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Manganese Regulation of Veratryl Alcohol in White Rot Fungi and Its Indirect Effect on Lignin Peroxidase

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Many white rot fungi are able to produce de novo veratryl alcohol, which is known to be a cofactor involved in the degradation of lignin, lignin model compounds, and xenobiotic pollutants by lignin peroxidase (LiP). In this study, Mn nutrition was shown to strongly influence the endogenous veratryl alcohol levels in the culture fluids of N-deregulated and N-regulated white rot fungi *Bjerkandera* sp. strain BOS55 and *Phanerochaete chrysosporium* BKM-F-1767, respectively. Endogenous veratryl alcohol levels as high as 0.75 mM in *Bjerkandera* sp. strain BOS55 and 2.5 mM in *P. chrysosporium* were observed under Mn-deficient conditions. In contrast, veratryl alcohol production was dramatically decreased in cultures supplemented with 33 or 264 μM Mn. The LiP titers, which were highest in Mn-deficient media, were shown to parallel the endogenous veratryl alcohol levels, indicating that these two parameters are related. When exogenous veratryl alcohol was added to Mn-sufficient media, high LiP titers were obtained. Consequently, we concluded that Mn does not regulate LiP expression directly. Instead, LiP titers are enhanced by the increased production of veratryl alcohol. The well-known role of veratryl alcohol in protecting LiP from inactivation by physiological levels of H_2O_2 is postulated to be the major reason why LiP is apparently regulated by Mn. Provided that Mn was absent, LiP titers in *Bjerkandera* sp. strain BOS55 increased with enhanced fungal growth obtained by increasing the nutrient N concentration while veratryl alcohol levels were similar in both N-limited and N-sufficient conditions.

White rot fungi and their ligninolytic enzymes have potential applications in biopulping and biobleaching (32, 40), as well as in the bioremediation of aromatic pollutants (1, 14). The extracellular lignin-degrading machinery is typically composed of lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP), which function together with H_2O_2-generating oxidases and secondary metabolites (26). Veratryl alcohol is an important secondary metabolite involved in the ligninolytic system.

Many white rot fungi, especially those which secrete LiP, have been shown to produce veratryl alcohol de novo (11). This metabolite is formed by white rot fungal cultures where glucose is the only carbon source (8, 31). Labelled phenylalanine was shown to be the precursor to veratryl alcohol, indicating that this metabolite is derived from the shikimate pathway (23).

The occurrence of veratryl alcohol biosynthesis coincides with the physiological events associated with the appearance of LiP and lignin mineralization (8, 22). Exogenous addition of veratryl alcohol to white rot fungal cultures has generally been shown to stimulate the extracellular titers of LiP (12, 30, 34). Veratryl alcohol has been shown to prevent inactivation of LiP by excess H_2O_2 (7, 43). For many substrates of LiP, such as benzo[*a*]pyrene (18), azo dyes (36), Poly R-478 (35), *p*-anisyl alcohol (44), 4-methoxymandelate (20), and lignin (19), veratryl alcohol was found behave as a beneficial or essential cosubstrate for in vitro catalyzed reactions. The role of veratryl alcohol in stabilizing LiP against H_2O_2 inactivation has been suggested to account for the stimulation (44). More recently, it has also been demonstrated that veratryl alcohol is responsible for the proper turnover of the enzyme cycle (e.g., by reducing compound II), enabling improved oxidation of nonphenolic substrates (27). Considering that veratryl alcohol is produced endogenously together with LiP and has demonstrable physiological roles both in vivo and in vitro, it therefore must be regarded as a physiological cofactor of LiP.

The ligninolytic machinery in white rot fungi is highly regulated by nutrients. In particular, Mn and N have been shown to have strong regulating effects (16, 26). There are many literature reports which indicate that Mn has a potent inducing effect on the expression MnP in many white rot fungi (3, 6, 45). While Mn is an essential cofactor for the proper functioning of the MnP protein (15), the molecular regulation of MnP expression is also signaled by Mn (5). Many studies indicate that Mn addition can severely decrease LiP titers in white rot fungi (3, 37, 38). However, Mn up to 180 μM did not decrease the transcription of *lip* mRNA in *Phanerochaete chrysosporium* (29), indicating that the negative impact of Mn on LiP titers is not related to regulation at the molecular level.

N-sufficient conditions repress expression of both LiP and MnP in *P. chrysosporium* (16, 29). Likewise, endogenous production of veratryl alcohol was repressed by N sufficiency (13). However, it should be noted that strains of the genus *Bjerkandera* are N deregulated, as evidenced by the stimulated production of LiP and MnP in N-sufficient compared with N-limited medium (24, 25). It was demonstrated that in *Bjerkandera* sp. strain BOS55, endogenous production of veratryl alcohol was much higher in N-sufficient glucose-yeast extract-peptone medium than in N-limited glucose BIII medium (8). Since the glucose-yeast extract medium does not contain Mn while BIII does, either N or Mn could have been responsible for the enhanced production of veratryl alcohol.

Here we report that Mn was responsible for regulating the

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endogenous production of veratryl alcohol. This type of regulation was discovered during our research evaluating the effect of Mn and N nutrients on the peroxidase titers in *Bjerkandera* sp. strain BOS55. Experiments were also carried out to demonstrate that the apparent regulation of LiP by Mn is an indirect result of the direct effect of this metal ion on veratryl alcohol biosynthesis.

MATERIALS AND METHODS

Microorganisms. *Bjerkandera* sp. strain BOS55 was isolated and determined as described before (9). This strain produces LiP, MnP, and manganese-independent peroxidase (MIP) (8, 10). *Bjerkandera* sp. strain BOS55 and *P. chrysosporium* BKM-F-1767 (ATCC 24725) were maintained at 4°C on peptone-yeast extract slants (per liter, 20 g of glucose, 5 g of mycological peptone, 2 g of yeast extract, 1 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 15 g of agar), from which they were transferred to malt extract plates (per liter, 15.0 g of agar, 3.5 g of malt extract, 5.0 g of glucose). They were incubated at 30°C for 4 to 6 days before use as the inoculum in experiments. The experiments were inoculated with agar plugs as described before (24).

