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# $Mn^{2+}$ Alters Peroxidase Profiles and Lignin Degradation by the White-Rot Fungus *Pleurotus ostreatus* Under Different Nutritional and Growth Conditions

**Roni Cohen, Limor Persky, Zahit Hazan-Eitan, Oded Yarden, and Yitzhak Hadar***

*Department of Plant Pathology and Microbiology and The Otto Warburg Center for Biotechnology in Agriculture, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, PO Box 12, Rehovot 76100, Israel, E-mail: hadar@agri.huji.ac.il*

## Abstract

The white-rot fungus *Pleurotus ostreatus* produces two types of extracellular peroxidases: manganese-dependent peroxidase (MnP) and versatile peroxidase (VP). The effect of $Mn^{2+}$ on fungal growth, peroxidase activity profiles, and lignin degradation by *P. ostreatus* was studied in liquid culture and under solid-state fermentation conditions on perlite, the latter resembling the natural growth conditions of this fungus. The fungus was grown in either a defined asparagine-containing basidiomycete selective medium (BSM) or in a rich peptone medium (PM). Biomass production, as determined by respiration experiments in solid-state fermentation and liquid cultures and fungal growth on Petri dishes, was higher in the PM than in the BSM. $Mn^{2+}$ affected biomass production only in the PM on Petri dishes. In the nonamended PM, high levels of MnP and VP activity were detected relative to the nonamended BSM. Nevertheless, a higher rate of $^{14}C$-lignin mineralization was measured in the $Mn^{2+}$-amended BSM, as determined during the course of 47 d of fermentation. $Mn^{2+}$ amendment of the PM increased mineralization rate to that obtained in the $Mn^{2+}$-amended BSM. The enzyme activity profiles of MnP and VP were studied in the BSM using anion-exchange chromatography. In the nonamended BSM, only minute levels of MnP and VP were detected. On $Mn^{2+}$ amendment, two MnP isoenzymes (B1 and B2) appeared. Isoenzyme B2 was purified and showed 100% identity with the MnP isoenzyme purified in our previous study from PM-solid-state fermentation (P6). P6 was found to be the dominant isoenzyme

*Author to whom all correspondence and reprint requests should be addressed.

in terms of activity level and gene expression compared with the VP isoenzymes. Based on these results, we concluded that $Mn^{2+}$ plays a key role in lignin degradation under different nutritional and growth conditions, since it is required for the production of MnP in *P. ostreatus*.

**Index Entries:** *Pleurotus ostreatus*; manganese peroxidase; versatile peroxidase; lignin degradation; solid-state fermentation.

## Introduction

In white-rot fungi, lignin depolymerization is catalyzed by extracellular oxidative enzymes. *Pleurotus* species preferentially degrades lignin in cotton stalks and wheat straw *(1,2)*. *Pleurotus* species has been found to produce laccase *(3,4)*, aryl-alcohol oxidase *(5)*, and two types of peroxidases: manganese-dependent peroxidase (MnP) *(6,7)* and versatile peroxidase (VP) *(8,9)*. No typical lignin peroxidase (LiP) has been described in this or any other *Pleurotus* species. MnP is produced during fungal degradation of lignocellulose and is induced by $Mn^{2+}$ *(10,11)*. Chelated $Mn^{3+}$ is a potential lignin-oxidizing mediator for the extensive delignification of wood and other lignocellulosic materials *(2,10,12)*.

Martinez et al. *(13)* studied the catalytic properties of MnP isoenzymes from *Pleurotus eryngii.* High MnP activity was obtained when peptone was used as the nitrogen source. In that study, conducted in liquid medium, $Mn^{2+}$ supplementation was not required to produce MnP. Two isoenzymes were purified and showed VP activity *(13)*. These isoenzymes were suggested to be an MnP-LiP "hybrid" *(8,14)*. In the current study, we use the term *VP* for enzymes that show $Mn^{2+}$-independent activity with phenolic substrates. Giardina et al. *(9)* described the production of MnP from *P. ostreatus* grown in solid stationary conditions on poplar sawdust; and $Mn^{2+}$ amendment increased the activity level of these isoenzymes. Two isoenzymes were purified and showed VP activity, and the gene encoding for MnP2 was cloned and sequenced *(9)*.

The effect of $Mn^{2+}$ on enhancing lignin degradation by *Pleurotus* species was shown, suggesting the importance of MnP in the process *(2)*. The degradation and mineralization of $^{14}C$-lignin under conditions of solid-state fermentation in basidiomycete selective medium (BSM) *(2)* were enhanced when $Mn^{2+}$ was added at concentrations ranging from 0 to 730 μ*M*. The same effects of $Mn^{2+}$ were shown in peptone medium (PM) under solid-state fermentation conditions *(15)*. Camarero et al. *(10)* described a strong stimulation of lignin mineralization (solid-state fermentation of wheat straw) by *Pleurotus pulmonarius* following the addition of $Mn^{2+}$.

