

## Lignin-Degrading Enzymes of the Commercial Button Mushroom, *Agaricus bisporus*

ALICE M. BONNEN,<sup>1\*</sup> LORI H. ANTON,<sup>1</sup> AND ANN B. ORTH<sup>2†</sup>

Departments of Plant Pathology<sup>1</sup> and Molecular and Cell Biology,<sup>2</sup> The Pennsylvania State University, University Park, Pennsylvania 16802

Received 6 August 1993/Accepted 1 January 1994

*Agaricus bisporus*, grown under standard composting conditions, was evaluated for its ability to produce lignin-degrading peroxidases, which have been shown to have an integral role in lignin degradation by wood-rotting fungi. The activity of manganese peroxidase was monitored throughout the production cycle of the fungus, from the time of colonization of the compost through the development of fruit bodies. Characterization of the enzyme was done with a crude compost extract. Manganese peroxidase was found to have a pI of 3.5 and a pH optimum of 5.4 to 5.5, with maximal activity during the initial stages of fruiting (pin stage). The activity declined considerably with fruit body maturation (first break). This apparent developmentally regulated pattern parallels that observed for laccase activity and for degradation of radiolabeled lignin and synthetic lignins by *A. bisporus*. Lignin peroxidase activity was not detected in the compost extracts. The correlation between the activities of manganese peroxidase and laccase and the degradation of lignin in *A. bisporus* suggests significant roles for these two enzymes in lignin degradation by this fungus.

The commercial button mushroom, *Agaricus bisporus* (*brunescens*), is grown on a straw-and-hay-based composted substrate. Recently, Lynch (26) proposed that mushroom compost could be separated into three major components: lignin, carbohydrates, and organic and inorganic nitrogen sources. The first indication that *A. bisporus* was capable of degrading the lignin contained in the compost came from the research of Waksman and Nissen (43), who found a significant reduction in the lignin component of compost by the end of the mushroom production cycle. Durrant et al. (9) showed that this loss of lignin from compost increased from the time of spawning up through the production of fruit body initials and then decreased with the onset of fruit body maturation. They also showed that *A. bisporus* is capable of degrading radioactively labeled lignin and synthetic lignin polymers and that this activity follows the pattern of lignin loss from the compost. However, the enzyme system responsible for lignin degradation by *A. bisporus* has not been defined.

Although laccase (polyphenol oxidase) is produced in copious amounts by *A. bisporus* (42, 47), its function in substrate utilization is unclear (33). The activity of this enzyme appears to be developmentally regulated in *A. bisporus* (42, 49) and correlates directly with the time of disappearance of lignin from the compost substrate (9, 43) and mineralization of labeled lignin to <sup>14</sup>CO<sub>2</sub> (9). In many wood-rotting fungi, the production of laccase corresponds with the presence of lignolytic activity, thus suggesting a role for this enzyme in lignin biodegradation (1, 5, 7). However, the role of laccase in biodegradation of lignin has not been well established. Laccases are believed to participate in lignin degradation through the oxidation of phenolics. This would imply a relatively limited role for these enzymes in lignin degradation, since phenolic subunits make up only a small proportion of the lignin

polymer. Recently, it has been reported that laccase produced by *Trametes versicolor* is able to oxidize nonphenolic substrates if provided with the proper substrate such as 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonate) (6). The ability to oxidize nonphenolic substrates would allow for a greater role in total lignin degradation. However, whether such substrates as 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonate) occur naturally and are present during lignin degradation is unknown. Other work indicating the involvement of laccase in lignin degradation includes that of Archibald and Roy (2), who have presented evidence that laccase produced by *T. versicolor* is capable of forming manganic chelates. This is a function usually ascribed to the lignin-degrading peroxidases (14) and is believed to be critical in the process of lignin depolymerization. In contrast, Haars and Hütterman (16) showed that laccase produced by *Fomes annosus* may be more important in repolymerization of low-molecular-weight lignin compounds than depolymerization. Additionally, lignin degradation by *T. versicolor* was shown to be unaffected by inhibition of laccase with specific antibodies (11). Thus, within the same fungal species such as *T. versicolor*, the role of laccase in lignin degradation is unclear.

In contrast to *A. bisporus*, lignin degradation by wood-rotting fungi has been well studied. The lignolytic system of the white rot fungus *Phanerochaete chrysosporium* has been particularly well characterized (24). It is presently known to consist of two families of peroxidases, lignin peroxidase (LP) and manganese peroxidase (MnP), each of which is represented by a number of isozymes (38). These enzymes are known to catalyze the initial depolymerization of lignin (24, 38). Hydrogen peroxide-producing enzymes such as glyoxyl oxidase are also considered important in the process of lignin biodegradation by *P. chrysosporium* (23). Other wood-rotting fungi produce lignin-degrading peroxidases but exhibit some variability compared with *P. chrysosporium*. The involvement of these types of peroxidases in lignin degradation by *A. bisporus* has not yet been investigated.

