Demonstration of Laccase in the White Rot Basidiomycete *Phanerochaete chrysosporium* BKM-F1767

C. SRINIVASAN,^{1,2} T. M. D'SOUZA,¹ K. BOOMINATHAN,^{1†} AND C. A. REDDY^{1,2*}

Department of Microbiology¹ and National Science Foundation Center for Microbial Ecology,²
Michigan State University, East Lansing, Michigan 48824-1101

Received 30 May 1995/Accepted 13 September 1995

It has been widely reported that the white rot basidiomycete *Phanerochaete chrysosporium*, unlike most other white rot fungi, does not produce laccase, an enzyme implicated in lignin biodegradation. Our results showed that *P. chrysosporium* BKM-F1767 produces extracellular laccase in a defined culture medium containing cellulose (10 g/liter) and either 2.4 or 24 mM ammonium tartrate. Laccase activity was demonstrated in the concentrated extracellular culture fluids of this organism as determined by a laccase plate assay as well as a spectrophotometric assay with ABTS [2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] as the substrate. Laccase activity was observed even after addition of excess catalase to the extracellular culture fluid to destroy the endogenously produced hydrogen peroxide, indicating that the observed activity is not due to a peroxidase. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by activity staining with ABTS revealed the presence of a laccase band with an estimated M_r of 46,500.

White rot basidiomycetous fungi produce three major classes of enzymes designated lignin peroxidases (LIPs), manganese-dependent peroxidases (MNP), and laccases, which play an important role in the fungal degradation of lignin (1, 4, 5, 9, 14, 16–19, 32). All three enzymes can oxidize phenolic compounds, thereby creating phenoxy radicals, while nonphenolic compounds are oxidized via cation radicals. Laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) can oxidize nonphenolic compounds with a relatively low ionization potential, while nonphenolics with high ionization potential are readily oxidized by LIPs and MNPs (5, 9, 17, 18). Some wood-degrading fungi contain all three classes of the lignin-modifying enzymes, while others contain only one or two of these enzymes (9, 16, 27). Several previous investigations indicated that laccase is widely distributed in many genera of white rot fungi (9, 16, 27). Catalytic mechanisms and properties of laccases have been reviewed recently (32, 36).

Phanerochaete chrysosporium has been extensively studied as a model organism for fungal lignin degradation (reviewed in references 4, 6, 13, and 19). The presence of LIPs and MNPs in *P. chrysosporium* is well established, but *P. chrysosporium* is widely quoted as an example of a white rot fungus that does not produce laccase (9, 16, 19, 32). It was not known whether the inability to demonstrate the presence of laccase in *P. chrysosporium* cultures was due to the use of culture conditions which are not favorable for laccase production by this organism or whether the organism lacks the genetic machinery for producing laccase. However, during our ongoing studies on regulation of expression of *lip* and *mnp* genes in cellulose- and wood-grown cultures of *P. chrysosporium*, we discovered that this fungus produces low but consistent levels of laccase. In this report, we present evidence for the presence of laccase in *P. chrysosporium* grown in low-nitrogen (2.4 mM) or high-nitrogen (24 mM) defined media containing cellulose as the carbon source.

MATERIALS AND METHODS

Fungal strains. Cultures of *P. chrysosporium* BKM-F1767 (ATCC 24725) were maintained in malt extract medium, and conidia were produced as previously described (3).

Media and cultures. Cultures were grown in defined liquid medium (33) containing dimethylsuccinate buffer (pH 4.2), ammonium tartrate (2.4 or 24 mM) as the nitrogen source, and cellulose powder (10 g/liter; Aldrich Chemical Co., Milwaukee, Wis.) replacing glucose as the carbon source. Liquid medium with 2.4 mM N is referred to as low-N-cellulose medium, and the one with 24 mM N is referred to as high-N-cellulose medium. Sterile aqueous conidial suspensions of *P. chrysosporium* strains were prepared as described previously (3) and were added to cellulose medium to give a final concentration of approximately 3.2×10^6 conidia per 10 ml of medium contained in 125-ml Erlenmeyer flasks. All cultures were incubated under static conditions at 25°C (ambient temperature), except in one experiment (see Table 1), in which cultures were also incubated at 37°C. Cultures were flushed with 100% oxygen (3) at the time of inoculation and every day thereafter for 4 or 6 days as stated in Results. The extracellular culture fluid was concentrated 40× as previously described (3) and was used in assays for LIP, MNP, and laccase activities.