Expression of MnP is regulated at the transcriptional level by various regulators. Ruiz-Duenas et al. *(16)* studied the regulation of *P. eryngii mnpl2* transcript levels in peptone liquid culture and in isolated mycelia. In liquid cultures, no transcript was detected when an $Mn^{2+}$ concentration of 25 μ*M* or higher was present in the medium. However, in isolated mycelia treated with $Mn^{2+}$, *mnpl2* transcript was present, most likely owing to induction of transcript, which had occurred when the samples were first cultured in PM

*(16)*. Previously, we have described *(15)* the regulation of peroxidase activity and *mnp* gene expression by $Mn^{2+}$, as well as the lignin mineralization rate by *P. ostreatus* in PM under solid-state fermentation conditions. The importance of one MnP isoenzyme (MnP3) was shown in activity and expression levels. The reduction in the abundance of VP gene transcript and the increase in *mnp3* transcript levels were colinear with the changes observed in the enzyme activity profiles, suggesting the importance of the $Mn^{2+}$-dependent peroxidase in lignin degradation.

In the present study, we describe the effect of $Mn^{2+}$ on fungal growth, peroxidase (MnP and VP) production, and lignin degradation in BSM and PM. We characterize MnP and VP enzymatic activity profiles and describe the purification of an MnP isoenzyme produced by *P. ostreatus* under solid-state fermentation conditions in BSM.

## Materials and Methods

### Solid-State Fermentation in BSM and PM

Stock cultures of *P. ostreatus* Florida F6 were maintained on PM or BSM. PM contained 20 g/L of glucose, 5 g/L of peptone (Bacto; Difco, Detroit, MI), 1 g/L of $KH_2PO_4$, 2 g/L of yeast extract, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 1.2 g/L of $Ca(NO_3)_2 \cdot 4H_2O$, and 0.012 g/L of $FeSO_4$, at pH 5.5. The BSM contained 5 g/L of glucose, 1 g/L of $K_2HPO_4$, 0.6 g/L of asparagine, 0.1 g/L of yeast extract (Difco), 0.5 g/L of KCl, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 0.003 g/L of $ZnNO_3$, 0.006 g/L of $Ca(NO_3)_2 \cdot 4H_2O$, and 0.003 g/L of $CuSO_4 \cdot 5H_2O$, at pH 5.5. $Mn^{2+}$ was added as $MnSO_4$ to the required concentrations.

The fungus was cultured in sterile cups (100 mL) containing 6 g of perlite and 24 mL of inoculum. The inoculum was prepared in BSM or PM by growing the fungal disks (20 disks of 6 mm and 10 disks of 10 mm diameter, respectively) for 6 and 3 d, respectively. The culture was then homogenized, and 10% of the culture volume was transferred to fresh media and cultured (28°C) for 24 and 48 h for BSM and PM, respectively. Culture samples (one cup each) were collected at different time points for enzyme activity analysis. Extraction was performed by adding 25 mL of 10 m*M* sodium acetate buffer (pH 6.0), followed by 20 min of shaking and filtration through a 0.45-µm filter.

To analyze the effect of $Mn^{2+}$ concentration on peroxidase activity, four $Mn^{2+}$ (added as $MnSO_4$) concentrations were tested: non-Mn amended, and amended with 73, 730, or 4500 µ*M*. $Mn^{2+}$ concentration in the non-amended medium (PM and BSM) was determined by atomic absorption spectroscopy and found to be <0.1 µ*M*.

Perlite (agricultural grade) was purchased from Agrical (Habonim, Israel) and used as the solid support for the solid-state fermentation. Prior to the addition of growth media, the solid particles were washed of impurities with 5% nitric acid, followed by distilled water.

### Mineralization of $^{14}C$-Lignin

$^{14}C$-lignin mineralization during solid-state fermentation was studied according to the methods described by Kerem and Hadar *(2)*. $^{14}C$-radiolabeled

substrate ($44 \times 10^4$ dpm of $^{14}$C-lignin, 20 ± 0.1 mg) was added to each of five replicates of 1.5 g of perlite in 20-mL polyethylene cups. Each polyethylene cup was then sterilized, inoculated with 8 mL of inoculum (prepared as already described), sealed in a 300-mL biometer flask with two gastight caps, and incubated at 28°C. The effect of $Mn^{2+}$ concentration on $^{14}$C-lignin mineralization was studied by $Mn^{2+}$ amendment, as already described.

The $^{14}CO_2$ evolved in each flask was trapped daily for 2 h, on GF/C 25-mm-diameter filter paper (Whatman, Maidstone, England) presoaked in 0.5 mL of 5 *N* NaOH. Following removal of the filters, the flasks were flushed for 1 min with moistened, sterile, atmospheric air. Subsequently, the amount of trapped $^{14}CO_2$ was measured by liquid scintillation counting (Hionic Fluor; Packard, Groningen, The Netherlands).

### Determination of Biomass Production

*P. ostreatus* was grown on BSM or PM. Two treatments were tested for each medium: non-Mn amended and medium containing 50 µ*M* $Mn^{2+}$. Two different experiments were conducted for biomass determination. In the first experiment, in Petri dishes, hyphal disks (5 mm diameter) were taken from the edge of a 5-d-old colony for each treatment. Petri dishes containing the same medium for each treatment were inoculated by placing one hyphal disk in the center of each dish. The Petri dishes were incubated at 28°C for 10 d. For each sample day, fungal hyphae were collected and dried at 60°C for 24 h, followed by determination of their dry weight. In the second experiment, in liquid cultures, 6 mL of the inoculum just described was transferred to new Erlenmeyer flasks (containing 60 mL of media). These flasks were incubated at 28°C, samples were collected for each sample day, and the dry weight of the fungal hyphae was determined.