Media. The standard basal medium used contained 2.2 mM N in the form of diammonium tartrate, Mn-free BIII mineral medium modified from that of Tien and Kirk (42), 10 g of glucose $liter^{-1}$, and 2 mg of thiamine $liter^{-1}$ in 20 mM 2,2-dimethylsuccinate (pH 4.5) buffer. The KH_2PO_4 content of standard basal medium was 40 mM. Different media were prepared identically with either 33 or 264 μM Mn and with extra N from various sources. The extra N sources were NH_4^+ in the form of diammonium tartrate, peptone, and a mixture of 20 L-amino acids (equal amounts of Ala, Arg, Asn, Asp, Cys, Gly, Glu, Gln, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val). The concentration of all extra N sources was 19.8 mM N, providing a total N content of 22 mM. In the experiment in which the effects of Mn and N additions (2, 6, 20, and 60 mM peptone-N) on peroxidase activity and on veratryl alcohol levels were studied, the basal medium did not contain diammonium tartrate. The pH of media used in the experiments with *P. chrysosporium* was 4.5, and the pH in media used in the experiments with *Bjerkandera* sp. strain BOS55 was adjusted to a value of 6 by NaOH addition.

All media were sterilized with FP 030/3 filters with a pore size of 0.2 μm (Schleicher & Schuell, Dassel, Germany).

The background levels of Mn in Mn-free basal and peptone media were measured with an atomic absorption spectrophotometer (SprectrAA 300/400 System; Varian, Houten, The Netherlands). Background levels of 3.2×10^{-5} and 4.3×10^{-5} μM Mn were detected in basal medium and this medium supplemented with 19.8 mM peptone-N, respectively.

Culture conditions. Aliquots (5 ml) of media were placed in 250-ml serum bottles. Each bottle was loosely capped for passive aeration. The bottles were incubated statically under an air atmosphere. The incubation temperatures were 30°C in experiments with *Bjerkandera* sp. strain BOS55 and 37°C in those with *P. chrysosporium*. For measuring CO_2 evolution, the 250-ml serum bottles were sealed with gas-impermeable rubber septa. After each sampling, the headspaces of the cultures were aseptically flushed with air.

Enzyme assays. All enzymes were determined spectrophotometrically (Lambda 1 UV/VIS; Perkin-Elmer, Norwalk, Conn.) at 30°C. For enzymatic assays, centrifuged (12,000 × *g* for 10 min) extracellular fluids were utilized. LiP activity was measured by oxidation of veratryl alcohol (42) with a correction for the background veratryl oxidase activity in the absence of H_2O_2 and in the presence of 55-fold-diluted catalase of *Aspergillus niger* (Sigma, St. Louis, Mo.). MnP and MIP activities were measured in a combined assay (8). The reaction mixture contained 50 mM sodium malonate (pH 4.5), 1 mM 2,6-dimethoxyphenol, 1 mM $MnSO_4$, and up to 600 μl of supernatant in a total volume of 1 ml. The reaction was initiated by adding 0.4 mM H_2O_2 and corrected for laccase activity. MIP activity was measured in the reaction mixture containing 50 mM sodium malonate (pH 4.5), 1 mM 2,6-dimethoxyphenol, 1 mM EDTA, and up to 550 μl of supernatant in a total volume of 1 ml. The reaction was initiated by adding 0.4 mM H_2O_2 and corrected for laccase activity. MnP activity is expressed as combined MnP-MIP activity minus MIP activity.

Carbon dioxide analysis. Carbon dioxide production was measured in the headspace by gas chromatography with a model 427 apparatus (Packard, Palo Alto, Calif.) fitted with a thermal conductivity detector (140°C). The column (Hayesep Q; Chrompack, Middelburg, The Netherlands) was maintained at 110°C, and the carrier gas was helium (30 ml min^{-1}). The injection port was maintained at 110°C. The injection volume was 100 μl.

Determination of the dry weight of mycelium. Mycelial mats were separated from the culture fluids by filtration. Mycelia were rinsed with distilled water and filtered through dried and tared glass fiber filters (GF 50; Schleicher & Schuell). Mycelial dry weights were determined after overnight drying at 105°C.

Determination of secondary metabolites. A 50-μl volume of centrifuged supernatant was analyzed by high-pressure liquid chromatography. A Pascal series HPLC ChemStation (Hewlett-Packard, Waldbronn, Germany) equipped with an HP1050 pumping station, an HP1040M series II diode array detector, and an HP9000-300 data processor was used. The column (200 by 3 mm) was filled with ChromoSpher C18-PAH (5-μm particles) (Chrompack). Aromatic metabolites were analyzed with the following gradient (0.4 ml min^{-1}, 30°C): 90:10, 0:100, and 0:100 H_2O-CH_3CN at 0, 15, and 20 min, respectively. Compound identifications were based on matching retention times and UV spectra with standards of veratryl alcohol and veratryl aldehyde.

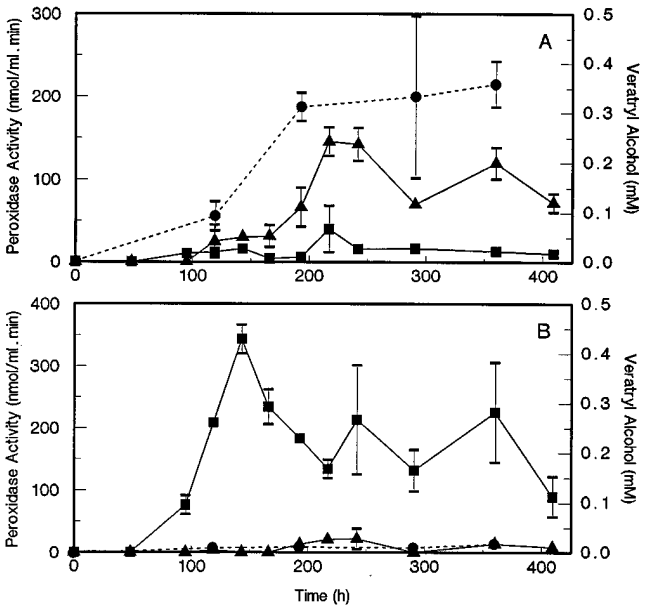

FIG. 1. Effect of Mn on the time course of peroxidase titers and endogenous veratryl alcohol concentrations in the extracellular fluid of *Bjerkandera* sp. strain BOS55. Panels: A, Mn-deficient medium; B, Mn-sufficient medium (264 μM Mn). Symbols: ■, MnP; ▲, LiP; ●, veratryl alcohol.