Plate assay of laccase activity. For the laccase plate assay, which allowed rapid determination of the presence of laccase in the extracellular culture fluid, 15 ml of sterile agarose (0.5%) medium containing 14 μmol of ABTS [2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); Sigma Chemical Co., St. Louis, Mo.] per ml in 50 mM glycine-HCl buffer (pH 3.0) was placed in a sterile petri dish (100 by 15 mm). Three evenly spaced 5-mm-diameter wells were made in the gel with the broad end of a sterile Pasteur pipette; 20 μl of 40×-concentrated extracellular fluid was placed in the first well, while the second well contained 20 μl of the same extracellular fluid boiled for 5 min and served as a negative control. As a positive control, 2 μl of a *Pyricularia oryzae* laccase (Sigma Chemical Co.) solution (2 mg/ml of glycine-HCl buffer) was placed in the third well. The development of an intense bluish green color around the wells was considered a positive test for laccase activity.

Laccase activity. Laccase activity was determined spectrophotometrically as described by Niku-Paavola et al. (24) with ABTS as the substrate, except where mentioned otherwise. All the assays were done at pH 3.0, the optimum pH for laccase of *P. chrysosporium* with ABTS as the substrate. The laccase reaction mixture (in a total volume of 1 ml) contained 0.5 ml of extracellular culture fluid and 14 μmol of ABTS in 50 mM glycine-HCl (pH 3.0). The reaction was monitored by measuring the change in A_{436} for 5 min. The laccase activity was then expressed as nanokatals (nanomoles/second) in 1 ml of 40×-concentrated extracellular fluid. The extinction coefficient of 29,300 M⁻¹ cm⁻¹ was used for oxidized ABTS. It is known that MNP present in the extracellular culture fluids of *P. chrysosporium* also shows activity with ABTS in the presence of H₂O₂. Since this organism produces low levels of H₂O₂ (about 4 μM) in the extracellular culture fluid (22), catalase (Sigma Chemical Co.) was added at 1,000 U/ml of extracellular fluid and incubated for 1 h at 37°C to remove any H₂O₂ present. This catalase-treated culture fluid failed to show MNP activity unless exogenous H₂O₂ was added, indicating that the catalase treatment was able to destroy the

* Corresponding author. Phone: (517) 355-6499. Fax: (517) 353-8767. Electronic mail address: reddy@cvm.msu.edu.

† Present address: Novo Nordisk Biotech, Inc., Davis, CA 95616.

FIG. 1. Plate assay for demonstration of laccase activity in extracellular culture fluids of *P. chrysosporium* BKM-F1767. Three evenly spaced 5-mm-diameter wells were made with the broad end of a Pasteur pipette in a petri dish (100 by 15 mm) containing 15 ml of agarose (0.5%) and 14 μmol of ABTS per ml of 50 mM glycine-HCl (pH 3.0) buffer. Photographs were taken after the blue color, indicating laccase activity, was allowed to develop for 1 h at room temperature. (A) 20 μl of 40 $\times$ -concentrated extracellular fluid from 6-day-old cultures of *P. chrysosporium* grown in high-N-cellulose medium; (B) 20 μl of 40 $\times$ -concentrated extracellular fluid of *P. chrysosporium* as in panel A except that it was boiled for 5 min (negative control); (C) 20 μl of *Pyricularia oryzae* laccase (Sigma Chemical Co.) at 2 mg/ml (positive control).

endogenous H_2O_2 . ABTS assay for laccase activity was repeated with this catalase-treated extracellular fluid to confirm the presence of laccase activity.

Laccase activity was also assayed with syringaldazine, a widely used substrate (15, 21), as well as 6-hydroxydopamine (2,4,5-trihydroxyphenethylamine) (both purchased from Aldrich Chemical Co.). Laccase assay with syringaldazine as the substrate was done as described by Szklarz et al. (31). The reaction mixture for this assay (in a total volume of 1 ml) consisted of 0.4 ml of McIlvaine buffer (pH 5.0), 0.1 ml of 1 mM syringaldazine in ethanol, and 0.5 ml of 40 $\times$ -concentrated extracellular culture fluid. Oxidation of syringaldazine to its quinone (molar absorptivity of 65,000 $\text{M}^{-1} \text{cm}^{-1}$) by laccase was measured by monitoring the increase in A_{525} . The laccase activity was expressed as nanokatals per milliliter of 40 $\times$ -concentrated culture fluid. The laccase assay with 6-hydroxydopamine was performed as described by Padiglia et al. (26). For this assay, a stock solution of 8 mM 6-hydroxydopamine was freshly made with 10 mM HCl (pH 2), and 50 μl of this stock solution was dissolved in 1 ml of 0.1 M sodium acetate buffer (pH 5.5) containing 500 μl of 40 $\times$ -concentrated extracellular culture fluid. The change in A_{490} was measured spectrophotometrically for 5 min. The extinction coefficient of the product (6-hydroxydopaminequinone) was reported to be 2,300 $\text{M}^{-1} \text{cm}^{-1}$ (26), and this was used to calculate the activity in nanokatals per milliliter of 40 $\times$ -concentrated extracellular culture fluid.