### Determination of $CO_2$ Evolution

A different preparation was used to monitor fungal respiration. This preparation consisted of 1.5 g of dry perlite in a 20-mL plastic polyethylene cup. Each polyethylene cup was attached to the bottom of a 250-mL flask and then autoclaved and inoculated with 8 mL of the inoculum described in the previous section. The flasks were sealed with rubber stoppers, periodically sampled, and then aerated to avoid $CO_2$ accumulation. The concentration of evolved $CO_2$ was determined by gas chromatography (GC model 580, equipped with a Poropak Q column and a TCD detector; Gow-Mac, Bridgewater, NJ).

### Enzyme Profiles Following Anion-Exchange Chromatography

The extracellular fluid of 14-d-old BSM solid-state fermentation cultures was extracted by soaking the perlite gently in 10 m*M* sodium acetate buffer (pH 6.0) for 20 min followed by 0.45-µm filtration and concentration by ultrafiltration (10 kDa; Amicon, Rotterdam, The Netherlands). The extracts were equilibrated with 10 m*M* sodium acetate (pH 6.0), and the concentrated supernatant was loaded onto a MonoQ anion-exchange column

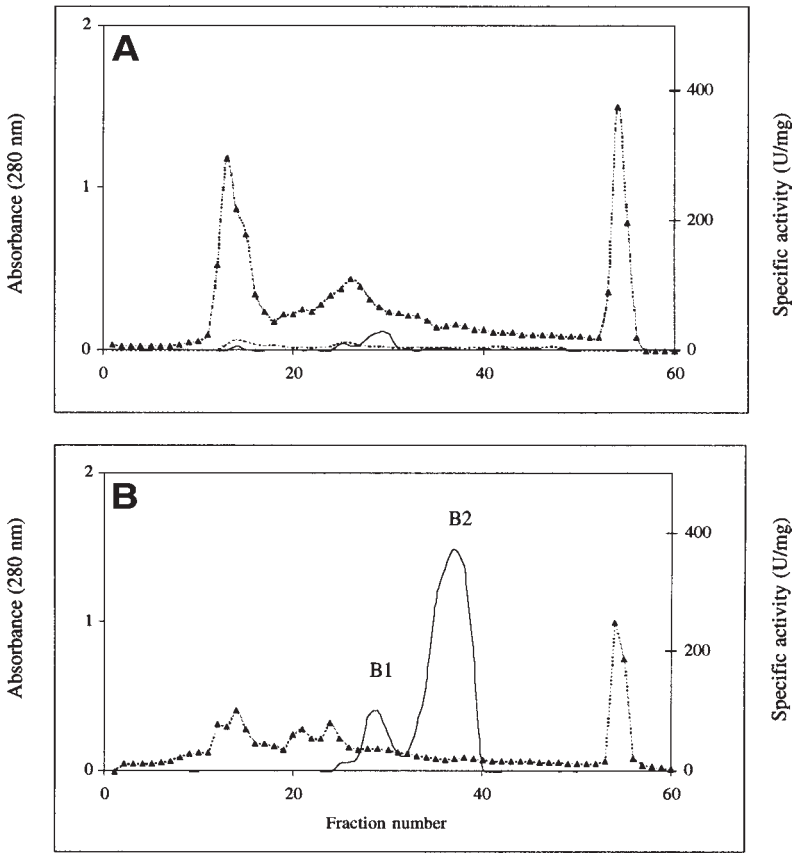


Fig. 1. Enzymatic profiles of MnP and VP after anion-exchange chromatography (MonoQ; fraction volume was 1 mL). *P. ostreatus* was grown under solid-state fermentation conditions on perlite on BSM in **(A)** nonamended manganese or **(B)** manganese-amended (500 μ*M*) media. The specific activities (U/mg protein) of MnP and VP were determined by the oxidation of phenol red with (——) or without (– – –) $Mn^{2+}$ in the reaction mixture, respectively. The absorbance at 280 nm is shown (– ▲ – ▲ –).

(Pharmacia, Uppsala, Sweden) using fast protein liquid chromatography (FPLC) (Äkta, Pharmacia): one-milliliter fractions were collected (at 1.5 mL/min). Proteins were eluted with a linear gradient of sodium acetate (pH 6.0) containing 1 *M* NaCl, and MnP and VP activities were determined in the eluted fractions using phenol red as the substrate (described next).

### *Purification and Characterization of MnP (B2)*

FPLC was used for the purification of MnP (B2), carried out on 14-d-old $Mn^{+2}$-amended (500 μ*M*) BSM solid-state fermentation cultures. The extraction and the first step of the purification (MonoQ) were performed as described for obtaining the enzyme profiles. Fractions of B2 (Fig. 1B) containing MnP activity were collected, washed, concentrated by ultrafiltration, and dialyzed against 10 m*M* sodium acetate (pH 3.6). After the concentrated fluid was loaded onto a MonoQ anion-exchange column

(Pharmacia), using 10 m*M* sodium acetate (pH 3.6), 1-mL fractions were collected (1.5 mL/min). Proteins were eluted with a linear gradient of the same buffer containing 1 *M* NaCl. The nonretained fractions containing MnP activity were collected, washed, and concentrated by ultrafiltration. The concentrated fluid was loaded onto a Superdex 200 HR 10/30 (10×300 mm, 24-mL vol) gel filtration column (Pharmacia). The enzyme was eluted with 10 m*M* sodium acetate buffer (pH 6.0) containing 150 m*M* NaCl, at a flow rate of 0.3 mL/min in 0.5-mL fractions.