Determination of the extracellular protein profile by fast protein liquid chromatography (FPLC). Extracellular fluid was centrifuged at 20,000 × *g* for 10 min at 4°C to remove the mycelium. A 15-ml volume of supernatant was concentrated threefold by ultrafiltration through PM-10 membrane (Amicon, Rotterdam, The Netherlands) with a cutoff of 10 kDa. The concentrated supernatant was analyzed at 405 nm by anion-exchange chromatography with an FPLC system (Mono-Q HR 5/5; Pharmacia, Uppsala, Sweden). The column was equilibrated with 10 mM KP_i, pH 6.0. The enzymes were eluted with a linear salt gradient from 0 to 300 mM KCl in starting buffer at a flow rate of 1 ml min^{-1} and 1 fraction min^{-1} for 40 min.

Chemicals. Mycological peptone was obtained from Oxoid Ltd. (Basingstoke, Hampshire, England). The L-amino acids were obtained from either Merck (Darmstadt, Germany) or Janssen Chimica (Geel, Belgium). Veratryl alcohol and veratryl aldehyde were purchased from Janssen Chimica. All other chemicals were commercially available and were used without further purification.

Statistical procedures. In all experiments, the measurements were carried out with triplicate parallel cultures. The values reported are means with standard deviations.

RESULTS

Effect of manganese on peroxidase and veratryl alcohol production in *Bjerkandera* sp. strain BOS55. Extracellular titers of LiP, MnP, and MIP and veratryl alcohol concentrations were measured for 18 days in cultures receiving three levels of Mn (0, 33, and 264 μM). Figure 1 shows a typical example of the time course of MnP, LiP, and veratryl alcohol production under Mn deficiency and Mn sufficiency. At a high Mn concentration, low LiP activity and traces of veratryl alcohol were detected; however, MnP was remarkably stimulated. On the other hand, MnP activity was very low in the absence of Mn, whereas LiP and veratryl alcohol production was enhanced.

The Mn regulation of veratryl alcohol and peroxidase production at different nitrogen levels was also tested. Four levels of peptone-N were studied (2, 6, 20, and 60 mM). Figure 2 presents the peak concentrations of endogenous veratryl alcohol production and the peak titers of MnP and LiP measured

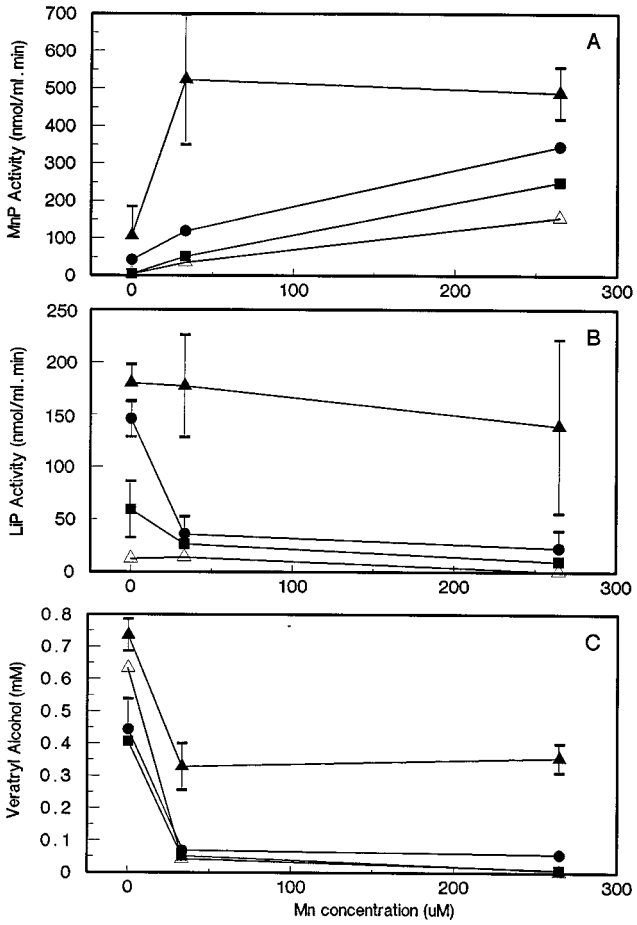

FIG. 2. Effect of Mn and peptone-N supplements on peak peroxidase titers and on the peak concentration of endogenous veratryl alcohol in *Bjerkandera* sp. strain BOS55. Panels: A, MnP; B, LiP; C, veratryl alcohol. Peptone-N was added at 2 (△), 6 (■), 20 (●), or 60 (▲) mM.

during 18 days of culturing in media with different nitrogen and manganese levels. Veratryl alcohol production was greatly influenced by manganese at any level of nitrogen. In the Mn-deficient media, irrespective of the peptone-N regimen, veratryl alcohol concentrations were high (0.40 to 0.75 mM) while they were much lower (<0.075 mM) in media containing added Mn. An exception to this trend was observed in the 60 mM peptone-N media, where the negative impact of Mn on veratryl alcohol production was partially reversed. During the experiment, the concentrations of veratryl aldehyde were also monitored. In Mn-deficient media, the veratryl aldehyde concentrations were very low (ranging from 0.007 to 0.044 mM) compared with the veratryl alcohol levels (Fig. 2C). The veratryl aldehyde levels were also significantly lowered by Mn supplementation.

Parallel to the trends observed for veratryl alcohol production, LiP titers were highest under Mn-deficient conditions provided the N content was 6 mM or greater. The LiP titers were remarkably reduced by Mn sufficiency. Again, it was observed that the negative effect of Mn could be diminished by 60 mM peptone-N. MnP activity was consistently enhanced by Mn addition at any level of nitrogen. The highest MnP activities were detected in the 264 μM Mn media.

The influence of Mn and nitrogen on the third type of peroxidase in *Bjerkandera* sp. strain BOS55, MIP, was also examined. Mn had no effect on MIP production at any level of nitrogen (result not shown). However, increasing nitrogen contents in the media gave increasing MIP titers at all levels of Mn. For example, at 33 μM Mn, 2, 6, 20, and 60 mM peptone-N gave peak MIP titers of 7.18 ± 1.08, 21.98 ± 12.62, 52.88 ± 11.08, and 198.08 ± 31.18 nmol ml^{-1} min^{-1}, respectively.