LIP and MNP assays. LIP activity in the extracellular fluid was determined as described by Tien and Kirk (33). The amount of LIP required for the oxidation of 1 μmol of veratryl alcohol into veratraldehyde per min is defined as 1 unit. MNP was assayed as described by Paszczynski et al. (28). One unit of MNP is defined as the amount of enzyme needed to oxidize 1 μmol of Mn(II) into Mn(III) in 1 min. Both LIP and MNP activities were expressed in units per liter.

PAGE of laccase proteins. Native polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate (SDS)-PAGE were performed at room temperature with 10% gels and Tris-glycine buffer (pH 8.3) at 125 V for 90 min as described previously (20). After electrophoresis, the gels were fixed for 10 min in a solution containing 10% (vol/vol) acetic acid and 40% (vol/vol) methanol and then stained by soaking in 50 mM glycine-HCl (pH 3.0) buffer containing 2.7 mg of ABTS per ml as described by Niku-Paavola et al. (25).

RESULTS AND DISCUSSION

Laccase plate assay. The results of the laccase plate assay (Fig. 1) showed the presence of laccase activity in the concentrated extracellular fluid of *P. chrysosporium* and in *Pyricularia oryzae* laccase (positive control) but not in heat-treated extracellular fluid of *P. chrysosporium* (negative control). These results also indicated that the plate assay can be used as a simple rapid assay for visual demonstration of the presence of laccase.

Laccase activity. The laccase activity in extracellular culture fluids of *P. chrysosporium* grown in cellulose medium at 25 and 37°C is shown in Table 1. Cultures that were grown in high-N-cellulose medium showed relatively more laccase activity

TABLE 1. Laccase activity of *P. chrysosporium* BKM-F1767 as affected by temperature and nitrogen concentration in the medium

Medium ^a	Days postinoculation	Laccase activity (nkat/ml) ^b at:	
		25°C	37°C
Low-N-cellulose	5	6.8	3.1
	7	3.4	1.1
High-N-cellulose	5	4.6	2.8
	7	27.4	5.7

^a Cultures were grown in low-N-cellulose medium (2.4 mM) or high-N-cellulose medium (24 mM) as described in the text and incubated at 25 or 37°C. The cultures were oxygenated at the time of inoculation and then on days 2, 3, and 4 after inoculation.

^b Laccase activity in the 40 $\times$ -concentrated extracellular culture fluid was assayed as previously described (24) with ABTS as the substrate.

than those grown in low-N-cellulose medium. This was particularly true for high-N-cellulose cultures incubated at 25°C. In contrast, laccase activity was not detectable when the organism was grown in low-N or high-N medium with glucose instead of cellulose as the carbon source (data not shown).

The time course of laccase activity in high-N-cellulose medium is shown in Fig. 2. The time course of LIP and MNP production in the same medium is also presented for comparison. Laccase activity first appeared on day 4, reached its peak on day 6, and began to decline from day 7 onward. It has been well established that in defined high-N medium containing glucose as the carbon source (35), LIP and MNP production is not seen (6, 35), while in low-N-glucose medium, production of both LIP and MNP is seen. In low-N-glucose medium, MNP usually peaks first around day 4 whereas LIP peaks around day 6. Our results showed that LIP and MNP, as well as laccase, activities peaked on day 6 in high-N-cellulose medium (Fig. 2). We observed a similar temporal pattern of laccase production in another widely studied strain of *P. chrysosporium*, strain ME446 (data not shown).

Oxygen appears to have a marked influence on the laccase activity of *P. chrysosporium*. Unoxxygenated cultures that were

FIG. 2. Time course of laccase, LIP, and MNP production by *P. chrysosporium* BKM-F1767. Cultures were grown in high-N-cellulose medium (10 ml contained in a 125-ml Erlenmeyer flask) under static conditions at 25°C. All cultures were flushed with oxygen for the first 6 days of incubation. Three replicate flasks were used each day from days 3 through 9, and the extracellular culture fluids were harvested and concentrated (40 $\times$) as previously described (3) and were used for assaying laccase (with ABTS as the substrate), LIP, and MNP as described in Materials and Methods.