In this study, we demonstrate the production of MnP by *A. bisporus*. We demonstrate that MnP activity in *A. bisporus* is regulated in a developmental manner similar to laccase activity

\* Corresponding author. Mailing address: Department of Plant Pathology, 311 Buckhout Laboratory, The Pennsylvania State University, University Park, PA 16802. Phone: (814) 863-7058. Electronic mail address: abonnen@psu.edu.

† Present address: Dow Elanco, Discovery Research, Indianapolis, IN 46268-1053.

and thus correlates with lignin loss from compost and with lignin-degrading activity.

### MATERIALS AND METHODS

**Materials.** All chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.) and Aldrich Chemical Co. (Milwaukee, Wis.), except as noted.

**Fungal strains and culture conditions.** *A. bisporus* MC 396 (The Pennsylvania State University Mushroom Culture Collection) was maintained on potato dextrose agar amended with 1.5 g of yeast extract per liter (PDYA). *P. chrysosporium* wild-type strain BKM-F-1767 (ATCC 24725) was maintained on PDYA and grown in potato dextrose broth amended with 1.5 g of yeast extract per liter (PDYB).

**Compost culture conditions.** Compost was prepared by standard mushroom composting techniques (37). *A. bisporus* spawn was prepared as previously described (20). Standard 10-in. (25.4-cm)-diameter flower pots were filled with 2.27 kg of compost and inoculated with 20 g of spawn. To initiate the fruiting process, 14 days after inoculation (spawn run), the fully colonized compost was covered with a layer of peat (casing), and the production room was flushed with fresh air to reduce both the temperature and the level of carbon dioxide (pinning). The first flush of mushrooms was produced 35 days after inoculation (first break). The second flush occurred approximately 7 days after the first flush (second break).

**Sample preparation.** Compost was sampled three times (2.27 kg each) at the following stages in the production cycle: (i) phase 1 (raw compost ingredients combined, wetted, and allowed to begin decomposition), collected 9 days after beginning decomposition; (ii) phase 2 (indoor decomposition and pasteurization of phase 1 compost under controlled conditions), collected 7 days after beginning phase 2, just prior to inoculation with *A. bisporus* spawn; (iii) spawn run, collected 14 days after inoculation; (iv) pinned (fruit body initials appearing), collected 28 days after inoculation; (v) first break, collected 35 days after inoculation; and (vi) second break, collected 7 days after first break. Casing was removed prior to sampling stages iv, v, and vi. In addition, samples of compost colonized with *A. bisporus* under axenic conditions were collected. Samples were pressed with a small hydraulic press (660 lb/in<sup>2</sup>), and the fluid was collected. The extract was filtered through several layers of cheesecloth, and the particulates were removed by centrifuging twice for 20 min at 26,000 × g (Beckman JA-14). The filtrate was concentrated by ultrafiltration (Amicon; 25,000-molecular-weight cutoff). The retentate was dialyzed against 0.01 M NaPO<sub>4</sub> (pH 6.8) and stored at -20°C. This compost extract was used for all subsequent assays.

**Protein determinations.** Protein concentrations were determined with the bicinchoninic acid protein reagent assay (Pierce, Rockford, Ill.).

**Enzyme assays.** All enzyme assays were performed at room temperature with compost extract and were repeated a minimum of three times. Results are reported as means of three separate assays.

MnP activity was measured by monitoring the oxidation of guaiacol spectrophotometrically at 465 nm ( $\epsilon = 12,100/\text{M}/\text{cm}$ ) (31). The reaction mixture contained 20 mM citric acid, 40 mM sodium phosphate (pH 5.5), 0.1 mM MnSO<sub>4</sub>, 50  $\mu\text{M}$  H<sub>2</sub>O<sub>2</sub>, and 1 mM guaiacol. The reaction was initiated by the addition of H<sub>2</sub>O<sub>2</sub>. Enzyme boiled for 5 min was used in the control. Since laccase can also utilize guaiacol as a substrate, MnP activity was determined by subtracting the H<sub>2</sub>O<sub>2</sub>- and MnSO<sub>4</sub>-independent activity from total activity.

TABLE 1. MnP and laccase activities in compost samples taken at various times during the mushroom production cycle<sup>a</sup>

Compost sample	MnP		Laccase	
	U · g of <sub>1,b</sub> compost	Sp act (nmol · min <sup>-1</sup> · mg <sup>-1</sup> of protein <sup>-1</sup> )	U · g of <sub>1,b</sub> compost	Sp act (nmol · min <sup>-1</sup> · mg <sup>-1</sup> of protein <sup>-1</sup> )
Phase I	0.0	0.0	0.0	0.0
Phase II	0.0	0.0	0.0	0.0
Spawn run	1.80 A	127.5 A	8.0 A	560.0 A
Pinned	2.40 B	201.5 B	8.5 A	717.0 B
First break	0.90 C	71.0 C	1.3 B	97.5 C
Second break	0.15 D	43.0 C	0.4 C	130.0 C
Spawn run, axenic	1.80 A	131.0 A	3.0 D	212.0 D

<sup>a</sup> Numbers followed by the same letter in a column are not significantly different. Statistical analysis is based on Duncan's multiple range test at  $P = 0.05$ .