TABLE 1. Effect of manganese on total CO_2 production after 18 days

N source (concn [mM])	Mean cumulative CO_2 production (mmol) ± SD at Mn concn of:		
	0 μM	33 μM	264 μM
Peptone-N (2)	0.218 ± 0.008	0.204 ± 0.015	0.326 ± 0.019
Ammonium-N (2.2)	0.258 ± 0.021	0.348 ± 0.015	0.429 ± 0.013
Peptone-N (6)	0.424 ± 0.015	0.508 ± 0.018	0.546 ± 0.012
Peptone-N (20)	1.104 ± 0.049	1.101 ± 0.014	1.030 ± 0.028
Amino acids-N (22)	1.065 ± 0.072	0.911 ± 0.033	0.961 ± 0.015
Peptone-N (60)	1.473 ± 0.011	1.380 ± 0.076	1.434 ± 0.078

Other nitrogen sources, NH_4^+ and the amino acid mixture at 22 mM N, were also studied to determine if the type of nitrogen source affects the Mn regulation of peroxidase and veratryl alcohol production. The trends described above for peptone-N were the same for the other nitrogen sources (results not shown). However, the maximum veratryl alcohol concentrations achieved (0.18 ± 0.02 to 0.24 ± 0.06 mM) in Mn-deficient cultures were somewhat lower than those observed with peptone-N. A part of the decrease could be accounted for by the fact that the veratryl aldehyde concentrations were higher (0.096 to 0.112 mM) when NH_4^+ and amino acids were used as the N sources. In the presence of Mn, both veratryl alcohol and veratryl aldehyde were only present at trace levels.

The differences observed in peroxidase and veratryl alcohol production due to Mn cannot be attributed to the role of manganese on fungal growth. Tables 1 and 2 compare data on total carbon dioxide production and mycelial yield as a function of Mn and N nutrition. No significant differences in either of these parameters were found when comparing Mn-deficient and -sufficient cultures under high-N conditions. Likewise, under low-N conditions, Mn had no effect on the biomass yield. However, Mn increased the CO_2 production to a small extent.

Effect of manganese on extracellular heme-protein composition in *Bjerkandera* sp. strain BOS55. The changes in extracellular heme-protein composition in the absence of Mn and in the presence of 264 μM Mn were monitored by FPLC on days 4, 7, 10, and 14 in 22 mM peptone-N-containing culture fluids. An example of these profiles is shown in Fig. 3, demonstrating that the heme-protein profiles on day 7 differed depending on the Mn concentration. In the absence of Mn, LiP activity was dominant in those fractions with higher A_{405} values. In the culture fluid containing 264 μM Mn, the absorbance of LiP-

TABLE 2. Effect of Mn on yield of mycelium dry weight on day 11

N source (concn [mM])	Mean mycelium dry wt (mg) ± SD at Mn concn of:		
	0 μM	33 μM[a]	264 μM
Ammonium-N (2.2)	10.650 ± 1.501	8.867 ± 0.660	10.525 ± 0.928
Peptone-N (20)	24.075 ± 4.412	26.400 ± 4.210	26.667 ± 0.329
Peptone-N (60)	33.900 ± 3.259	33.633 ± 1.144	34.100 ± 1.778

[a] The dry weight of the mycelium for cultures receiving 33 μM Mn was determined in an experiment separate from those using 0 and 264 μM Mn.

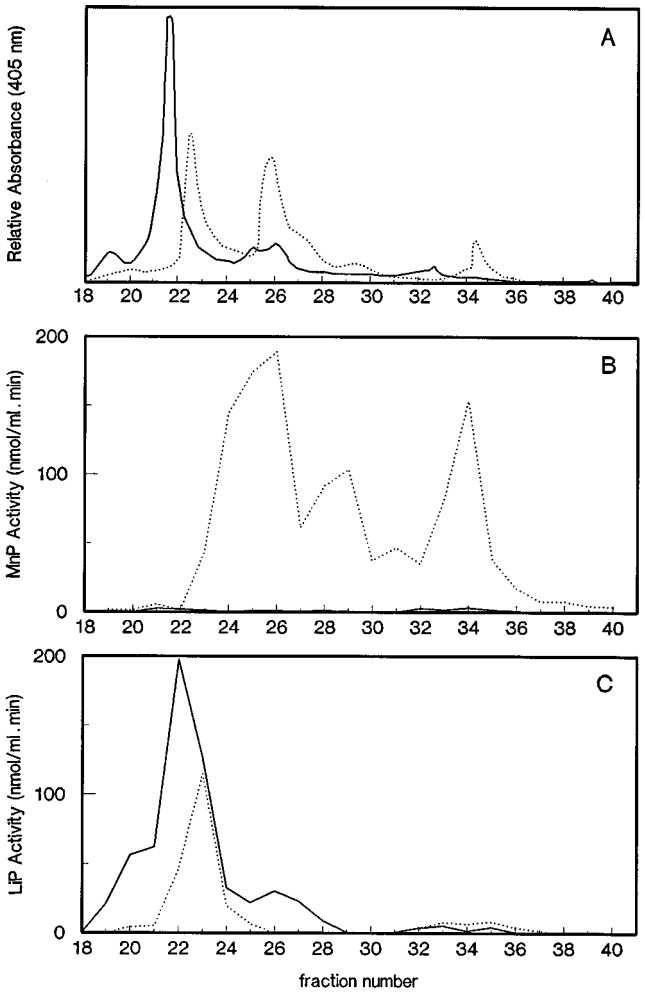

FIG. 3. FPLC protein profile of 7-day-old extracellular fluids of *Bjerkandera* sp. strain BOS55 grown in medium containing 22 mM peptone-N. Panels: A, A_{405}; B, MnP activity; C, LiP activity. ——, Mn not added; . . . . . ., Mn added (264 μM).