Protein concentration was determined by means of Bradford reagent (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard. Protein homogeneity was verified by native polyacrylamide gel electrophoresis (PAGE) in 12% polyacrylamide gels. Protein bands were stained with Coomassie blue R-250.

The molecular mass of the enzyme was determined by gel filtration using a Superdex 200 HR 10/30 column and calibration kit (Pharmacia) and was subsequently confirmed by sodium dodecyl sulfate PAGE analysis with marker size (Sigma, Israel).

The internal sequence of the enzymatically active band of the purified protein was analyzed. In-gel proteolysis using trypsin was performed followed by peptide separation using reverse-phase chromatography. The liquid from the column was electrosprayed into an ion-trap mass spectrometer (LCQ; Finnigan, San Jose, CA). Mass spectrometry (MS) was performed in the positive ion mode using a repetitive full MS scan followed by collision-induced dissociation (CID) of the most dominant ion selected from the first MS scan. The MS data were compared to simulated proteolysis and CID of the proteins in the "genpept" database using Sequest software (J. Eng and J. Yates, University of Washington and Finnigan, San Jose). N-terminal sequencing was performed on a peptide sequencer (494A; Perkin-Elmer).

### *Enzyme Assays*

Enzyme activity was determined as described by Martinez et al. *(13)*. MnP activity was determined by the production of an $Mn^{3+}$-tartrate complex ($\varepsilon_{238}$ = 6.5 m$M^{-1}$ $cm^{-1}$) from 0.1 m*M* $MnSO_4$ with 0.1 m*M* $H_2O_2$ in 0.1 *M* sodium tartrate (pH 5.0). MnP was also determined with 0.01% (w/v) phenol red (610 nm) as the substrate ($\varepsilon_{610}$ = 22,000 $M^{-1}$ $cm^{-1}$) *(17)* under the conditions described by Kuwahara et al. *(18)*. VP activity was determined with phenol red (as described for MnP) or with 0.1 m*M* 2,6-dimethoxyphenol (Sigma) in a reaction mixture that did not contain $Mn^{2+}$. One unit of enzymatic activity was defined as the amount of enzyme that transformed 1 μmol of substrate/min.

## Results

### *Mineralization of $^{14}$C-Lignin in BSM and PM*

Mineralization of $^{14}$C-lignin (prepared from cotton branches) was studied in BSM and PM under solid-state fermentation conditions in biometer flasks using perlite as the inert support. We compared non-Mn-amended

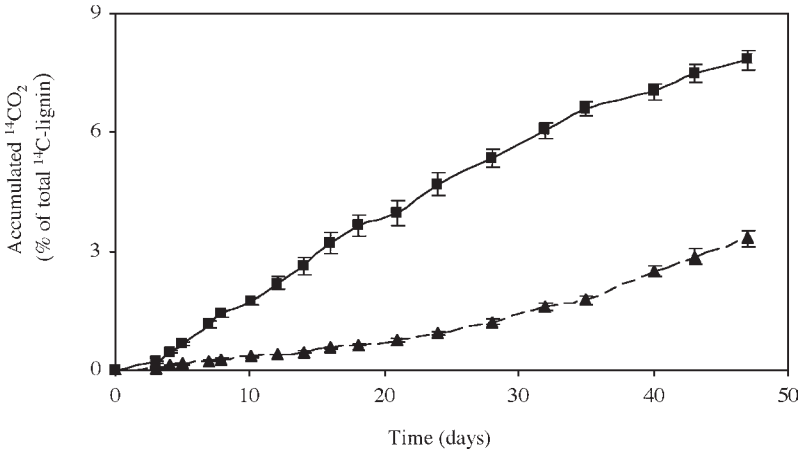


Fig. 2. Mineralization of $^{14}$C-lignin by *P. ostreatus* grown under solid-state fermentation conditions in BSM (containing 50 µ*M* $Mn^{2+}$) (—■—) and PM (nonamended $Mn^{2+}$) (—▲—). Accumulated $^{14}CO_2$ emitted from the total initial radiolabeled $^{14}$C-lignin is shown. Bars represent SEs and when not visible are smaller than the symbol.

PM with BSM containing 50 µ*M* $Mn^{2+}$, based on a report that *P. eryngii* MnP is inhibited by $Mn^{2+}$ amendment when the fungus is grown in liquid PM *(13)*. This remarkable phenomenon raises some interesting questions, since most white-rot fungi require $Mn^{2+}$ for *mnp* gene expression and protein production. The results (Fig. 2) indicated that after 47 d, approx 9% of the initial lignin had been mineralized in the BSM, compared with only 3% in the PM—despite the high level of MnP and VP activity in the crude supernatant of the PM and the high level of biomass production.

To determine whether the low level of mineralization in PM was the result of $Mn^{2+}$ deficiency, the level of $^{14}$C-lignin mineralization was determined after 45 d following the addition of 73 or 730 µ*M* $Mn^{2+}$ (data not shown). The addition of 73 and 730 µ*M* $Mn^{2+}$ increased lignin degradation significantly, 1.72- and 1.6-fold, respectively. The degradation level was close to that obtained in BSM containing 50 µ*M* $Mn^{2+}$. These results suggest an important role for $Mn^{2+}$ in lignin degradation in both growth media.