<sup>b</sup> One unit of enzyme activity is equivalent to 1 nmol of product produced per min.

Laccase activity was determined by measuring the change in  $A_{525}$  with syringaldazine as the substrate ( $\epsilon = 65,000/\text{M}/\text{cm}$ ) (4). The reaction mixture contained 0.18 M citric acid, 0.36 M phosphate (pH 6.0), and 0.075 mM syringaldazine. Enzyme boiled for 5 min was used in the control.

LP activity was measured by monitoring the oxidation of veratryl alcohol to veratraldehyde as indicated by an increase in  $A_{310}$  ( $\epsilon = 9,300/\text{M}/\text{cm}$ ) (40). The assay mixture contained 25 mM sodium tartrate (pH 2.5), 2 mM veratryl alcohol, and 0.4 mM H<sub>2</sub>O<sub>2</sub>. The reaction was initiated by the addition of H<sub>2</sub>O<sub>2</sub>. Enzyme boiled for 5 min was used in the control. *P. chrysosporium* LP (H2) purified from liquid culture (41) was used as the positive control (provided by M. Tien, The Pennsylvania State University).

**IEF.** Compost extract samples were dialyzed in distilled water and focused on 5.0% acrylamide gels with a pH range of 2.5 to 5.0 (Pharmalytes; Sigma) with a 2117 Multiphor II electrophoresis system (Pharmacia LKB, Uppsala, Sweden). MnP bands on the isoelectric focusing (IEF) gels were visualized with the phenol red activity stain (15). The stain mixture contained 50 mM sodium lactate (pH 4.5), 50 mM sodium tartrate (pH 4.5), 0.1 mM MnSO<sub>4</sub>, 3 mg of gelatin per ml, 50  $\mu\text{M}$  H<sub>2</sub>O<sub>2</sub>, and 0.1 mM phenol red. Control IEF gel activity stain did not contain H<sub>2</sub>O<sub>2</sub> or MnSO<sub>4</sub>. Total proteins were stained with Coomassie blue R 250 according to the manufacturer's protocol (34). Standards had pIs of 3.55 (amyloglucosidase), 3.75 (methyl red), 4.2 (glucose oxidase), 4.6 (trypsin inhibitor), and 5.13 ( $\beta$ -lactoglobulin A).

### RESULTS

**Enzyme activity in compost extracts.** MnP, LP, and laccase activity assays of the compost extracts were performed. Laccase activity for *A. bisporus* in both liquid culture and compost substrate has been well characterized (42, 47, 48). For this reason, we chose to use laccase as a positive control for enzyme activity.

A comparison of the MnP and laccase activities found in compost throughout the mushroom production cycle is shown in Table 1. Neither activity was observed in the compost collected prior to inoculation with *A. bisporus* (phases 1 and 2). Following initial colonization by *A. bisporus*, the specific activity for both enzymes increased through the pinning stage and then decreased with fruit body maturation (first break). Enzyme activity at second break was similar to that observed at first break. Although the two enzymes exhibited similar pat-

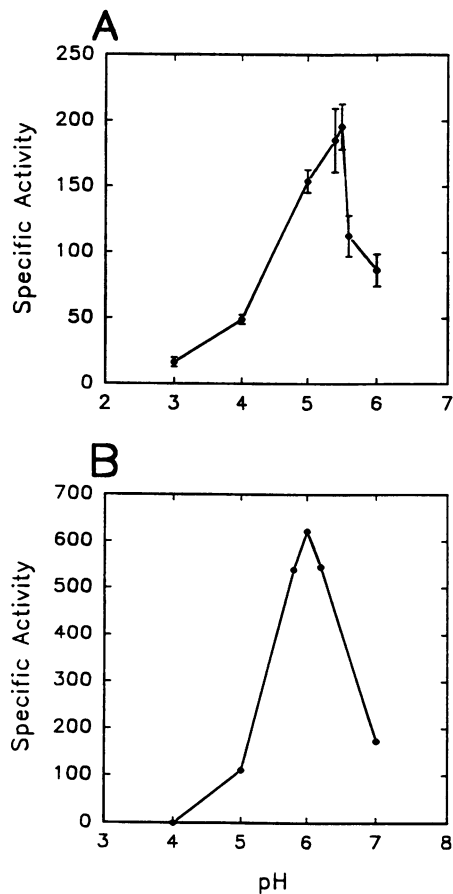


FIG. 1. The effect of pH on the activity of *A. bisporus* MnP (A) and laccase (B) from compost extract. Specific activity is defined as nanomoles per minute per milligram of protein. Substrates were guaiacol (A) and syringaldazine (B). Vertical bars in panel A represent the standard error of the mean. Error bars in panel B are obscured by the symbols.

terns of regulation, the decrease in activity upon fruit body maturation was greater for laccase than for MnP, with a loss in activity of 87% for laccase compared with 65% for MnP. This dramatic loss in activity occurred within 7 days, between the time of production of the first fruit body initials and first break.