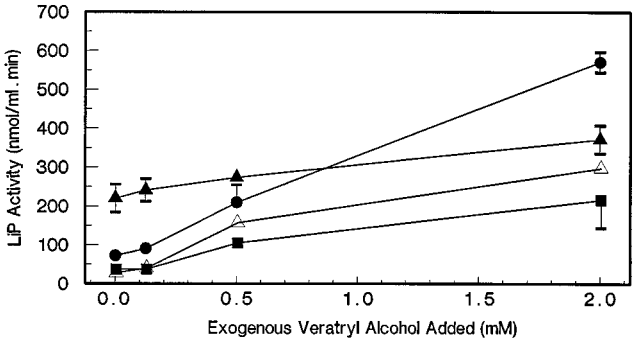

FIG. 4. Effect of exogenous veratryl alcohol additions on peak LiP titers of *Bjerkandera* sp. strain BOS55 grown in media containing 33 μM Mn under varied conditions of N nutrition. Symbols: △, 2.2 mM NH_4^+-N; ■, 22 mM NH_4^+-N; ●, 22 mM amino acids-N; ▲, 22 mM peptone-N.

containing fractions decreased and at the same time, new peaks were evident where mainly MnP activity occurred. During the entire time course, FPLC peaks corresponding mainly to LiP activity were dominant in Mn-free media whereas peaks with MnP activities remained dominant in the high-Mn medium.

Effect of exogenous veratryl alcohol addition on LiP production in *Bjerkandera* sp. strain BOS55. The effect of exogenous veratryl alcohol addition on LiP production was tested in the presence of 33 μM Mn. Veratryl alcohol was added at concentrations of 0.125, 0.5, and 2 mM at the time of inoculation, and LiP activity was measured for 18 days and compared to that of control cultures without veratryl alcohol addition.

LiP production was greatly enhanced by increasing veratryl alcohol additions in Mn-sufficient media irrespective of the nitrogen source and the concentration (Fig. 4). Surprisingly, high LiP titers were obtained in low-N media (2.2 mM NH_4^+-N) with addition of 0.5 and 2 mM veratryl alcohol. The most remarkable increase in LiP activity was measured in media with the amino acid mixture as the nitrogen source. Peptone medium gave the highest LiP activity in the controls (no veratryl alcohol added) in comparison with the other media, and with addition of veratryl alcohol, the increases in LiP production were less dramatic than with the other nitrogen sources.

The fate of the exogenous veratryl alcohol was monitored after 2 mM veratryl alcohol addition (data not shown). The veratryl alcohol concentration was reduced to about 1 mM on day 8. This level of veratryl alcohol remained more or less the same during the rest of the experiment in media with organic N sources, while veratryl alcohol decreased further to 0.22 mM or less in media with NH_4^+ as the N source. In almost all cases, approximately half of the eliminated veratryl alcohol was recovered in the form of veratryl aldehyde.

Effect of manganese on LiP and veratryl alcohol production in *P. chrysosporium*. The effect of Mn on LiP production by another white rot fungus was also investigated. *P. chrysosporium* was cultured under N-limited conditions either in Mn-deficient or in Mn-containing media (33 μM) to compare LiP titers and veratryl alcohol concentrations for 14 days. During the first 8 days, only slight increases in LiP activity and veratryl alcohol levels could be attributed to the Mn deficiency. However, after day 8, both the LiP titers and the veratryl alcohol concentrations started to become remarkably higher in the absence of Mn (Fig. 5). The endogenous veratryl alcohol reached a maximum concentration of 2.5 mM on day 11.

DISCUSSION

Mn regulation of peroxidases. The results of this study demonstrate that Mn has a strong regulatory effect on the appearance of MnP and LiP titers in the extracellular fluids of *Bjerkandera* sp. strain BOS55. In contrast to these peroxidases, the titer of the third type of peroxidase in *Bjerkandera* sp. strain BOS55, MIP, was not influenced by Mn nutrition.

The MnP titers in *Bjerkandera* sp. strain BOS55 were highly stimulated in media containing 33 and 264 μM Mn(II) compared with those in medium lacking Mn(II). Similar results have been reported previously for a wide variety of white rot fungi (3). Addition of Mn(II) to Mn-deficient cultures results in detectable *mnp* mRNA in *P. chrysosporium* (16), indicating that Mn has a regulatory role at the molecular level in white rot fungi.

In contrast to MnP, LiP titers in the extracellular fluids of *Bjerkandera* sp. strain BOS55 were dramatically lowered when the organism was cultivated in medium containing 33 or 264 μM Mn(II) compared with those of organisms cultivated in Mn-deficient medium. However, in 264 μM Mn medium, the LiP protein peak areas in FPLC profiles and the LiP activities

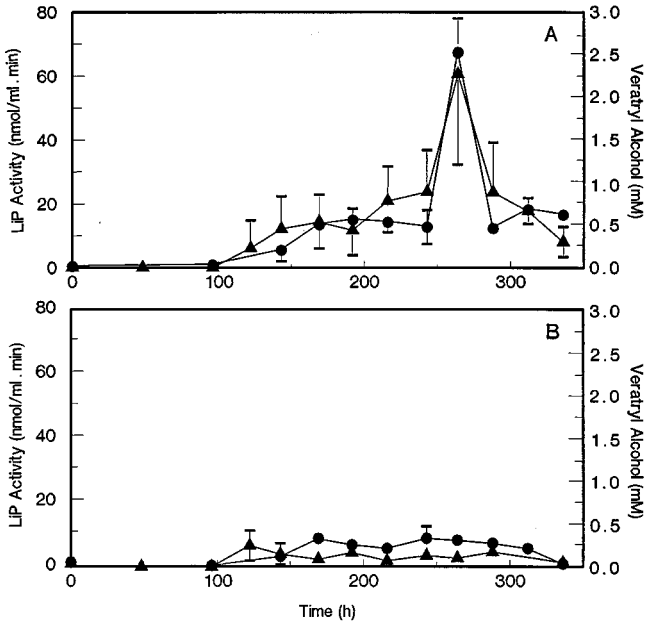

FIG. 5. Effect of Mn on the time course of LiP titers and endogenous veratryl alcohol concentrations in extracellular fluids of *P. chrysosporium* BKM-F-1767. Panels: A, Mn-deficient medium; B, Mn-sufficient medium (33 μM Mn). Symbols: ▲, LiP; ●, veratryl alcohol.