## *Fungal Respiration and Biomass Production in BSM and PM*

One possible mechanistic explanation for the difference in $Mn^{2+}$-affected lignin degradation in BSM vs PM could be higher growth rate, biomass production, and general peroxidase activity, under any of the studied conditions. Thus, a comparison between BSM and PM as growth media for *P. ostreatus* was conducted.

$CO_2$ accumulation was used as a fungal growth parameter and was determined in cultures grown for 20 d under solid-state fermentation in BSM and PM with different concentrations of $Mn^{2+}$ amendment (0, 73, 730, and 4500 µ*M*). Accumulated $CO_2$ was significantly higher in the PM (data not shown), at all time points tested: on d 20, $CO_2$ production in this media

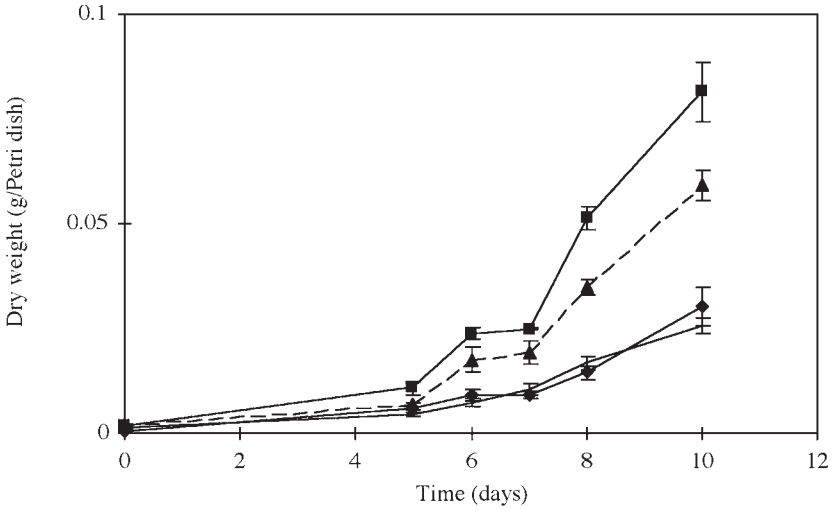


Fig. 3. Biomass production of *P. ostreatus* grown on Petri dishes containing nonamended $Mn^{2+}$ (—▲— and ——) or $Mn^{2+}$-amended (50 μ*M*) (—■— and —◆—) PM and BSM, respectively. Dry weight was determined on each sample day. Bars represent SEs and when not visible are smaller than the symbol.

was about 50 times higher than that in the BSM. There was no significant effect of $Mn^{2+}$ amendment on *P. ostreatus* respiration in either BSM or PM.

The effect of $Mn^{2+}$ amendment (50 μ*M*) on fungal biomass production was studied in Petri dishes and liquid cultures. In Petri dishes, on each sampling day, the fungal hyphae dry weight was determined (Fig. 3). After 10 d of growth, fungal dry weight (82 mg) was significantly higher in the $Mn^{2+}$-amended PM compared with its nonamended counterpart (60 mg). In the BSM, biomass production was lower (28 mg) than in the PM, and there was no significant effect of $Mn^{2+}$ amendment. These results were confirmed in liquid cultures of BSM and PM (Fig. 4). After 7 d, biomass production in the PM was about four times higher than that in the BSM. $Mn^{2+}$ amendment increased biomass production very slightly on different days in both media. Such vigorous growth in the PM is most likely the result of the higher concentration of glucose (20 g/L) relative to that present in BSM (5 g/L) and the enriched nitrogen source (peptone) relative to that in BSM (asparagine).

## *Effect of $Mn^{2+}$ on Enzyme Activity in BSM and PM*

The specific activities of MnP and VP produced by *P. ostreatus* in the crude supernatant of BSM and PM were determined in solid-state fermentation cultures harvested when MnP activity had peaked (6 and 14 d for PM and BSM, respectively; data not shown). In the nonamended PM, a high level of VP activity (559 U/mg of protein) was detected. MnP activity was higher (481 U/mg of protein) in this medium than in that containing 500 μ*M* $Mn^{2+}$ (334 U/mg of protein). These results support those described

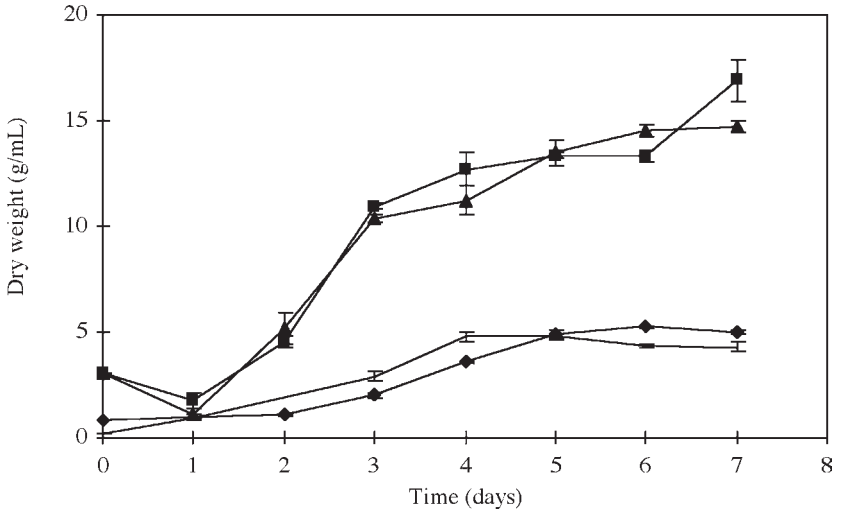


Fig. 4. Biomass production of *P. ostreatus* grown in liquid cultures containing nonamended $Mn^{2+}$ (– –▲– – and ——) or $Mn^{2+}$-amended (50 µ*M*) (—■— and —◆—) PM and BSM, respectively. Dry weight was determined on each sample day. Bars represent SEs and when not visible are smaller than the symbol.