Axenically grown *A. bisporus* spawn-run compost was found to have the same level of MnP activity as that observed for the nonaxenic cultures (Table 1). However, 62% less laccase activity was found in the axenic spawn-run compost than in the nonaxenic spawn-run compost.

Expressed on a per-gram-of-compost basis, the same general pattern of activity was observed throughout the production cycle for both enzymes as when expressed on a per-milligram-of-protein basis, except that laccase activity was maximal by the end of spawn run rather than increasing through pinning (Table 1).

The MnP activity, with guaiacol as the substrate, was maximal in all compost extracts at a pH of 5.4 to 5.5 (Fig. 1A). Because of the ability of laccase to utilize guaiacol as a substrate, it was necessary to subtract the activity which was not dependent on the presence of  $H_2O_2$  and manganese. The MnP activity constituted approximately 30% of the total activity in the pinned compost extract at the optimal pH of 5.4 to 5.5. By

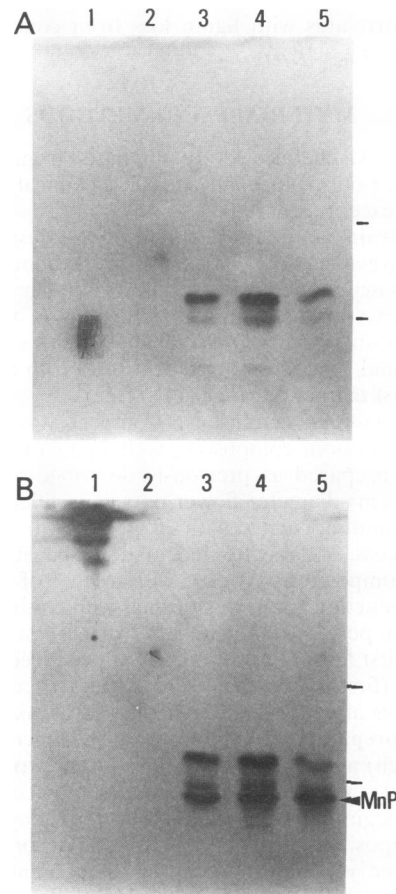


FIG. 2. Analytical IEF gel stained with phenol red (15) in the absence (A) or presence (B) of  $H_2O_2$  and  $MnSO_4$ . Lines next to gels indicate positions of two standards, glucose oxidase (pI 4.2 [upper line]) and amyloglucosidase (pI 3.55 [lower line]). Lanes: 1, MnP standard (H3) from *P. chrysosporium*; 2, phase 2 compost extract; 3, spawn-run compost extract; 4, pinned compost extract; 5, axenic spawn-run compost extract.

first break, MnP activity constituted a greater percentage of total activity (50 to 60%) because of a larger reduction in laccase activity relative to MnP activity (Table 1). A pH optimum of 6.0 was observed for laccase with syringaldazine (Fig. 1B) or guaiacol (unpublished data) as the substrate.

We were unable to detect LP activity in any of the compost extracts. In a test to determine whether the lack of activity could be due to inhibition of LP in the compost extracts, purified LP (H2) from *P. chrysosporium* was assayed for activity in the presence and absence of compost extract. In the absence of compost extract, the specific activity of *P. chrysosporium* LP was found to be  $7.5 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}$  of protein $^{-1}$ . However, the activity of the *P. chrysosporium* LP was completely abolished upon addition of compost extract.

**IEF of compost extract proteins.** Because of the similarities in the types of substrates utilized by laccase and MnP, activity staining was performed with isoelectrically focused proteins to demonstrate that these activities in the compost extracts are due to distinct proteins. Phenol red activity staining of the analytical IEF gel revealed multiple bands for laccase (Fig. 2A and B) and a single prominent band for MnP (Fig. 2B). The MnP band was not present on the control gel stained in the absence of  $H_2O_2$  and  $MnSO_4$  (Fig. 2A). pIs were calculated

from standards stained for total protein with Coomassie blue (data not shown). The calculated pI for MnP was 3.5. The laccase isozymes had pIs ranging from 3.3 to 3.65. There may be an additional MnP isozyme overlapping with one of the laccase isozymes at a pI of 3.55, as suggested by the darkening of the band at that site upon addition of H<sub>2</sub>O<sub>2</sub> and MnSO<sub>4</sub>. The phase 2 extract did not exhibit any detectable laccase or MnP activity bands. The other compost extract samples examined (spawn run, pinned, and axenic spawn run) exhibited similar banding patterns for both laccase and MnP.

