGE gel to a polyvinylidene difluoride membrane (Immobilon-P; Millipore), and sequence determination was made at the Molecular Genetics Instrumentation Facility, University of Georgia, using an automated protein sequencing system (Applied Biosystems Inc., Foster City, Calif.). Estimation of the neutral sugar content and determination of the glycosyl composition of the enzyme (from 50 μg of protein) were performed according to Merkle and Poppe (30).

Substrate specificity and inhibition studies. Spectrophotometric measurements of substrate oxidation by *P. cinnabarinus* laccase and the commercially available laccase from *Coriolus hirsutus* (120 U/mg, dry weight; Calbiochem, San Diego, Calif.) were carried out at 30°C in a 1-ml reaction volume containing the test substrates in 50 mM sodium tartrate buffer (pH 4.0). The concentrations of both enzymes were adjusted to give the same oxidation rate of ABTS. The effect of potential inhibitors of the laccase activity was monitored with 5 mM guaiacol as substrate in sodium tartrate buffer (pH 4.0).

Temperature stability and pH optimum. The above assay with guaiacol as substrate was also used to determine the temperature stability of the *P. cinnabarinus* laccase after different preincubation times at temperatures of up to 80°C in 50 mM sodium tartrate buffer (pH 4.0). To estimate the pH optimum of the enzyme, activity was measured with 5 mM guaiacol in 50 mM glycine HCl (pH 2.0 to 3.0)–50 mM sodium acetate (pH 3.0 to 6.0)–50 mM sodium citrate (pH 4.0 to 7.0)–50 mM MOPS (morpholinepropanesulfonic acid)–NaOH buffer (pH 5.0 to 6.5). All determinations were performed in duplicate, with an average sample mean deviation of the reported values of less than 3%.

RESULTS

Production of phenoloxidases by *P. cinnabarinus*: effect of nitrogen levels. In preliminary experiments, *P. cinnabarinus* was grown on agar plates containing basal medium and lignosulfonate (1% [wt/vol]) to induce ligninolytic enzymes. After 7 days, the mycelium was removed and the plates were flooded with guaiacol (10 mM) to detect extracellular phenoloxidase activity. Laccase was found to be the predominant phenoloxidase, since color development, indicating guaiacol oxidation,

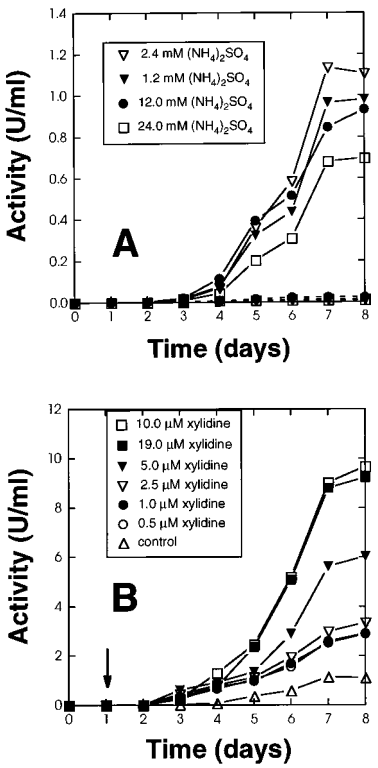

FIG. 1. (A) Influence of nitrogen concentration on extracellular laccase (solid lines) and peroxidase (dashed lines) production by *P. cinnabarinus* grown in shake flask cultures (5 g of glucose per liter as carbon source). (B) Stimulation of laccase production in cultures of *P. cinnabarinus* [2.4 mM $(NH_4)_2SO_4$; 5 g of glucose per liter] by addition of various amounts of 2,5-xylidine. The arrow indicates the time of 2,5-xylidine addition. Values represent the averages from three independent experiments, with a sample mean deviation of less than ±7% of the reported values.

was not dependent on the addition of H_2O_2 and was not inhibited by the addition of catalase.

In accordance with these observations, laccase also appeared to constitute the major phenoloxidase activity in the culture fluid when *P. cinnabarinus* was grown in liquid cultures. Growth and enzyme production in shake flasks were superior to those found in standing cultures, and shaking conditions were therefore chosen for routine cultivation. Laccase activity in the culture fluid was detectable after 48 h and reached a maximum of 1.2 U/ml on day 7 in a medium with a C/N ratio of about 15 [3 g of glucose per liter; 2.4 mM $(NH_4)_2SO_4$] (Fig. 1A). At 1.2 mM $(NH_4)_2SO_4$ in the culture medium, only 80% of the maximum levels of mycelial biomass was produced. The specific laccase activity (based on milligrams of mycelial dry weight) produced at the lower nitrogen level was comparable to that produced at the optimal concentration of 2.4 mM $(NH_4)_2SO_4$, but the total laccase activity (0.9 U/liter) was lower. At higher concentrations of $(NH_4)_2SO_4$, i.e., lower C/N ratios, production of laccase was also reduced, although an almost constant yield of fungal biomass was obtained. However, even at a C/N ratio of 1.5 [3 g of glucose per liter; 24 mM $(NH_4)_2SO_4$], about 60% of maximal laccase production (0.7 U/ml) was achieved.