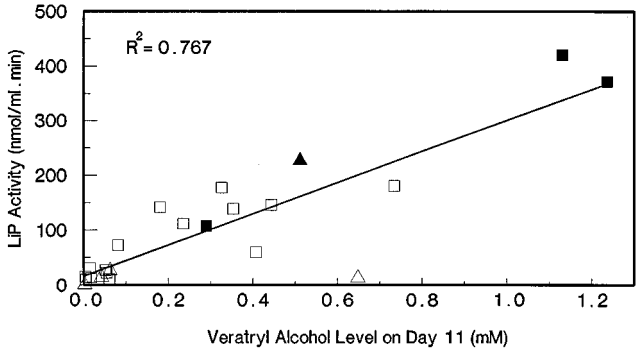

FIG. 6. Correlation between LiP titers and veratryl alcohol concentrations in the extracellular fluids of an 11-day-old *Bjerkandera* sp. strain BOS55 culture. Shown are endogenous veratryl alcohol concentrations in response to various Mn concentrations in media supplemented with 2 (△) or 20 to 60 (□) mM N and exogenous veratryl alcohol concentrations remaining in Mn-sufficient cultures supplemented with 2.2 (▲) or 22 (■) mM N.

in the extracellular protein mixture washed free of low-molecular-weight material were equal to about half of those obtained from cultures grown under Mn deficiency. Similar observations were also reported in experiments with *Phlebia tremellosa* and *P. chrysosporium* (37, 47). These findings, together with the fact that Mn had no demonstratable effect in lowering *lip* mRNA levels in *P. chrysosporium* (29), suggest that Mn(II) additions somehow inhibit LiP activity but do not necessarily prevent LiP expression. Mn(II) itself is not toxic to LiP in vitro at concentrations below 1,000 μM (21). Thus, the toxic effect of Mn(II) addition is most likely related to a product generated from Mn(II). One obvious candidate is Mn(III), which is known to rapidly deactivate LiP (39).

Mn regulation of veratryl alcohol biosynthesis. The absence of Mn(II) in the medium was found to strongly increase the physiological levels of endogenous veratryl alcohol in the two white rot fungi tested, *Bjerkandera* sp. strain BOS55 and *P. chrysosporium* BKM-F 1767. This is the first time that Mn nutrition has been shown to affect the veratryl alcohol levels in white rot fungi. Previously, it was claimed that Mn(II) nutrition has no influence on the endogenous veratryl alcohol levels in *P. chrysosporium* (6). However, this claim was based on a spot check on a 6-day-old culture. In our experiments, Mn-deficient medium provided an unprecedented high level of endogenously produced veratryl alcohol (2.5 mM) on day 11. Mn deficiency under a wide variety of experimental conditions consistently stimulated endogenous veratryl alcohol pools in *Bjerkandera* sp. strain BOS55. The appearance of veratryl alcohol was earlier; however, the peak levels of around 0.75 mM were not as high as those in *P. chrysosporium.*

The fact that Mn had such an important impact on the endogenous veratryl alcohol levels in white rot fungi suggests that it regulates enzymes involved in either the degradation, recycling, or biosynthesis of veratryl alcohol. Even though veratryl alcohol is a de novo metabolite, it is also degraded by white rot fungi (11, 31). Intracellular reductases have been shown to occur in *Bjerkandera* sp. BOS55, which can recycle veratryl aldehyde back to veratryl alcohol (8). In this study, veratryl aldehyde was observed to be an important degradation product when high levels of exogenous veratryl alcohol were added to the culture medium. Endogenous veratryl aldehyde remained at trace levels and was not observed to increase as a result of the lowered endogenous veratryl alcohol levels in Mn-sufficient culture conditions. On the contrary, veratryl aldehyde levels were even lower in Mn-sufficient medium. Consequently, we must conclude that Mn is not likely involved in the regulation of veratryl alcohol degradation or recycling. Therefore, a more plausible location for the regulation would be during the biosynthesis of veratryl alcohol. Veratryl alcohol is synthesized via phenylalanine, cinnamic acid, benzoic acid, and benzylaldehyde (23). The regulation probably occurs in a step after phenylalanine. Mn had little or no effect on fungal growth in NH_4^+-N media lacking phenylalanine. Therefore, the fungus was apparently able to synthesize this essential amino acid in the presence of Mn.

Indirect effect of veratryl alcohol on LiP titers. The endogenous levels of veratryl alcohol were generally highly correlated with LiP titers. This finding suggested that the higher LiP titers found in Mn-deficient cultures were an indirect result of the enhanced veratryl alcohol production. LiP isozymes are known to be rapidly inactivated by physiological levels of H_2O_2 (7, 21, 43), and veratryl alcohol is known to protect LiP from this kind of inactivation (7, 18, 43, 44). Adding exogenous veratryl alcohol to Mn-sufficient cultures of *Bjerkandera* sp. strain BOS55 resulted in high LiP titers. The LiP titers were highly statistically correlated ($R^2 = 0.767$, $P < 0.001$) with the veratryl alcohol concentration, irrespective of whether it was produced endogenously in Mn-deficient cultures or added exogenously in Mn-sufficient medium (Fig. 6), confirming that Mn(II) has no direct regulating effect on LiP expression. Instead, LiP titers are only apparently higher because of the enhanced endogenous production of veratryl alcohol.

Exogenous veratryl alcohol supplements are commonly used in studies evaluating LiP production by white rot fungi. For this reason, Mn at concentrations of up to approximately 200 μM had no major role in lowering LiP titers (3, 33, 38, 46). However, when abnormally high concentrations of Mn were used (720 to 3618 μM), little or no LiP activity was detected (3, 33, 38). In these cases, Mn probably had an inhibitory effect on LiP protein activity, as suggested above.

While veratryl alcohol apparently induces LiP expression in

Bjerkandera sp. strain BOS55 and many other white rot fungi (12, 34), no increases in *lip* mRNA levels were found to result from adding veratryl alcohol to cultures of *P. chrysosporium* (7, 41). Thus, LiP secretion must be signalled by another mechanism. Under the N-sufficient conditions used here, a likely signal would be the cyclic AMP that results from limited energy resources during C starvation and is known to induce *lip* gene expression in *P. chrysosporium* (4). The first appearance of LiP activity in *Bjerkandera* sp. strain BOS55 generally coincided with the cessation of growth, indicating that it occurred when the readily available substrates were being depleted.