TABLE 2. Extracellular laccase activity assayed with different substrates^a

Time post-inoculation (days)	Laccase activity (nkat/ml) with:	
	ABTS	6-Hydroxydopamine
3	0	0
4	3.9	0
5	11.4	8.7
6	51.1	33.6
7	47.9	31.0
8	37.8	22.5
9	22.7	14.2

^a Cultures of *P. chrysosporium* BKM-F1767 were grown in high-N-cellulose medium (24 mM) (described in Materials and Methods) at 25°C and were oxygenated at the time of inoculation and then on days 2 through 6 of incubation. Extracellular culture fluid was concentrated 40× and treated with catalase (1,000 U/ml) to destroy H₂O₂. These catalase-treated culture fluids were then assayed for laccase activity with ABTS and 6-hydroxydopamine as described in the text (24, 26).

grown in high-N-cellulose medium seldom showed detectable levels of laccase activity (data not shown). In contrast, cultures that were oxygenated daily for the first 4 days of incubation (Table 1) or the first 6 days of incubation (Fig. 2; Table 2) showed relatively high levels of activity. Our results also show that cultures which were flushed with oxygen on each of the first 6 days of incubation (Fig. 2; Table 2) had higher laccase activity than did cultures which were oxygenated for only the first 4 days (Table 1).

Laccase production in *P. chrysosporium* appears to be relatively low (1.7 nkat/ml of concentrated extracellular fluid) even with ABTS, which is considered one of the more sensitive substrates for laccase assay (12, 25). *Phlebia radiata* was reported to produce 4.9 to 61 nkat/ml, while several other white rot fungi were shown to produce <100 nkat/ml (27, 32, 36). Some basidiomycetous fungi, on the other hand, show extremely high levels of laccase activity. For example, *Ganoderma lucidum* showed 47,500 nkat/ml (11) with ABTS as the substrate while another basidiomycete, strain PM1, showed 26,000 nkat/ml with guaiacol as the substrate (7). The plant-pathogenic fungus *Botrytis cinerea* showed laccase activity of 350,000 nkat/ml with 2,6-dimethoxyphenol as the assay substrate (30). The low level of laccase production in *P. chrysosporium* plus the lack of detectable laccase activity in low-N or high-N medium with glucose as the substrate might be the reasons why previous investigators failed to see laccase activity in this organism. Since this is the first report of laccase activity in *P. chrysosporium*, we also tested activity with 6-hydroxydopamine as an alternate substrate. Laccase activity was substantially lower with 6-hydroxydopamine than with ABTS (Table 2). The use of syringaldazine, another widely used substrate (15, 21, 31) for assaying laccase, led to even lower activity than that obtained with 6-hydroxydopamine (data not shown).

It has been well established that N and C concentrations in the medium, as well as the type of C and N source in the medium, have a profound effect on the production of LIP and MNP (6, 19, 29, 35). For example, with glucose as the carbon source, LIP and MNP production was seen in low-N (2.4 mM) medium but was completely suppressed in high-N (24 mM) medium (6, 29). However, when the glucose in the above medium was replaced by a less readily available carbon source such as cellulose or glycerol, LIP and MNP production was observed in the presence of low or high levels of N (6, 34). Our results show that laccase activity is not detectable even in

40×-concentrated extracellular fluid of *P. chrysosporium* grown in low-N or high-N medium with glucose as the carbon source (data not shown). On the other hand, laccase activity was readily demonstrable when the organism was grown in low-N- or high-N-cellulose medium.