## DISCUSSION

*A. bisporus* is typically grown on a substrate in which straw and hay are major components. As a consequence, lignocellulose makes up a significant proportion of the substrate. It has been suggested that cultivation of *A. bisporus* may be one of the few economically feasible modes of bioconversion of agricultural lignocellulosic waste (9). The ability of *A. bisporus* to degrade lignin has been demonstrated convincingly by Durrant et al. (9), using radiolabeled wheat lignin and synthetic lignin polymers. We have now demonstrated that *A. bisporus* has a lignin-degrading system similar to that of the wood-rotting fungi, with MnP being the predominant peroxidase enzyme detected.

Activity staining of the analytical IEF gels demonstrates not only the presence of MnP in *A. bisporus*-colonized compost extracts but also its dependence on manganese and hydrogen peroxide for activity (Fig. 2). The pI of the *A. bisporus* MnP is lower than the reported pIs of the MnP isozymes from *P. chrysosporium* (4.2 to 4.9) (39) but is comparable to the pIs reported for MnP from *Phlebia radiata*, *Lentinula edodes*, and *Panus tigrinus* (3.8, 3.2, and 2.95 [3.2], respectively) (12, 21, 27). The pIs for the laccase isozymes from *A. bisporus* are in the same range as those previously reported (3.4 to 4.0) (47). We have tentatively identified a second MnP isozyme overlapping with a laccase isozyme at a pI of 3.55. However, purification of the proteins will be necessary to determine this for certain.

LP enzyme activity could not be detected in the compost extracts. However, we believe this is due to an inhibitory component in the concentrated extracts, as demonstrated by inhibition of purified *P. chrysosporium* LP (H2) with compost extract. Immunoblots of compost extract proteins probed with an antibody raised against *P. chrysosporium* H2 isozyme detected a single, weakly reacting protein approximately 40 kDa in size (unpublished results). No bands were detected on the control blot treated with preimmune serum or in any of the compost extracts sampled prior to colonization by *A. bisporus* (phases 1 and 2). Although no LP activity could be detected, the information from the immunoblots indicates the presence of an LP-like protein produced by *A. bisporus* during growth on compost.

The pH optimum of the MnP from *A. bisporus* was slightly higher than the pH optima reported for MnP from a variety of wood-rotting fungi (pH 2.6 to 5.0) (12–14, 22, 27). The compost substrate maintains a pH of about 6.0 (5.8 to 6.2 in our experiments) throughout the mushroom production cycle, which may explain the slightly higher pH optimum for the *A. bisporus* enzyme. The pH optimum determined for laccase from compost with syringaldazine (or guaiacol) as the substrate was slightly higher than the pH optimum previously determined for this enzyme (pH 5.6) (47). The discrepancy may be due to the fact that the enzymes were isolated from different substrates (compost versus liquid media) and were in different states of purification (crude versus purified).

Lignin-degrading activity in the wood-rotting fungi, particu-

larly *P. chrysosporium*, has been studied extensively. Two families of peroxidases have been identified as having a significant role in lignolysis, LP and MnP. The importance of these enzymes in lignin biodegradation has been shown by the ability of the isolated enzymes to depolymerize synthetic lignin polymers (17, 45). As the lignolytic systems of other wood-rotting fungi are investigated, it is becoming clear that there is variability in the types of enzyme systems utilized for lignin biodegradation (29, 30, 44). Many produce an array of lignin-degrading enzymes similar to those in *P. chrysosporium*, but others produce one or the other of the peroxidase enzymes, often in combination with laccase, which is also believed to have a critical role in lignolysis by certain fungi. For example, *P. radiata* (19), *Phlebia brevispora* (32), *Trametes gibbosa* (29), and *T. versicolor* (2, 8) produce laccase in addition to both types of lignin-degrading peroxidases. *P. tigrinus* (27), *Stereum hirsutum*, *Dichomitus squalens*, *Coriolopsis polyzona*, and *Ganoderma valesiacum* (29) produce only MnP in combination with laccase and do not produce LP. *Pleurotus ostreatus* has been reported to produce only laccase during solid-state fermentation, but no assays were performed to test for the presence of MnP activity (22). *Ceriporiopsis subvermispora* (36) has been shown to produce both MnP and laccase. Although unable to detect LP activity, Rüttiman et al. (36) were able to detect an LP-like gene by using a radiolabeled probe of an LP clone (H8). A common feature among nearly all of these fungi, including *A. bisporus*, is the production of at least one type of lignin-degrading peroxidase.