by Martinez et al. *(13)* for *P. eryngii.* $Mn^{2+}$ amendment to the PM resulted in a sharp decrease in VP activity (238 U/mg of protein). Peroxidase levels were very low in the BSM relative to those detected in the PM. In the nonamended BSM, MnP activity was 51 U/mg of protein and VP was 10 U/mg of protein; however, in the medium containing 500 µ*M* $Mn^{2+}$, MnP activity was about ninefold higher, whereas VP activity was hardly detectable. Figure 5 describes the effect of $Mn^{2+}$ on the enzymatic activities of MnP and VP in liquid PM and BSM. In the nonamended BSM, VP and MnP activities were hardly detectable; however, in the $Mn^{2+}$-amended BSM, MnP and VP activities could be detected at very low levels on d 6 (Fig. 5A). MnP activity was much higher in the PM during all the days tested (Fig. 5B), relative to BSM. VP activity was low during the 8 d of the experiment, except on d 2, when it peaked. These results show the effect of media composition (in terms of nitrogen source and $Mn^{2+}$ concentration) and growth conditions (solid-state fermentation or liquid cultures) on the activities of the different peroxidases.

## *Peroxidase Activity Profiles in BSM*

To determine the relationship between enzyme activity in the different media and lignin degradation, and to confirm the importance of $Mn^{2+}$ and the activity of MnP and VP in this process, enzymatic profiles were determined. The effect of $Mn^{2+}$ on the presence of MnP and VP isoenzymes in BSM was tested by determining the enzymes' specific activities after anion-exchange chromatography. Protein (5 mg) was passed through an anion-exchange column, the eluted fractions were tested for MnP and VP activities, and their specific activities were calculated.

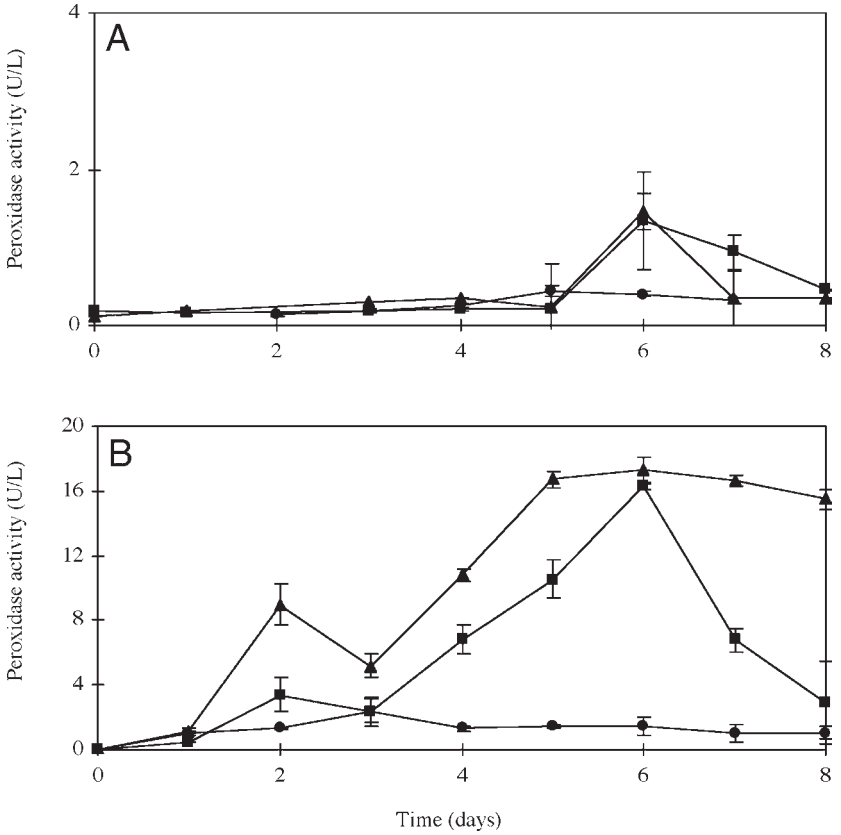


Fig. 5. Enzymatic activity of MnP and VP in liquid cultures of *P. ostreatus* in **(A)** BSM and **(B)** PM: VP activity in nonamended media (—●—); MnP activity in nonamended (—▲—) or manganese-amended (500 µ*M*) (—■—) media. Bars represent SEs and when not visible are smaller than the symbol.

In the nonamended BSM (Fig. 1A), only very low levels of peroxidase activity (MnP and VP) were detected following anion-exchange chromatography. In the BSM containing 500 µ*M* $Mn^{2+}$ (Fig. 1B), no VP activity was observed, but two isoenzymes (B1 and B2) appeared showing only MnP activity. The MnP activity of B2 was much higher than that of B1. We concluded that these isoenzymes play a key role in lignin degradation by *P. ostreatus* under solid-state fermentation conditions. The same trend in enzymatic profiles was observed with the PM *(15)*.