SDS-PAGE and native PAGE. Fractionation of concentrated extracellular fluid from cultures grown on high-N-cellulose medium in a native PAGE gel showed two prominent protein bands, which stained positive for laccase activity, indicating the presence of at least two isoforms of laccase in *P. chrysosporium* (Fig. 3A). However, SDS-PAGE followed by activity staining showed a single dominant band, indicating that the isoforms are similar in molecular weight (Fig. 3B). The faint band seen below the major band in the SDS-PAGE gel probably represents a minor form of the enzyme; this became apparent only after an extended period of staining. Laccase is one of a relatively small number of proteins which retain enzymatic activity even after the SDS-PAGE procedure (2, 8, 25, 30). The SDS-PAGE results show that *P. chrysosporium* laccase has a molecular weight of 46,500, which is in the range reported for other basidiomycetes (23, 36). Molecular weights of most fungal laccase proteins fall between 43,000 and 110,000 (32, 36). However, it was suggested that the high molecular weight reported for some laccases may be due to incomplete dissociation of these proteins during the SDS-PAGE procedure. A majority of laccases from basidiomycete fungi were reported to have molecular weights in the range of 55,000 to 72,000 (32, 36), but several isoforms of laccases from the well-studied white rot fungus *Trametes versicolor* were reported to be 43,000 to 66,000 (23, 36) and five isoforms of *Schizophyllum commune* laccases ranged in molecular weight from 36,000 to 48,000 (10, 36).

The results show that *P. chrysosporium* produces laccase, contrary to the widely held belief (9, 16, 19, 32) that laccase is absent in this organism. Evidence suggests that there are two isoforms of laccase in *P. chrysosporium*. It is of interest that laccase activity is found in low-N- or high-N-cellulose medium but not in low-N- or high-N-glucose medium.

FIG. 3. Native PAGE (A) and SDS-PAGE (B) of extracellular culture fluid of *P. chrysosporium* BKM-F1767 cultures grown in high-N-cellulose medium for 6 days. Cultures were oxygenated every day during incubation. Gels were stained for laccase activity with ABTS as the substrate (24). A 20-μl volume of extracellular culture fluid (40× concentrated) containing 208 μg of protein was loaded on SDS-PAGE or native PAGE gels, and the gels were run as described in the text.

ACKNOWLEDGMENTS

This work was supported in part by grant DE-FGO2-85 ER 13369 from the U.S. Department of Energy and grant B I R 912-006 from the NSF Center for Microbial Ecology, Michigan State University.