Laccase activity was found to be greater for *A. bisporus* grown under nonaxenic conditions than for *A. bisporus* grown under axenic conditions (Table 1). This higher laccase activity is not likely to be a result of the presence of other microorganisms, since uninoculated compost, maintained under the same conditions as colonized, spawned compost, exhibits no laccase activity (unpublished results). Laccase has been suggested as being a good indicator of *A. bisporus* mycelial biomass in compost (28). While *A. bisporus* grows well in sterile compost, its growth is slower than in nonsterile compost over the same period of time. Thus, the lower level of laccase in the axenic compost cultures may reflect the presence of a lower total biomass.

Although the role of laccase in lignin biodegradation is not fully understood, it has been suggested as being necessary for lignin degradation (1). The importance of laccase in this process is supported by work by Galliano et al. (13) showing that *Rigidiporus lignosus* MnP and laccase act synergistically during lignin degradation. They showed an additional increase in lignolytic activity with the inclusion of glucose oxidase, a hydrogen peroxide-producing enzyme. Other studies by Bourbonnais and Paice (6), indicating that the activity of laccases may extend beyond the ability to oxidize phenolic substrates to nonphenolic substrates, and that of Archibald and Roy (2), showing the production of manganic chelates by laccase, allow for a much greater role for laccases in total lignin degradation than first suggested. Laccase is the predominant extracellular protein produced by *A. bisporus* (48). We have found, as others have (42, 48, 49), that laccase activity is highest during colonization and initiation of fruit body development and lowest with fruit body maturation. Loss of lignin from the compost and lignin-degrading activity have also been shown to increase up through the production of fruit body initials and then to decrease with the onset of fruit body maturation (9, 43). Thus, the level of laccase activity directly correlates with lignin-degrading activity and loss of lignin from the compost substrate when colonized by *A. bisporus*. In addition to laccase, we have now shown that *A. bisporus* produces MnP activity during

growth on the compost substrate. We have demonstrated that MnP activity parallels laccase activity, and thus parallels the loss of lignin from the compost and correlates directly with degradation of labeled lignin and labeled synthetic lignin polymers during growth on compost. Thus, both laccase and MnP appear to be similarly and developmentally regulated in *A. bisporus* and to be directly correlated with lignin degradation.

It may be of significance that *A. bisporus*, like many wood-rotting fungi, produces large amounts of oxalate crystals during growth on compost (3, 10). The function of the oxalate crystals in *A. bisporus* is not known (18). However, it has been suggested recently that oxalate production may be important in lignin biodegradation by *P. chrysosporium* through chelation of the Mn(III) formed by MnP (25, 46). Chelators such as oxalate and other organic acids appear to increase the rate of dissociation of the manganese-enzyme complex and to stabilize the Mn(III) formed (25, 46). Oxalate may also be important in LP-mediated lignin biodegradation either through involvement in production of hydroxyl radicals (4) or through a series of reactions initiated by the formation of an oxalate radical leading to the oxidation of manganese (35). These studies, along with the present work identifying MnP production by *A. bisporus*, may indicate at least one role for the large amounts of oxalate found in association with *A. bisporus* mycelium.

Although *A. bisporus* is capable of degrading the lignin in compost, the specific components of the enzyme system utilized by this fungus during the biodegradation of lignin are not known with certainty. However, significant roles for MnP and laccase in lignin degradation by *A. bisporus* are suggested by the correlation between the activities of laccase and MnP with lignin degradation (loss of lignin from the compost and degradation of radiolabeled lignin) and the roles identified for these enzymes in lignin degradation by other organisms. Purification of the MnP and LP proteins, isolation of their respective genes, and identification of other enzymes involved in lignolysis will be important for further characterization of the lignin-degrading system of *A. bisporus*.

#### ACKNOWLEDGMENT

We gratefully acknowledge Ming Tien and his laboratory group for valuable assistance.