The laccase/peroxidase activity ratio varied with the nitrogen concentration, ranging between 48 [2.4 mM $(NH_4)_2SO_4$] and 90 [24 mM $(NH_4)_2SO_4$]. The peroxidase activity, as determined by ABTS oxidation in the presence of H_2O_2, was only slightly stimulated by lower nitrogen levels. No LiP activity (H_2O_2-dependent veratryl alcohol oxidation) was detectable under various conditions known to stimulate production of the enzyme. LiP assays, carried out with addition of whole mycelial pellets so as to include cell-bound activities, were also negative. Moreover, the peroxidase activity could not be ascribed to an MnP-type enzyme either since it was not dependent on the presence of free Mn(II) and could not oxidize phenol red, a standard substrate for MnP's. No aryl alcohol oxidase activity was found in *P. cinnabarinus* cultures, in contrast to *Pleurotus ostreatus* cultures in which laccase and veratryl alcohol oxidase, but no LiP or MnP, were identified (40).

Induction of laccase. To enhance laccase production, several putative laccase inducers were tested. Lignosulfonate (1%, wt/vol; Na salt; Roth, Karlsruhe, Germany) and veratryl alcohol (1 mM) increased laccase activity 2.8- and 2.0-fold, respectively, whereas addition of guaiacol (1 mM) did not stimulate enzyme production. Addition of 2,5-xylidine showed the most pronounced effect, with the highest laccase production occurring at concentrations between 10 and 19 μM, when added after 24 h to shaken cultures (Fig. 1B). Under these conditions, laccase activity could be enhanced about ninefold, i.e., to approximately 9.6 U/ml, compared with the basal level obtained in 2.4 mM $(NH_4)_2SO_4$ cultures without inducer. At higher concentrations of 2,5-xylidine, less stimulation was observed, most likely due to a toxic effect on the fungus. Induction of laccase by 2,5-xylidine did not alter the isoenzyme pattern of the enzyme, as determined from activity staining of crude enzyme samples subjected to IEF-PAGE (data not shown). Also, addition of laccase inducers to the culture medium did not stimulate peroxidase production.

Large-scale production and purification of laccase. Cultivation of *P. cinnabarinus* in a fermentor increased laccase activity twofold to about 18 U/ml compared with shake flask cultures, and maximum activity was also reached 3 days earlier. The fungus was harvested on day 5, when laccase activity reached its maximum, and accounted for about 70% of the total extracellular protein.

The laccase was purified to homogeneity according to the procedure summarized in Table 1. The resultant enzyme pool was found to contain a single polypeptide (Fig. 2A) with a molecular mass of approximately 76,500 Da as determined by SDS-PAGE or 81,000 Da as determined by calibrated gel fil-

TABLE 1. Purification of extracellular laccase from *P. cinnabarinus*

Purification step	Vol (ml)	Total protein (mg)	Protein (mg/ml)	Total activity (U)	Sp act (U/mg)	Yield (%)	Purification factor (fold)
Culture filtrate	67,000	2,500	0.04	43,500	17	100	
Concentrated ultrafiltrate (10-kDa filter)	125	320	2.60	37,900	120	87	7
DEAE-M eluate	55	100	1.80	32,600	330	75	20
Butyl-Toyopearl eluate	40	44	1.10	24,400	560	56	32
S-400 eluate	10	33	3.30	20,200	610	47	36

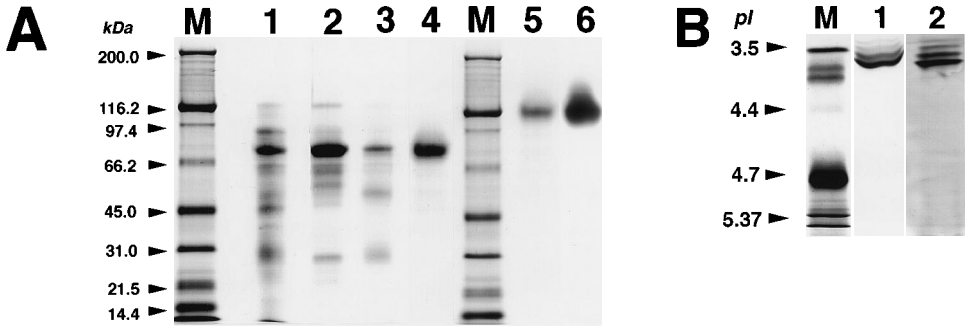

FIG. 2. Electrophoresis of *P. cinnabarinus* laccase after different purification steps. Protein was stained with Coomassie brilliant blue R-250. Laccase activity was detected by zymogram staining (0.6 mg of diaminobenzidine ml^{-1} in 50 mM sodium tartrate, pH 4.0). (A) SDS-PAGE (10% T; 0.1% SDS). Lanes: M, protein marker; 1, concentrated culture supernatant (track loaded with approximately 25 μg of protein); 2, DEAE-M column eluate (25 μg); 3, butyl-Toyopearl column eluate (10 μg); 4, S-400 gel filtration eluate (25 μg); 5, S-400 gel filtration eluate (15 μg; unboiled); 6, laccase activity staining of S-400 gel filtration eluate (15 μg; unboiled). (B) IEF (pH 3 to 10; Serva). Only part of the gel is shown. Lanes: M, protein marker; 1, protein-stained laccase after gel filtration; 2, activity stained laccase after gel filtration. Gels were scanned (Scan Jet IIc; Hewlett-Packard) and labeled in Photoshop (Adobe), using a Macintosh Powerbook 520.