Nitrogen regulation. *Bjerkandera* sp. strain BOS55 and other *Bjerkandera* strains produce LiP and MnP in N-sufficient conditions (8, 24, 25). Therefore, *Bjerkandera* spp. can be regarded as N deregulated, in strong contrast to the model fungus, *P. chrysosporium* (16, 26). In the absence of N regulation, increasing N would be expected to increase peroxidase production as a result of increased cell yields. *Bjerkandera* sp. strain BOS55 followed this behavior. N sufficiency remarkably increased growth with a parallel increase in LiP titers in Mn-deficient medium or with a parallel increase in MnP titers in Mn-sufficient medium. On the other hand, veratryl alcohol levels in any given Mn regimen were more or less similar, independently of the N supplied. Therefore, N has no regulatory effect on veratryl alcohol biosynthesis in *Bjerkandera* sp. BOS55, in contrast to *P. chrysosporium* (13, 26).

Previously, it was reported that Mn regulation and N regulation function independently of each other in both wild-type *P. chrysosporium* and N-deregulated mutants (45). Between 2 and 20 mM N, this pattern was also observed to be the case with *Bjerkandera* sp. strain BOS55. However, when 60 mM peptone-N was used, the Mn-sufficient media were no longer able to repress veratryl alcohol production or lower LiP titers. This effect may be due to the ability of some peptides in peptone to chelate Mn. Peptides containing glycine, cysteine, and glutamate are powerful metal-binding agents (17). In one study, Mn(II) chelated by polyglutamate was unable to enter the active site of MnP (28). Perhaps peptides can also lower the bioavailability of Mn(II) for the molecular regulation of a key enzyme involved in veratryl alcohol biosynthesis or for a key enzyme that requires Mn as a cofactor.

Importance of veratryl alcohol regulation by Mn. Veratryl alcohol is an essential cofactor for the proper functioning of LiP. Nonphenolic lignin models and lignin itself can be degraded in vitro with LiP only if veratryl alcohol is present (19, 20, 44). Therefore, the biosynthesis of veratryl alcohol in response to Mn deficiency must be regarded as a key physiological event enabling LiP to function as a ligninolytic enzyme. Mn deficiency would be expected to result from decreases in available Mn lost to insoluble MnO_2 deposits occurring during white rot decay (2, 39).

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REFERENCES

1. **Barr, D. P., and S. D. Aust.** 1994. Mechanisms white rot fungi use to degrade pollutants. Environ. Sci. Technol. **28:**78–87.
2. **Blanchette, R. A.** 1984. Manganese in wood decay by white rot fungi. Phytopathology **74:**725–730.
3. **Bonnarme, P., and T. W. Jeffries.** 1990. Mn(II) regulation of lignin peroxidases and manganese-dependent peroxidases from lignin-degrading white-rot fungi. Appl. Environ. Microbiol. **56:**210–217.
4. **Boominathan, K., and C. A. Reddy.** 1992. cAMP-mediated differential regulation of lignin peroxidase and manganese-dependent peroxidase production in the white-rot basidiomycete *Phanerochaete chrysosporium*. Proc. Natl. Acad. Sci. USA **89:**5586–5590.
5. **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.
6. **Brown, J. A., J. K. Glenn, and M. H. Gold.** 1990. Manganese regulates expression of manganese peroxidase by *Phanerochaete chrysosporium*. J. Bacteriol. **172:**3125–3130.
7. **Cancel, A. M., A. B. Orth, and M. Tien.** 1993. Lignin and veratryl alcohol are not inducers of the ligninolytic system of *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **59:**2909–2913.
8. **De Jong, E., A. E. Cazemier, J. A. Field, and J. A. M. de Bont.** 1994. Physiological role of chlorinated aryl alcohols biosynthesized de novo by the white rot fungus *Bjerkandera* sp. strain BOS55. Appl. Environ. Microbiol. **60:**271–277.
9. **De Jong, E., F. P. de Vries, J. A. Field, R. P. van der Zwan, and J. A. M. de Bont.** 1992. Isolation and screening of basidiomycetes with high peroxidative activity. Mycol. Res. **96:**1098–1104.
10. **De Jong, E., J. A. Field, and J. A. M. Bont.** 1992. Evidence for a new extracellular peroxidase: manganese inhibited peroxidase from the white-rot fungus *Bjerkandera* sp. BOS55. FEBS Lett. **299:**107–110.
11. **De Jong, E., J. A. Field, and J. A. M. de Bont.** 1994. Aryl alcohols in the physiology of white-rot fungi. FEMS Microbiol. Rev. **13:**153–188.
12. **Faison, B. D., T. K. Kirk, and R. L. Farrell.** 1986. Role of veratryl alcohol in regulating ligninase activity in *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **52:**251–254.
13. **Fenn, P., and T. K. Kirk.** 1981. Relationship of nitrogen to the onset and suppression of ligninolytic activity and secondary metabolism in *Phanerochaete chrysosporium*. Arch. Microbiol. **130:**59–65.
14. **Field, J. A., E. de Jong, G. Feijoo Costa, and J. A. M. de Bont.** 1993. Screening for ligninolytic fungi applicable to the biodegradation of xenobiotics. Trends Biotechnol. **14:**44–49.
15. **Glenn, J. K., L. Akileswaran, and M. H. Gold.** 1986. Mn(II) oxidation is the principal function of the extracellular Mn-peroxidase from *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **251:**688–696.
16. **Gold, M. H., and M. Alic.** 1993. Molecular biology of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Microbiol. Rev. **57:**605–622.
17. **Grill, E., E.-L. Winnacker, and M. H. Zenk.** 1986. Synthesis of seven different homologous phytochelatins in metal-exposed *Schizosaccharomyces pombe* cells. FEBS Lett. **197:**115–120.
18. **Haemmerli, S. D., M. S. A. Leisola, D. Sanglard, and A. Fiechter.** 1986. Oxidation of benzo(a)pyrene by extracellular ligninase of *Phanerochaete chrysosporium*. J. Biol. Chem. **261:**6900–6903.
19. **Hammel, K. E., and M. A. Moen.** 1991. Depolymerization of a synthetic lignin in vitro by lignin peroxidase. Enzyme Microb. Technol. **13:**15–18.
20. **Harvey, P. J., H. E. Schoenmaker, and J. M. Palmer.** 1986. Veratryl alcohol as a mediator and the role of radical cations in lignin biodegradation by *Phanerochaete chrysosporium*. FEBS Lett. **195:**242–246.
21. **Hu, Z. C., R. A. Korus, C. R. Venkataramu, and R. L. Crawford.** 1993. Deactivation kinetics of lignin peroxidase from *Phanerochaete chrysosporium*. Enzyme Microb. Technol. **15:**567–574.
22. **Jeffries, T. W., S. Choi, and T. K. Kirk.** 1981. Nutritional regulation of lignin degradation by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **42:**290–296.
23. **Jensen, K. A., Jr., K. M. C. Evans, T. K. Kirk, and K. E. Hammel.** 1994. Biosynthetic pathway for veratryl alcohol in ligninolytic fungus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **60:**709–714.
24. **Kaal, E. E. J., E. de Jong, and J. A. Field.** 1993. Stimulation of ligninolytic peroxidase activity by nitrogen nutrients in the white rot fungus *Bjerkandera* sp. strain BOS55. Appl. Environ. Microbiol. **59:**4031–4036.
25. **Kimura, Y., Y. Asada, and M. Kuwahara.** 1990. Screening of basidiomycetes for lignin peroxidase genes using a DNA probe. Appl. Microbiol. Biotechnol. **32:**436–442.
26. **Kirk, T. K., and R. L. Farrell.** 1987. Enzymatic "combustion": the microbial degradation of lignin. Annu. Rev. Microbiol. **41:**465–505.
27. **Koduri, R. S., and M. Tien.** 1994. Kinetic analysis of lignin peroxidase. Explanation for the mediation phenomenon by veratryl alcohol. Biochemistry **33:**4225–4230.
28. **Kuan, I.-C., K. A. Johnson, and M. Tien.** 1993. Kinetic analysis of manganese peroxidase. The reaction with manganese complexes. J. Biol. Chem. **268:**20064–20070.
29. **Li, D., M. Alic, and M. H. Gold.** 1994. Nitrogen regulation of lignin peroxidase gene transcription. Appl. Environ. Microbiol. **60:**3447–3449.
30. **Liebskind, M., H. Hocker, C. Wandrey, and A. C. Jager.** 1990. Strategies for improved lignin peroxidase production in agitated pellet cultures of *Phanerochaete chrysosporium* and the use of a novel inducer. FEMS Microbiol. Lett. **71:**325–330.
31. **Lundquist, K., and T. K. Kirk.** 1978. De novo synthesis and decomposition of veratryl alcohol by a lignin-degrading basidiomycete. Phytochemistry **17:**1676.