#### REFERENCES

- 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.
- Archibald, F., and B. Roy. 1992. Production of manganic chelates by laccase from the lignin-degrading fungus *Trametes (Coriolus) versicolor*. Appl. Environ. Microbiol. **58**:1496-1499.
- Atkey, P. T., and D. A. Wood. 1983. An electron microscope study of wheat straw composted as a substrate for the cultivation of the edible mushroom (*Agaricus bisporus*). J. Appl. Bacteriol. **55**:293-304.
- Barr, D. P., M. M. Shah, T. A. Grover, and S. D. Aust. 1992. Production of hydroxyl radical by lignin peroxidase from *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **298**:480-485.
- Bollag, J.-M., and A. Leonowicz. 1984. Comparative studies of extracellular fungal laccases. Appl. Environ. Microbiol. **48**:849-854.
- 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.
- Davidson, R. W., L. E. Campbell, and D. J. Blaisdell. 1938. Differentiation of wood-decay fungi by their reactions on gallic or tannic acid medium. J. Agric. Res. **57**:683-695.
- 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 catalyzes C<sub>α</sub>-C<sub>β</sub> cleavage in a lignin model compound. FEMS Microbiol. Lett. **42**:17-22.
- Durrant, A. J., D. A. Wood, and R. B. Cain. 1991. Lignocellulose biodegradation by *Agaricus bisporus* during solid substrate fermentation. J. Gen. Microbiol. **137**:751-755.
- Dutton, M. V., C. S. Evans, P. T. Atkey, and D. A. Wood. 1993. Oxalate production by basidiomycetes, including the white-rot species *Coriolus versicolor* and *Phanerochaete chrysosporium*. Appl. Microbiol. Biotechnol. **39**:5-10.
- Evans, C. S. 1985. Laccase activity in lignin degradation by *Coriolus versicolor* in vivo and in vitro studies. FEMS Microbiol. Lett. **27**:339-343.
- Forrester, I. T., A. T. Grabski, C. Mishra, B. D. Kelley, W. N. Strickland, G. F. Leatham, and R. R. Burgess. 1990. Characteristics and N-terminal amino acid sequence of a manganese peroxidase purified from *Lentinus edodes* cultures grown on a commercial wood substrate. Appl. Microbiol. Biotechnol. **33**:359-365.
- Galliano, H., G. Gas, J. L. Seris, and A. M. Boudet. 1991. Lignin degradation by *Rigidoporus lignosus* involves synergistic action of two oxidizing enzymes: Mn peroxidase and laccase. Enzyme Microb. Technol. **13**:478-482.
- Glenn, J. K., L. Akileswaran, and M. Gold. 1986. Mn(II) oxidation is the principal function of the extracellular Mn-peroxidase from *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **251**:688-696.
- 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*. Arch. Biochem. Biophys. **242**:329-341.
- Haars, A., and A. Hüttermann. 1980. Function of laccase in the white-rot fungus *Fomes annosus*. Arch. Microbiol. **125**:233-237.
- Hammel, K. E., and M. A. Moen. 1991. Depolymerization of a synthetic lignin *in vitro* by lignin peroxidase. Enzyme Microb. Technol. **13**:15-18.
- Hammond, J. B. W., and D. A. Wood. 1985. Metabolism, biochemistry and physiology, p. 63-80. In P. B. Flegg, D. M. Spencer, and D. A. Wood (ed.), The biology and technology of the cultivated mushroom. Wiley and Sons, New York.
- Hatakka, A. I., T. K. Lundell, A. L. M. Tervilä-Wilo, and G. Brunow. 1991. Metabolism of non-phenolic β-O-4 lignin model compounds by the white-rot fungus *Phlebia radiata*. Appl. Microbiol. Biotechnol. **36**:270-277.
- Jodon, M. H., and D. J. Royse. 1979. Care and handling of cultures of the cultivated mushroom. The Pennsylvania Agricultural Experiment Station bulletin no. 258. The Pennsylvania State University, University Park.
- Karhunen, E., A. Kantelinen, and M.-L. Niku-Paavola. 1990. Mn-dependent peroxidase from the lignin-degrading white-rot fungus *Phlebia radiata*. Arch. Biochem. Biophys. **279**:25-31.
- Kerem, Z., D. Friesem, and Y. Hadar. 1992. Lignocellulose degradation during solid-state fermentation: *Pleurotus ostreatus* versus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **58**:1121-1127.
- Kersten, P. J., and T. K. Kirk. 1987. Involvement of a new enzyme, glyoxyl oxidase, in extracellular H<sub>2</sub>O<sub>2</sub> production by *Phanerochaete chrysosporium*. J. Bacteriol. **169**:2195-2201.
- Kirk, T. K., and R. L. Farrell. 1987. Enzymatic "combustion." The microbial degradation of lignin. Annu. Rev. Microbiol. **41**:465-505.
- Kuan, I.-C., and M. Tien. 1993. Stimulation of Mn peroxidase activity: a possible role for oxalate in lignin biodegradation. Proc. Natl. Acad. Sci. USA **90**:1242-1246.
- Lynch, J. M. 1993. Substrate availability in the production of composts, p. 24-35. In H. A. J. Hoitink and H. M. Keener (ed.), Science and engineering of composting: design, environmental, microbiological and utilization aspects. Renaissance Publications, Worthington, Ill.
- Maltseva, O. V., M.-L. Niku-Paavola, A. A. Leontievsky, N. M. Myasoedova, and L. A. Golovleva. 1991. Lignolytic enzymes of the white rot fungus *Panus tigrinus*. Biotechnol. Appl. Biochem. **13**:291-302.
- Matcham, S. E., B. R. Jordan, and D. A. Wood. 1985. Estimation of fungal biomass in a solid substrate by three independent