## *Purification and Characterization of MnP Isoenzyme B2*

To determine the molecular nature of the B2 isoenzyme, and to compare it to other known MnPs, we first purified it from *P. ostreatus* cultures grown on BSM solid-state fermentation containing 500 µ*M* $Mn^{2+}$. Fractions of isoenzyme B2 (Fig. 1B) containing MnP activity from the first purification step (MonoQ; pH 6.0) were further purified in another anion-exchange

Table 1
Purification of MnP Isoenzyme (B2) from Solid-State Fermentation Culture of *P. ostreatus* Grown on BSM with 500 µ*M* $Mn^{2+}$

| Purification step | Volume (mL) | Protein concentration (mg/mL) | Specific activity (U/mg)[a] | Yield (%) | Purification factor |
|---|---|---|---|---|---|
| Crude extract | 11 | 8.44 | 0.008 | 100 | 1 |
| After MonoQ (pH 6.0) | 2.4 | 0.23 | 0.41 | 29 | 49 |
| After MonoQ (pH 3.6) | 1.3 | 0.05 | 0.43 | 4.11 | 52 |
| After S-200 | 0.5 | 0.004 | 0.63 | 0.15 | 76 |

[a]One unit of enzymatic activity was defined as the amount of enzyme that transformed 1 µmol of substrate/min.

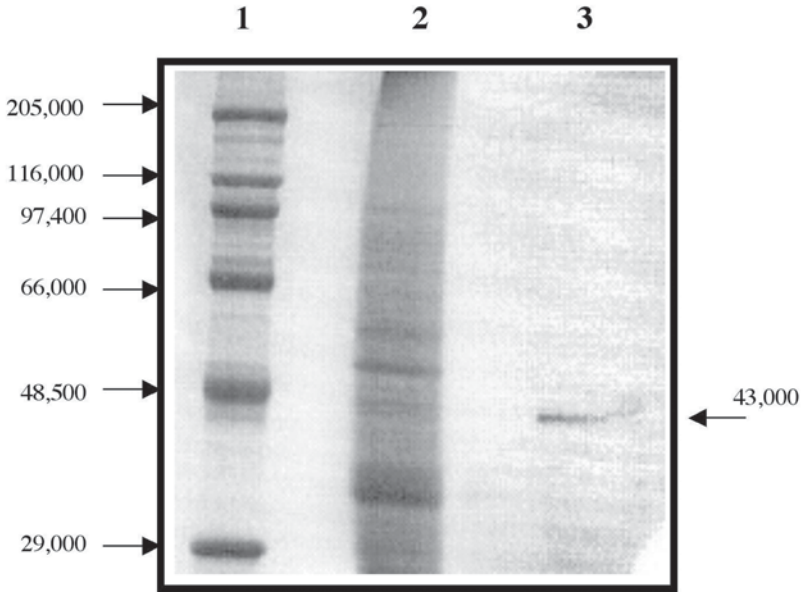


Fig. 6. Native PAGE of samples from purification process of B2 isoenzyme from BSM solid-state fermentation culture of *P. ostreatus* (Coomassie blue staining). B2 was purified by three purification steps as described in Results. Lane 1, marker sizes; lane 2, 10 µg of protein of crude supernatant; lane 3, 10 µL of purified B2.

chromatography step (MonoQ; pH 3.6), in order to concentrate the enzyme in the nonretained fractions while trapping other proteins on the column. MnP activity was detected in the nonretained fractions, which were subsequently subjected to the last purification step, gel filtration chromatography (Superdex 200HR). Table 1 summarizes the purification steps for the B2 isoenzyme. A purification factor of 76 was determined after the three purification steps. Although the second purification step (MonoQ; pH 3.6) was not highly efficient, this step was essential to obtain a pure enzyme preparation. In Fig. 6 the native PAGE of samples of the crude supernatant

(lane 2) and the purified protein (lane 3) are visualized. Based on its elution from the gel filtration column and the native PAGE results, the molecular mass of B2 was determined to be 43 kDa, which is identical to the isoenzyme that was purified in our previous study *(15)*. B2 oxidized $Mn^{2+}$ to $Mn^{3+}$ in the presence of $H_2O_2$ and exhibited $Mn^{2+}$-dependent activity with phenol red and 2,6-dimethoxyphenol as substrates. Activity gels using native PAGE in 12% polyacrylamide gels and 4-chloro-1-naphthol as the substrate confirmed that the purified B2 was indeed that of an MnP, since activity staining was detected only in reactions containing $Mn^{2+}$ and $H_2O_2$ (data not shown).

Since the N-terminal sequences of the different MnPs featured in the public databases are very similar, we obtained an internal sequence of the purified B2 in order to determine its subfamily grouping. The two internal sequence peptides obtained corresponded (100% identity) to the amino acid residues 231–253 and 301–317 of isoenzyme P6 that we have previously purified from PM solid-state fermentation cultures *(15)*. P6 isoenzyme was found to be identical to MnP3 from *P. ostreatus* grown on peptone-containing medium *(19)*, in a study in which the *mnp3* gene and cDNA were isolated. Thus, we concluded that B2 and P6 isoenzymes are probably the same enzymes, and that *mnp3* is the gene (GenBank AB016519) encoding them.