tration chromatography. This is comparable to other fungal laccases (15). After IEF, one major activity band was observed; however, five minor bands having regular spacing and containing decreasing amounts of protein were also apparent (Fig. 2B). This unusual IEF pattern was found throughout the entire purification procedure and also appeared on IEF gels with different pH ranges. To investigate the significance of the multiple bands, the major band was excised from one gel, and the enzyme was reapplied to a second IEF gel on which it split once again into minor bands of decreasing concentration. This result suggests that under the studied cultivation conditions *P. cinnabarinus* produces only a single isoform of laccase having a pI of about 3.7.

Spectroscopy. To determine the nature of the catalytic center of the *P. cinnabarinus* laccase, the enzyme was characterized spectroscopically. This provided a sensitive indication of the state of its copper ions and circumvented some problems associated with inaccuracies in copper content measurements, which require very exact protein determinations. The UV-visible spectrum of the purified *P. cinnabarinus* laccase (Fig. 3) showed a peak of absorption at around 614 nm, typical for the type I Cu(II), that is responsible for the deep blue color of the enzyme. A shoulder at around 325 nm suggests the presence of the type III binuclear Cu(II) pair. The EPR spectrum showed the superimposed signals from two Cu(II) ions, each in a different coordination environment. The unusually low hyperfine coupling constant, IA_zI, of $\leq 95 \times 10^{-4}$ cm^{-1} characterized the type I Cu(II) center, while the absorbance at $IA_zI > 140 \times 10^{-4}$ cm^{-1} indicated the presence of a type II Cu(II) center, which does not have an optical absorption signal (Fig. 4).

Degree of glycosylation. The enzyme bound to concanavalin A-Sepharose, and its subsequent elution with α-methyl-mannopyranoside was evidence that the enzyme was glycosylated. This was confirmed by carbohydrate analysis showing that the enzyme contained about 9% of total carbohydrate, of which mannose was found to be the predominant glycosyl residue (>70%).

Temperature stability and pH optimum. *P. cinnabarinus* laccase (24 U/ml) was very stable below 50°C (Fig. 5). At 70°C, the half-life of the enzyme was about 60 min, whereas at 80°C, the laccase was completely inactivated within this time period. No activating effects were observed at higher temperatures as had been reported for a laccase isolated from an unidentified basidiomycete, PM1 (9).

The pH profile for *P. cinnabarinus* laccase activity showed one peak of optimal activity at pH 4.0 (Fig. 6). This lies well within the range determined for other fungal laccases isolated from ligninolytic cultures. On the other hand, laccases from the cultivated mushroom *Agaricus bisporus* and the ascomycete *Monocillium saxena* each display two distinct pH optima (46, 52).

Substrate specificity and inhibition pattern. A series of differently substituted phenols were examined as potential substrates for laccase (Table 2). The enzyme oxidized a variety of *o*- and *p*-phenols, as well as aromatic amines. Laccase from the white rot fungus *C. hirsutus* was included in the characterization because this fungus produces LiP in addition to laccase (27). We were interested in studying whether a laccase from a white rot fungus, such as *P. cinnabarinus*, lacking the high redox potential of LiP might secrete a laccase having different catalytic properties. However, comparison of the two laccases did not indicate major differences in their catalytic activities. The *C. hirsutus* enzyme showed a slightly higher reactivity towards hydroquinone, guaiacol, and 4-hydroxyindole, whereas *N*,*N*-dimethyl-1,4-phenylenediamine was a better substrate for the *P. cinnabarinus* laccase. Hydroxyindoles have recently been suggested to be a new class of laccase substrates (7). Indeed, both laccases reacted with 4-hydroxyindole at an even higher rate than with a number of more common laccase substrates. As expected for a laccase-like enzyme, no activity towards tyrosine was observed.

The sensitivity of *P. cinnabarinus* laccase towards several

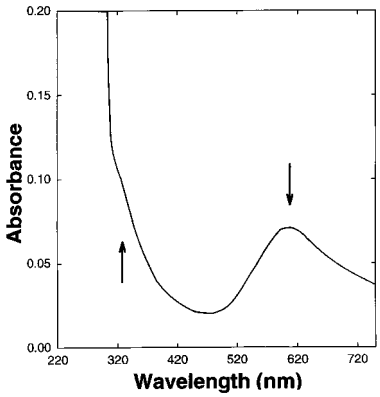

FIG. 3. Absorbance spectrum of laccase from *P. cinnabarinus* (0.2 mg in 25 mM sodium acetate, pH 5.0) at 25°C. Arrows indicate the characteristic absorbance peaks corresponding to type I (ca. 614 nm) and III (ca. 325 nm) Cu(II).