- methods. *Appl. Microbiol. Biotechnol.* **21**:108–112.
29. **Nerud, F., Z. Zouchova, and Z. Misurcova.** 1991. Lignolytic properties of different white-rot fungi. *Biotechnol. Lett.* **9**:657–660.
  30. **Orth, A. B., D. J. Royse, and M. Tien.** 1993. Ubiquity of lignin-degrading peroxidases among various wood-degrading fungi. *Appl. Environ. Microbiol.* **59**:4017–4023.
  31. **Paszczynski, A., R. L. Crawford, and V.-B. Huynh.** 1988. Manganese peroxidase of *Phanerochaete chrysosporium*: purification. *Methods Enzymol.* **161**:264–270.
  32. **Perez, J., and T. W. Jeffries.** 1990. Mineralization of <sup>14</sup>C-ring-labeled synthetic lignin correlates with the production of lignin peroxidase, not of manganese peroxidase or laccase. *Appl. Environ. Microbiol.* **56**:1806–1812.
  33. **Perry, C. R., S. E. Matcham, D. A. Wood, and C. F. Thurston.** 1993. The structure of laccase protein and its synthesis by the commercial button mushroom *Agaricus bisporus*. *J. Gen. Microbiol.* **139**:171–178.
  34. **Pharmacia LKB.** 1989. 2117 Multiphor II electrophoresis system handbook. Pharmacia LKB Biotechnology, Uppsala, Sweden.
  35. **Popp, J. L., B. Kalyanaraman, and T. K. Kirk.** 1990. Lignin peroxidase oxidation of Mn<sup>2+</sup> in the presence of veratryl alcohol, malonic or oxalic acid and oxygen. *Biochemistry* **29**:10475–10480.
  36. **Rüttiman, C., E. Schwember, L. Salas, D. Cullen, and R. Vicuña.** 1992. Lignolytic enzymes of the white-rot basidiomycetes *Phlebia brevispora* and *Ceriporiopsis subvermispora*. *Biotechnol. Appl. Biochem.* **16**:64–76.
  37. **Schisler, L. C.** 1967. Stimulation in yield in the cultivated mushroom by vegetable oils. *Appl. Microbiol.* **15**:844–850.
  38. **Tien, M.** 1987. Properties of ligninase from *Phanerochaete chrysosporium* and their possible applications. *Crit. Rev. Microbiol.* **15**:141–168.
  39. **Tien, M., and D. Cai.** 1990. Lignin-degrading peroxidases from *Phanerochaete chrysosporium*, p. 433–451. In C. C. Reddy, G. A. Hamilton, and K. M. Madyastha (ed.), *Biological oxidation systems*. Academic Press, San Diego, Calif.
  40. **Tien, M., and T. K. Kirk.** 1984. Lignin-degrading enzyme from *Phanerochaete chrysosporium*: purification, characterization and catalytic properties of a unique H<sub>2</sub>O<sub>2</sub>-requiring oxygenase. *Proc. Natl. Acad. Sci. USA* **81**:2280–2284.
  41. **Tien, M., and T. K. Kirk.** 1988. Lignin peroxidase of *Phanerochaete chrysosporium*. *Methods Enzymol.* **161**:238–249.
  42. **Turner, E. M.** 1974. Phenoloxidase activity in relation to substrate and development stage in the mushroom, *Agaricus bisporus*. *Trans. Br. Mycol. Soc.* **63**:541–547.
  43. **Waksman, S., and W. Nissen.** 1932. On the nutrition of the cultivated mushroom *Agaricus campestris*, and the chemical changes brought about by this organism in the manure compost. *Am. J. Bot.* **19**:514–537.
  44. **Waldner, R., M. S. A. Leisola, and A. Fletcher.** 1988. Comparison of lignolytic activities of selected white-rot fungi. *Appl. Microbiol. Biotechnol.* **29**:400–407.
  45. **Wariishi, H., K. Valli, and M. H. Gold.** 1991. *In vitro* depolymerization of lignin by manganese peroxidase of *Phanerochaete chrysosporium*. *Biochem. Biophys. Res. Commun.* **176**:269–275.
  46. **Wariishi, H., K. Valli, and M. H. Gold.** 1992. Manganese(II) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*—kinetic mechanism and role of chelators. *J. Biol. Chem.* **267**:23688–23695.
  47. **Wood, D. A.** 1980. Production, purification and properties of extracellular laccase of *Agaricus bisporus*. *J. Gen. Microbiol.* **117**:327–338.
  48. **Wood, D. A.** 1980. Inactivation of extracellular laccase during fruiting of *Agaricus bisporus*. *J. Gen. Microbiol.* **117**:339–345.
  49. **Wood, D. A., and P. W. Goodenough.** 1977. Fruiting of *Agaricus bisporus*. Changes in extracellular enzyme activities during growth and fruiting. *Arch. Microbiol.* **114**:161–165.