## Discussion

In recent years, more emphasis has been placed on analyzing MnP, because this enzyme is produced by most white-rot fungi, including species that lack LiP. Moreover, the significance of $Mn^{3+}$ in lignin degradation has been clearly demonstrated *(8,15,16,20,21)*. Although different types of peroxidases have been characterized, including MnP and VP, their role in lignin degradation is not well understood. In the present study, we evaluated the effect of $Mn^{2+}$ and peroxidase activities on $^{14}C$-lignin degradation by the white-rot fungus *P. ostreatus*. We have described the effect of $Mn^{2+}$ on $^{14}C$-lignin degradation and characterized the ligninolytic enzyme activity profiles of MnP and VP in this fungus in BSM and PM under solid-state fermentation conditions.

MnP is expressed during fungal growth on its natural substrates under solid-state conditions. However, lignocellulosic substrates such as cotton stalks and wheat straw interfere with the extraction of active enzymes. Thus, the use of perlite as an inert support facilitates the study of ligninolytic enzymes under solid-state conditions *(2)* and was therefore adopted in the current study.

*Phanerochaete chrysosporium*—the most widely studied white-rot fungus—which produces LiP and MnP, synthesizes MnP in medium containing ammonium tartrate as the nitrogen source *(18)*, whereas *Bjerkandera adusta* and *P. eryngii* produce MnP in nitrogen-rich medium containing peptone *(13,22)*. In our study, higher peroxidase activity was detected in

the PM than in the BSM and was positively correlated with increased biomass production. The predominant activity in the non-$Mn^{2+}$-amended PM was that of VP, whereas in the nonamended BSM, both MnP and VP activities were very low. The first report of VP activity was in *Bjerkandera* sp. BOS55 *(23)*. Later, Martinez et al. *(13)* studied the catalytic properties of MnP isoenzymes of *P. eryngii* from liquid PM. High MnP activity was detected in this medium, even though peroxidase production was strongly inhibited by $Mn^{2+}$ concentrations >10 μ*M (13)*. Two isoenzymes were purified and described as VP, capable of oxidizing $Mn^{2+}$ as well as phenolic and nonphenolic aromatic substrates *(13)*.

Despite the high levels of biomass and peroxidase activities, less lignin mineralization was obtained in the nonamended PM than in the BSM. Kerem and Hadar *(2)* described the positive effect of $Mn^{2+}$ amendment on $^{14}$C-lignin in BSM. In the present study, the addition of 73 and 730 μ*M* $Mn^{2+}$ to the PM resulted in lignin degradation levels approximating those obtained in BSM containing 50 μ*M* $Mn^{2+}$. In the nonamended PM, only 3% of the $^{14}$C-lignin was mineralized, compared with 8% in the PM containing 73 or 730 μ*M* $Mn^{2+}$. The improvement in $^{14}$C-lignin mineralization in $Mn^{2+}$-containing PM could be the result of an increase in MnP activity, the higher level of biomass, or both.

The effect of $Mn^{2+}$ on the enzyme activity profiles of MnP and VP in BSM was studied using anion-exchange chromatography of the crude supernatants. In nonamended BSM, extremely low levels of MnP and VP were found. With an increase in $Mn^{2+}$ concentration, two isoenzymes (B1 and B2) appeared showing only MnP activity. A different profile was found in our previous study for the nonamended PM *(15)*. This medium contains a high level of VP activity relative to the BSM. In this medium, six isoenzymes exhibiting both VP and MnP activities were detected. $Mn^{2+}$ amendment decreased the total activity of these isoenzymes but increased the level of one of the MnP isoenzymes (P6). The positive effect of $Mn^{2+}$ amendment on the activity levels of peroxidases that are dependent on $Mn^{2+}$ was similar in both BSM and PM. We purified the MnP isoenzyme B2, and its internal sequence showed that it and P6 are the same enzyme, with deduced amino acid sequences matching those published for *P. ostreatus* MnP3 *(19)*. In our previous study, we described the effect of $Mn^{2+}$ on the relative abundance of gene transcripts of three VPs and one MnP (P6) from *P. ostreatus* grown on PM solid-state fermentation. Using reverse transcriptase polymerase chain reaction (RT-PCR) with oligonucleotide primer sets synthesized on the basis of nonconserved sequences of the different peroxidases, we showed the reduction in VP gene transcript abundance and the increase in *mnp3* transcript level, which were colinear with the changes observed in the enzyme activity profiles. Those results indicated that peroxidase activity is regulated at the transcriptional level, and we suggest that the expressions of MnP and VP may be differentially regulated by the presence of $Mn^{2+}$. We assume that the effect of $Mn^{2+}$ on the abundance of MnP3 (P6 and B2) transcript in the different media is similar. However, this assumption requires further verification using RT-PCR experiments with the BSM.

The results described herein confirm the hypothesis that $Mn^{2+}$ is important for the induction of MnP, and thus plays a significant role in lignin degradation by *P. ostreatus* under solid-state fermentation conditions on perlite. Media composition and growth conditions are important factors affecting extracellular ligninolytic enzyme production and lignin degradation. $Mn^{2+}$ is known as a mediator, inducer, and substrate for MnP and the positive effect of $Mn^{2+}$ on lignin degradation and MnP expression suggests the importance of MnP, as opposed to VP isoenzymes, in the process.

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