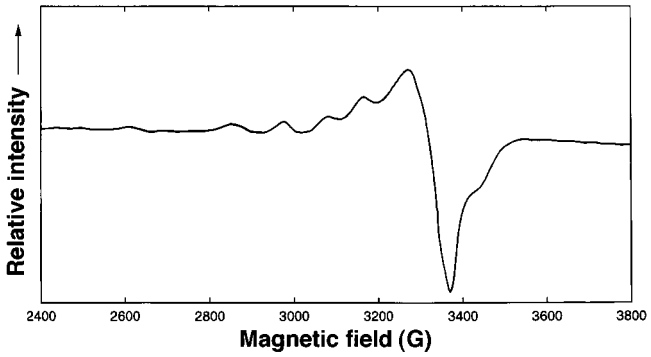

FIG. 4. EPR spectrum of purified *P. cinnabarinus* laccase (0.2 mg in 25 mM sodium acetate, pH 5.0) at 1 GHz and 20 K.

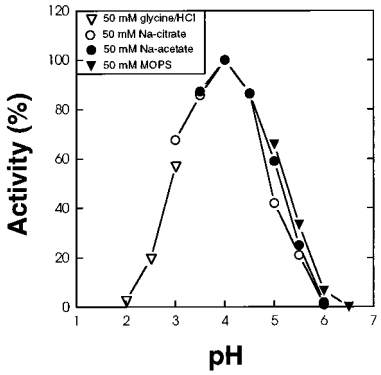

FIG. 6. pH optimum curve of purified *P. cinnabarinus* laccase. One hundred percent activity refers to 24 U/ml, using 5 mM guaiacol (50 mM sodium tartrate, pH 4.0) as the substrate.

putative laccase inhibitors was very similar to that seen with the laccase from *C. hirsutus*. Both were completely inhibited by 0.1 mM Na azide, the most effective inhibitor; 1 mM L-cysteine; 1 mM dithiothreitol; and 2 mM diethyldithiocarbamic acid (Table 3). In contrast to Murao et al. (32), who reported a complete inhibition of *C. versicolor* laccase by 0.35 μM kojic acid, we observed little or no inhibition of the *P. cinnabarinus* and *C. hirsutus* laccases even at much higher inhibitor concentrations. The metal chelator tropolone and *p*-coumaric acid caused only slight inhibition, whereas acetylacetone and EDTA had no inhibitory effect. As has been observed previously (12), at low concentrations, SDS had an enhancing effect on the apparent oxidation rate of guaiacol. However, this was at least partly due to a shift of the absorbance spectrum of the substrate in the presence of SDS and not to an activation of the enzyme.

N-terminal amino acid sequence. N-terminal amino acid sequence analysis of the purified laccase showed only a single polypeptide sequence (Table 4). This result supported our contention that the unusual band pattern observed after IEF of the enzyme was not the result of multiple isoenzymes. We have obtained a gene fragment which encodes a protein matching the N-terminal protein sequence data and are currently isolating a full-length laccase cDNA clone.

Comparison of the N-terminal sequence of the *P. cinnabarinus* laccase with those of other fungal laccases showed closest similarity to laccase II from *T. versicolor* (86%). About 76% similarity was found in comparison to the laccases from *C. hirsutus* and the basidiomycete PM1 and laccase IIIc from *T. versicolor*; 70% similarity to *Ceriporiopsis subvermispora* laccase and 64% similarity to *Phlebia radiata* laccase were found. All of these fungi are wood-rotting basidiomycetes belonging to the class of white rot fungi. In contrast, the N-terminal sequences of laccases isolated from non-wood-rotting fungi such as the commercial mushroom *A. bisporus*, the ascomycete *Neurospora crassa*, and the yeastlike fungus *Cryptococcus neoformans* were significantly different (similarities of 13, 18, and 0%, respectively).

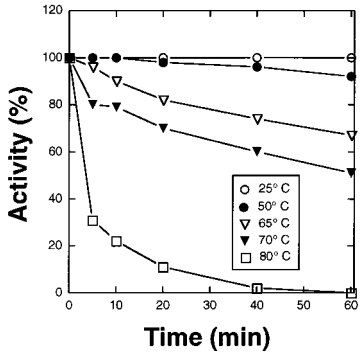

FIG. 5. Activity of purified *P. cinnabarinus* laccase after preincubation at different temperatures. One hundred percent activity refers to 24 U/ml, using 5 mM guaiacol (50 mM sodium tartrate, pH 4.0) as the substrate.

DISCUSSION

In this paper, we have described the physiological requirements for laccase production by *P. cinnabarinus* and characterized some of the enzyme properties after its large-scale production and purification. The fungus has been found to produce an unusual set of extracellular phenoloxidases consisting of a single isoform of laccase and a peroxidase that is neither an LiP nor an MnP. Our results confirm an earlier observation by Nerud et al. (33), who when screening for ligninolytic enzymes in several white rot fungi, detected H_2O_2-dependent ABTS oxidation but no LiP or MnP in a strain of *P. cinnabarinus*. A novel enzyme, tentatively named Mn-inhibited peroxidase, was recently detected in *Bjerkandera* sp. (11), but in contrast to this Mn-inhibited peroxidase, the *P. cinnabarinus*