32. **Messner, K., and E. Srebotnik.** 1994. Biopulping: an overview of developments in an environmentally safe paper-making technology. FEMS Microbiol. Rev. **13:**351–364.
33. **Michel, F. C., Jr., S. B. Dass, E. A. Grulke, and C. A. Reddy.** 1991. Role of manganese peroxidase and lignin peroxidases of *Phanerochaete chrysosporium* in the decolorization of kraft bleach plant effluent. Appl. Environ. Microbiol. **57:**2368–2375.
34. **Niku-Paavola, M.-L., E. Karhunen, A. Kantelinen, L. Viikari, T. Lundell, and A. Hatakka.** 1990. The effect of culture conditions on the production of lignin modifying enzymes by the white-rot fungus *Phlebia radiata*. J. Biotechnol. **13:**211–221.
35. **Ollikka, P., K. Alhonmäki, V.-M. Leppänen, T. Glumoff, T. Raijola, and I. Suominen.** 1993. Decolorization of azo, triphenyl methane, heterocyclic, and polymeric dyes by lignin peroxidase isoenzymes from *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **59:**4010–4016.
36. **Paszczynski, A., and R. L. Crawford.** 1991. Degradation of azo compounds by ligninase from *Phanerochaete chrysosporium*: involvement of veratryl alcohol. Biochem. Biophys. Res. Commun. **178:**1056–1063.
37. **Perez, J., and T. W. Jeffries.** 1990. Mineralization of ^{14}C-ring-labeled synthetic lignin correlates with the production of lignin peroxidase, not of manganese peroxidase or laccase. Appl. Environ. Microbiol. **56:**1806–1812.
38. **Perez, J., and T. W. Jeffries.** 1992. Role of manganese and organic acid chelators in regulating lignin degradation and biosynthesis of peroxidases by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **58:**2402–2409.
39. **Perez, J., and T. W. Jeffries.** 1993. Role of organic chelators in manganese regulation of lignin degradation by *Phanerochaete chrysosporium*. Appl. Biochem. Biotechnol. **39/40:**227–238.
40. **Reid, I. D., and M. G. Paice.** 1994. Biological bleaching of the kraft pulps by white-rot fungi and their enzymes. FEMS Microbiol. Rev. **13:**369–376.
41. **Reiser, J., I. S. Walther, C. Fraefel, and A. Fiechter.** 1993. Methods to investigate the expression of the lignin peroxidase genes by the white rot fungus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **59:**2897–2903.
42. **Tien, M., and T. K. Kirk.** 1988. Lignin peroxidase of *Phanerochaete chrysosporium*. Methods Enzymol. **161B:**238–248.
43. **Tonon, F., and E. Odier.** 1988. Influence of veratryl alcohol and hydrogen peroxide on ligninase activity and ligninase production by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **54:**466–472.
44. **Valli, K., H. Wariishi, and M. H. Gold.** 1990. Oxidation of monomethoxylated aromatic compounds by lignin peroxidase: role of veratryl alcohol in lignin biodegradation. Biochemistry **29:**8535–8539.
45. **Van der Woude, M. W., K. Boominathan, and C. Adimarayana-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.
46. **Vares, T., T. K. Lundell, and A. I. Hatakka.** 1993. Production of multiple lignin peroxidases by the white-rot fungus *Phlebia ochraceofulva*. Enzyme Microb. Technol. **15:**664–669.
47. **Vares, T., O. Niemenmaa, and A. Hatakka.** 1994. Secretion of ligninolytic enzymes and mineralization of ^{14}C-ring-labelled synthetic lignin by three *Phlebia tremellosa* strains. Appl. Environ. Microbiol. **60:**569–575.