REFERENCES

1. Ander, P., and K. E. Eriksson. 1976. The importance of phenol oxidase activity in lignin degradation by the white-rot fungus *Sporotrichum pulverulentum*. Arch. Microbiol. **109**:1–8.
2. Bao, W., D. M. O'Malley, R. Whetten, and R. R. Sederoff. 1993. A laccase associated with lignification in loblolly pine xylem. Science **260**:672–674.
3. Boominathan, K., S. B. Dass, T. A. Randall, and C. A. Reddy. 1990. Lignin peroxidase-negative mutant of the white rot basidiomycete *Phanerochaete chrysosporium*. J. Bacteriol. **172**:260–265.
4. Boominathan, K., and C. A. Reddy. 1992. Fungal degradation of lignin, p. 763–822. In D. K. Arora, R. P. Elander, and K. G. Mukerji (ed.), Handbook of applied mycology, vol. 4. Fungal biotechnology. Marcel Dekker, Inc., New York.
5. 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.
6. Buswell, J. A., and E. Odier. 1987. Lignin biodegradation. Crit. Rev. Biotechnol. **6**:1–60.
7. Coll, P. M., J. M. Fernandez-Abalos, J. M. Villanueva, R. Santamaria, and P. Perez. 1993. Purification and characterization of a phenol oxidase from the lignin-degrading basidiomycete PM1 (CECT 2971). Appl. Environ. Microbiol. **59**:2607–2613.
8. Dass, S. B., C. G. Desoretz, C. A. Reddy, and H. E. Grethlein. 1995. Extracellular proteases produced by the wood-degrading fungus *Phanerochaete chrysosporium* under ligninolytic and non-ligninolytic conditions. Arch. Microbiol. **163**:254–258.
9. de Jong, E., J. M. Field, and J. A. M. de Bont. 1994. Aryl alcohols in the physiology of ligninolytic fungi. FEMS Microbiol. Rev. **13**:153–188.
10. De Vries, O. M. H., W. H. C. F. Kooistra, and G. H. Wessels. 1986. Formation of an extracellular laccase by *Schizophyllum commune* dikaryon. J. Gen. Microbiol. **132**:2817–2836.
11. D'Souza, T. M., K. Boominathan, and C. A. Reddy. Unpublished data.
12. Fukushima, Y., and T. K. Kirk. 1995. Laccase component of the *Ceriporiopsis subvermispora* lignin-degrading system. Appl. Environ. Microbiol. **61**:872–876.
13. Gold, M. H., and M. Alic. 1993. Molecular biology of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Microbiol. Rev. **57**:605–622.
14. Gold, M. H., H. Wariishi, and K. Valli. 1989. Extracellular peroxidases involved in lignin degradation by the white-rot basidiomycete, *Phanerochaete chrysosporium*. ACS Symp. Ser. **389**:127–140.
15. Harkin, J. M., and J. R. Obst. 1974. Use of syringaldazine for detection of laccase in sporophores of wood rotting fungi. Mycologia **66**:469–476.
16. Hatakka, A. 1994. Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. FEMS Microbiol. Rev. **13**:125–135.
17. Higuchi, T. 1993. Biodegradation mechanism of lignin by white-rot basidiomycetes. J. Biotechnol. **30**:1–8.
18. Ishihara, T. 1980. The role of laccase in lignin degradation, p. 17–31. In T. K. Kirk, T. Higuchi, and H. M. Chang (ed.), Lignin degradation: microbiology, chemistry and potential applications, vol. 2. CRC Press, Inc., Boca Raton, Fla.
19. Kirk, T. K., and R. L. Farrell. 1987. Enzymatic "combustion": the microbial degradation of lignin. Annu. Rev. Microbiol. **41**:465–505.
20. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
21. Leonowicz, A., and K. Grzywnowicz. 1981. Quantitative estimation of laccase forms in some white-rot fungi using syringaldazine as a substrate. Enzyme Microb. Technol. **3**:55–58.
22. Li, D., M. Alic, J. A. Brown, and M. H. Gold. 1995. Regulation of manganese peroxidase gene transcription by hydrogen peroxide, chemical stress, and molecular oxygen. Appl. Environ. Microbiol. **61**:341–345.
23. Milstein, O., B. Nicklas, and A. Hutterman. 1989. Oxidation of aromatic compounds in organic solvents with laccase from *Trametes versicolor*. Appl. Microbiol. Biotechnol. **31**:70–74.
24. Niku-Paavola, M.-L., E. Karhunen, P. Salola, and V. Raunio. 1988. Ligninolytic enzymes of the white-rot fungus *Phlebia radiata*. Biochem. J. **254**:877–884.
25. Niku-Paavola, M. L., L. Raaska, and M. Itavaara. 1990. Detection of white-rot fungi by a non-toxic stain. Mycol. Res. **94**:27–31.
26. Padiglia, A., R. Medda, A. Rescigno, and G. Floris. 1994. On the use of 2,4,5-trihydroxyphenethylamine as a rapid method for laccase quantification. Ital. J. Biochem. **43**:24–26.
27. Paláez, F., M. J. Martínez, and A. T. Martínez. 1995. Screening of 68 species of basidiomycetes for enzymes involved in lignin degradation. Mycol. Res. **99**:37–42.
28. Paszczynski, A., R. L. Crawford, and V. B. Huynh. 1988. Manganese peroxidase of *Phanerochaete chrysosporium*: purification. Methods Enzymol. **161B**:264–274.
29. Reddy, C. A. 1993. An overview of the recent advances on the physiology and molecular biology of lignin peroxidases of *Phanerochaete chrysosporium*. J. Biotechnol. **30**:91–107.
30. Slomczynski, D., J. P. Nakas, and S. W. Tanenbaum. 1995. Production and characterization of laccase from *Botrytis cinerea* 61-34. Appl. Environ. Microbiol. **61**:907–912.
31. Szklarz, G., R. Antibus, R. Sinsabaugh, and A. Linkins. 1989. Production of phenol oxidases and peroxidases by wood-rotting fungi. Mycologia **81**:234–240.
32. Thurston, C. F. 1994. The structure and function of fungal laccases. Microbiology **140**:19–21.
33. Tien, M., and T. K. Kirk. 1988. Lignin peroxidase of *Phanerochaete chrysosporium*. Methods Enzymol. **161B**:238–249.
34. Tonon, F., C. P. de Castro, and E. Odier. 1990. Nitrogen and carbon regulation of lignin peroxidase and enzymes of nitrogen metabolism in *Phanerochaete chrysosporium*. Exp. Mycol. **14**:243–254.
35. Van der Woude, M. W., K. Boominathan, and C. A. Reddy. 1993. Nitrogen regulation of lignin peroxidase and manganese-dependent peroxidase production is independent of carbon and manganese regulation in *Phanerochaete chrysosporium*. Arch. Microbiol. **160**:1–4.
36. Yaropolov, A. I., O. V. Skorobogat'ko, S. S. Vartanov, and S. D. Varfolomeyev. 1994. Laccase: properties, catalytic mechanism, and applicability. Appl. Biochem. Biotechnol. **49**:257–280.