TABLE 2. Activities of laccase from *P. cinnabarinus* and *C. hirsutus* assessed against various substrates[a]

Substrate	Concn (mM)	ε_{max} (M^{-1} cm^{-1})	Wavelength (nm)	Laccase (U/ml)	
				P. cinnabarinus	*C. hirsutus*
ABTS	5.0	36,000	420	77.3	76.9
Guaiacol	5.0	6,400	436	24.1	30.5
4-Hydroxyindole	5.0	2,685	615	18.4	24.8
4-Methylcatechol	5.0	2,091	420	17.7	19.6
2,6-Dimethoxy-phenol	5.0	35,645	470	17.1	28.3
Ferulic acid	5.0	12,483	287	13.0	13.7
Catechol	5.0	2,211	450	12.7	12.7
Hydroquinone	5.0	17,252	248	10.6	29.8
Pyrogallol	5.0	4,400	450	5.9	6.8
N,*N*-Dimethyl-1,4-phenylenediamine	5.0	43,160	515	3.4	0.5
Tyrosine	3.0	ND[b]	280	0	0

[a] Assays were done in 50 mM sodium tartrate (pH 4.0). For each assay equal amounts of both laccases, based on their activity with ABTS, were used. All values represent the mean of duplicate measurements with a sample mean deviation of less than 3%.

[b] ND, not determined.

TABLE 3. Effect of putative laccase inhibitors on oxidation of guaiacol by purified *P. cinnabarinus* laccase[a]

Compound	Concn (mM)	Inhibition (%)	
		P. cinnabarinus	*C. hirsutus*
L-Cysteine	1.0	100	100
NaN_3	0.1	100	100
DTT[b]	1.0	100	100
DDC[c]	0.1	64	77
	1.0	91	100
	2.0	100	
Tropolone	1.0	3	1
	4.0	22	20
	6.0	29	28
p-Coumaric acid	1.0	0	0
	3.0	3	5
Kojic acid	1.0	0	0
	10.0	4	0
Acetylacetone	1.0	0	0
	10.0	0	0
	50.0	0	0
EDTA	4.0	0	0

[a] Guaiacol was used at 5 mM in 50 mM sodium acetate, pH 4.0. Zero inhibition refers to 24 U of laccase activity per ml, using 5 mM guaiacol in a control assay.
[b] DTT, dithiothreitol.
[c] DCC, diethyldithiocarbamic acid.

peroxidase did not oxidize phenol red. In that respect, it more closely resembles another non-LiP, non-MnP peroxidase produced by the white rot fungus *Junghunia separabilima* (50). However, unlike the *J. separabilima* peroxidase, the *P. cinnabarinus* peroxidase was not active in the absence of H_2O_2. Detailed characterization of the *P. cinnabarinus* peroxidase is under way.

P. cinnabarinus represents a common type of white rot fungus devoid of LiP but in possession of laccase in combination with a peroxidase of lower redox potential (10). Although *P. cinnabarinus* appears to lack enzymes having the high oxidation potential thought to be necessary for the depolymerization of the major, nonphenolic structures of lignin, it very efficiently degrades the lignin in wood (22). In *P. cinnabarinus*, the lack of LiP does not seem to require compensation by a laccase having an unusually high redox potential. Our comparison of the laccases from *P. cinnabarinus* and *C. hirsutus*, a fungus that also secretes LiP and MnP, did not show significant differences in their oxidation capacity for various substrates. This result could be taken as support for the possible presence of a physiological enzyme-mediator system allowing for the oxidation of nonphenolic lignin structures by laccase (3, 8).

Laccase production by *P. cinnabarinus* was less sensitive to high concentrations of nitrogen than is known for production of phenoloxidases in ligninolytic systems of most other white rot fungi (29). High concentrations of glucose (≥5 g/liter) in low-nitrogen cultures (C/N ratio, >25) led to accumulation of an extracellular polysaccharide which strongly interfered with enzyme extraction from the medium. A similar effect has been demonstrated in *P. chrysosporium*, in which low-nitrogen, high-glucose conditions trigger the synthesis of an extracellular β-1,3-glucan (1, 14). When grown in liquid culture, *P. cinnabarinus* secreted laccase in concentrations comparable to those produced by *T. versicolor* and *Pleurotus ostreatus*, two fungi considered prodigious laccase producers.

The *P. cinnabarinus* laccase appears to occur in only one single acidic isoform, an unusual feature among fungal laccases. For instance, the well-characterized laccase from *T. versicolor* can be separated chromatographically into two major isoforms, which can be further split by IEF-PAGE into numerous isoelectric forms (24). Recent evidence suggests that these *T. versicolor* laccases represent true isoenzymes in that they are transcribed from separate genes under ligninolytic conditions (23, 27). A single laccase has been purified from *Pycnoporus coccineus*; however, it is unclear whether it was the only isoform or just the major expressed form (34). The multiband pattern observed on IEF gels for the *P. cinnabarinus* laccase isoenzyme adds to a number of previous reports of unusual behavior of purified laccases after electrophoresis. A multiple band pattern on SDS-PAGE gels has been reported for purified laccases from *A. bisporus* and *N. crassa* (16, 36) and is also known for the highly glycosylated plant laccases (44). Post-translational modifications of the extracellular polypeptide by proteolytic activity or differences in the degree of glycosylation may account for some of these banding patterns. However, in our case the faint bands are obviously an artifact that arose during IEF.

The *P. cinnabarinus* enzyme has all of the characteristics of a typical laccase, i.e., blue color due to its A_{610} and two Cu(II) ions in the type 3 configuration that show ca. A_{320}. The EPR signature is similar to those of other laccases, e.g., laccases from *Rhus vernificera* (36) and *Podospora anserina* (31). Thus, the spectroscopic data strongly suggest the presence of all four copper(II) ions typical of laccases in the *P. cinnabarinus* enzyme. The *Phlebia radiata* laccase has been reported to be lacking the type III binuclear Cu(II) ion pair (25).

Despite the highly conserved catalytic copper sites found in all known laccase gene sequences (47), differences in the reactivity of laccases from different species towards various substrates have been observed (12, 45). This was demonstrated

TABLE 4. Comparison of N-terminal amino acid sequences of *P. cinnabarinus* laccase and other fungal laccases

Microorganism	N-terminal amino acid sequence
P. cinnabarinus	A I G P V A D L T L T N A A V S P D G F S
T. (Coriolus) versicolor[a]	
I	A I G P V A S L V V A N A P V S P D G F L
II	G I G P V A D L T I T D A A V S P D G F S
III	G I G P V A D L T I T D A E V S P D G L S
Basidiomycete PM1[b]	S I G P V A D L T I S N G A V S P
C. hirsutus[c]	A I G P T A D L T I S N A E V S P D G F A
C. subvermispora[d]	A I G P V T D L E I T D A F V S P D G P
Phlebia radiata[e]	S I G P V T D F H I V N A A V S P
A. bisporus[f]	D T – K T F N F D L V N T R L A –
N. crassa[g]	G G G G G C N S P T N R Q C W S P
Cryptococcus neoformans[h]	X K T D E S P E A V S D N Y M P K

[a] Bourbonnais et al. (4).
[b] Coll et al. (9).
[c] Kojima et al. (27).
[d] Fukushima and Kirk (17).
[e] Saloheimo et al. (39).
[f] Perry et al. (36).
[g] Germann et al. (18).
[h] Williamson (51).

here for the *P. cinnabarinus* and *C. hirsutus* laccases. More laccase gene sequences will help to develop a better understanding of the structure-function relationships that govern substrate specificities and functions of laccases in different biological systems.

White rot fungi are diverse in their response to putative laccase inducers, and differences in the regulation of gene transcription may reflect different physiological functions for fungal laccases. In *P. cinnabarinus*, the eliciting effects of 2,5-xylidine, lignosulfonate, and veratric acid had no influence on the isozyme pattern, whereas in other fungi, new laccase isoforms are often induced (2). The elicitation effect most likely represents a response of the fungus to toxic compounds which could resemble either breakdown products of lignin or antimicrobial agents secreted by competing fungi. Fungi can often eliminate such toxic compounds by enzymatic transformation, i.e., oxidative polymerization by laccase or other phenoloxidases. Fungi, like the unidentified basidiomycete PM1, in which laccase activity was not stimulated by several putative laccase inducers (9), might not require such an enhanced defense reaction in their natural habitat.

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REFERENCES

1. **Bes, B., B. Petterson, H. Lennholm, T. Iversen, and K.-E. Eriksson.** 1987. Synthesis, structure and enzymatic degradation of an extracellular glucan produced in nitrogen-starved cultures of the white-rot fungus *Phanerochaete chrysosporium*. Biotechnol. Appl. Biochem. **9:**310–318.
2. **Bollag, J.-M., and A. Leonowicz.** 1984. Comparative studies of extracellular fungal laccases. Appl. Environ. Microbiol. **48:**849–854.
3. **Bourbonnais, R., and M. G. Paice.** 1990. Oxidation of non-phenolic substrates. An expanded role for laccase in lignin biodegradation. FEBS Lett. **167:**99–102.
4. **Bourbonnais, R., M. G. Paice, I. A. 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.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
6. **Brown, J. A., M. Alic, and M. H. Gold.** 1991. Manganese peroxidase gene transcription in *Phanerochaete chrysosporium*: activation by manganese. J. Bacteriol. **173:**4101–4106.
7. **Cai, W., R. Martin, B. Lemaure, J.-L. Leuba, and V. Petiard.** 1993. Hydroxyindoles: a new class of laccase substrates. Plant Physiol. Biochem. **31:** 441–445.
8. **Call, H. P.** December 1994. Process for modifying, breaking down or bleaching lignin, materials containing lignin or like substances. World patent application WO 94/29510.
9. **Coll, P. M., J. M. Fernandez-Abalos, J. R. Villanueva, R. Santamaria, and P. Perez.** 1993. Purification and characterization of a phenoloxidase (laccase) from the lignin-degrading basidiomycete PM1 (CECT 2971). Appl. Environ. Microbiol. **59:**2607–2613.
10. **De Jong, E., F. P. de Vries, J. A. Field, R. P. van der Zwan, and A. M. de Bont.** 1992. Isolation and screening of basidiomycetes with high peroxidase activity. Mycol. Res. **96:**1098–1104.
11. **De Jong, E., J. A. Field, and J. A. M. de Bont.** 1992. Evidence for a new extracellular peroxidase. Manganese-inhibited peroxidase from the white-rot fungus *Bjerkandera* sp. BOS 55. FEBS Lett. **1:**107–110.
12. **De Vries, O. M. H., W. H. C. F. Kooistra, and J. G. H. Wessels.** 1986. Formation of an extracellular laccase by a *Schizophyllum commune* dikaryon. J. Gen. Microbiol. **132:**2817–2826.
13. **Dodson, P. J., C. S. Evans, P. J. Harvey, and J. M. Palmer.** 1987. Production and properties of an extracellular peroxidase from *Coriolus versicolor* which catalyses Cα-Cβ-cleavage in a lignin model compound. FEMS Microbiol. Lett. **42:**17–22.
14. **Eriksson, K.-E., P. Ander, and B. Petterson.** 1986. Regulation of lignin degradation in *Phanerochaete chrysosporium*, p. 24–27. *In* Proceedings of the 3rd International Conference on Biotechnology in the Pulp and Paper Industry, Stockholm, Sweden.
15. **Eriksson, K.-E. L., R. A. Blanchette, and P. Ander.** 1990. Biodegradation of lignin. Springer-Verlag KG, Berlin.
16. **Froehner, S. C., and K.-E. Eriksson.** 1974. Purification and properties of *Neurospora crassa* laccase. J. Bacteriol. **120:**458–465.
17. **Fukushima, Y., and T. K. Kirk.** 1995. Laccase component of the *Ceriporiopsis subvermispora* lignin-degrading enzyme. Appl. Environ. Microbiol. **61:**872–876.
18. **Germann, U. A., G. Müller, P. E. Hunziker, and K. Lerch.** 1988. Characterization of two allelic forms of *Neurospora crassa* laccase. J. Biol. Chem. **263:** 885–896.
19. **Glenn, J. K., and M. H. Gold.** 1985. Purification and characterization of an extracellular Mn(II)-dependent peroxidase from the lignin-degrading basidiomycete, *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun. **114:**1077–1083.
20. **Glenn, J. K., M. A. Morgan, M. B. Mayfield, M. Kuwahara, and M. H. Gold.** 1983. An extracellular-H_2O_2-requiring enzyme preparation involved in lignin degradation by the white-rot basidiomycete *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun. **114:**1077–1083.
21. **Harlow, E., and D. Lane.** 1988. Antibodies—a laboratory manual, p. 596. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Hatakka, A., and A. K. Uusi-Rauva.** 1983. Degradation of ^{14}C-labelled poplar wood lignin by selected white-rot fungi. Eur. J. Appl. Microbiol. Biotechnol. **17:**235–242.
23. **Iumura, Y., K. Takenouchi, M. Nakamura, S. Kawai, Y. Katayama, and N. Morohoshi.** 1992. Cloning and sequence analysis of laccase genes and its use for an expression vector in *Coriolus versicolor*, p. 27–30. *In* M. Kuwahara and M. Shimada (ed.), Biotechnology in the pulp and paper industry. Uni Publisher, Kyoto, Japan.
24. **Jonsson, M., E. Pettersson, and B. Reinhammar.** 1968. Isoelectric fractionation, analysis, and characterization of ampholytes in natural pH gradients. VII. The spectra of fungal laccase A and B. Acta Chem. Scand. **22:**2135.
25. **Karhunen, E., M.-E. Niku-Paavola, L. Viikari, T. Haltia, R. A. van der Meer, and J. A. Duine.** 1990. A novel combination of prosthetic groups in a fungal laccase; PQQ and two copper atoms. FEBS Lett. **267:**6–8.
26. **Kirk, T. K., E. Schultz, W. J. Connors, L. F. Lorenz, and J. G. Zeikus.** 1978. Influence of culture parameters on lignin metabolism by *Phanerochaete chrysosporium*. Arch. Microbiol. **117:**277–285.
27. **Kojima, Y., Y. Tsukuda, Y. Kawai, A. Tsukamoto, J. Sugiura, M. Sakaino, and Y. Kita.** 1990. Cloning, sequence analysis, and expression of ligninolytic phenoloxidase genes of the white-rot basidiomycete *Coriolus hirsutus*. J. Bacteriol. **265:**15224–15230.
28. **Kuwahara, M. J., M. K. Glenn, M. A. Morgan, and M. H. Gold.** 1984. Separation and characterization of two extracellular H_2O_2-dependent oxidases from ligninolytic cultures of *Phanerochaete chrysosporium*. FEBS Lett. **169:**247–250.
29. **Leatham, G. F., and T. K. Kirk.** 1983. Regulation of ligninolytic activity by nutrient nitrogen in white-rot basidiomycetes. FEMS Microbiol. Lett. **16:** 65–67.
30. **Merkle, R. K., and I. Poppe.** 1994. Carbohydrate-composition analysis of glucoconjugates by gas-liquid-chromatography mass-spectrometry. Methods Enzymol. **230:**1–15.
31. **Molitoris, P., and B. Reinhammar.** 1975. Phenoloxidases of the ascomycete *Podospora anserina*. The state of copper of laccases I, II and III. Biochim. Biophys. Acta **386:**493–502.
32. **Murao, S., Y. Hinode, E. Matsumura, A. Numata, K. Kawai, H. Ohishi, H. Jin, H. Oyama, and T. Shin.** 1992. A novel laccase inhibitor, N-hydroxyglycine, produced by *Penicillium citrinum* YH-31. Biosci. Biotechnol. Biochem. **56:**987–988.
33. **Nerud, F., Z. Zouchova, and Z. Misurcova.** 1991. Ligninolytic properties of different white-rot fungi. Biotechnol. Lett. **13:**657–660.
34. **Oda, Y., K. Adachi, I. Aita, M. Ito, Y. Aso, and H. Igarashi.** 1991. Production and properties of laccase secreted by *Pycnoporus coccineus*. Agric. Biol. Chem. **55:**1393–1395.
35. **Perie, F. H., and M. H. Gold.** 1991. Manganese regulation of manganese peroxidase expression and lignin degradation by the white-rot fungus *Dichomitus squalens*. Appl. Environ. Microbiol. **57:**2240–2245.
36. **Perry, C. R., S. E. Matcham, D. A. Wood, and C. F. Thurston.** 1993. The structure and function of laccase protein and its synthesis by the commercial mushroom *Agaricus bisporus*. J. Gen. Microbiol. **139:**171–178.
37. **Rheinhammar, B.** 1984. Laccase, p. 4–10. *In* R. Lontie (ed.), Copper proteins and copper enzymes. CRC Press, Inc., Boca Raton, Fla.
38. **Rüttimann, C., E. Schwember, D. Salas, D. Cullen, and R. Vicuna.** 1992. Ligninolytic enzymes of the white-rot basidiomycetes *Phlebia radiata* and *Cerioporiopsis subvermispora*. Biotechnol. Appl. Biochem. **16:**64–76.
39. **Saloheimo, M., M.-L. Niku-Paavola, and J. K. C. Knowles.** 1991. Isolation

and structural analysis of the laccase gene from the lignin-degrading fungus *Phlebia radiata*. J. Gen. Microbiol. **137:**1537–1544.
40. **Sannia, G., E. Limongi, F. Cocca, G. Buonocore, G. Nitti, and P. Giardina.** 1991. Purification and characterization of veratryl alcohol oxidase enzyme from the lignin degrading basidiomycete *Pleurotus ostreatus*. Biochim. Biophys. Acta **1073:**114–119.
41. **Schägger, H., and G. von Jagow.** 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. **166:**368–379.
42. **Schliephake, K., G. T. Lonergan, C. L. Jones, and D. E. Mainwaring.** 1993. Decolorization of a pigment plant effluent by *Pycnoporus cinnabarinus* in a packed-bed bioreactor. Biotechnol. Lett. **15:**1185–1188.
43. **Srinivasan, C., T. M. D'Souza, K. Boominathan, and C. A. Reddy.** 1995. Demonstration of laccase in the white rot basidiomycete *Phanerochaete chrysosporium* BKM-F1767. Appl. Environ. Microbiol. **61:**4274–4277.
44. **Sterjiades, R., J. F. D. Dean, and K.-E. L. Eriksson.** 1992. Laccase from Sycamore maple (*Acer pseudoplatanus*) polymerizes monolignols. Plant Physiol. **99:**1162–1168.
45. **Sterjiades, R., J. F. D. Dean, G. Gamble, D. S. Himmelsbach, and K.-E. L. Eriksson.** 1993. Extracellular laccases and peroxidases from sycamore maple (*Acer pseudoplatanus*) cell suspension cultures. Planta **190:**75–87.
46. **Thakker, G., C. S. Evans, and K. K. Rao.** 1992. Purification and characterization of laccase from *Monocillium indicum* Saxena. Appl. Microbiol. Biotechnol. **37:**321–323.
47. **Thurston, C. F.** 1994. The structure and function of fungal laccases. Microbiology **140:**19–26.
48. **Tien, M., and T. K. Kirk.** 1983. Lignin-degrading enzyme from the hymenomycete *Phanerochaete chrysosporium*. Science **326:**520–523.
49. **Tien, M., and T. K. Kirk.** 1984. Lignin-degrading enzyme from *Phanerochaete chrysosporium*: purification, characterization, and catalytic properties of a unique H_2O_2-requiring oxygenase. Proc. Natl. Acad. Sci. USA **81:** 2280–2284.
50. **Vares, T., T. K. Lundell, and A. I. Hatakka.** 1992. Novel heme-containing enzyme possibly involved in lignin degradation by the white-rot fungus *Junghunia separabilima*. FEMS Microbiol. Lett. **99:**53–58.
51. **Williamson, P. R.** 1994. Biochemical and molecular characterization of the diphenol oxidase of *Cryptococcus neoformans*: identification as a laccase. J. Bacteriol. **176:**656–664.
52. **Wood, D. A.** 1980. Production, purification and properties of extracellular laccase of *Agaricus bisporus*. J. Gen. Microbiol. **117:**327–338.