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Effect of Manganese on Lignin Degradation by *Pleurotus ostreatus* during Solid-State Fermentation

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Lignin degradation by *Pleurotus ostreatus* was studied under solid-state fermentation (SSF) in chemically defined medium containing various levels of Mn. Degradation of [^{14}C]lignin prepared from cotton branches to soluble products, as well as its mineralization to $^{14}CO_2$, was enhanced by the addition of Mn. The effect of malonate on lignin mineralization was most marked during the first 10 days of SSF, in a treatment amended with 73 μM Mn. A high concentration of Mn (4.5 mM) caused inhibition of both fungal growth and mineralization rates during the first 2 weeks of incubation. Addition of malonate reversed this effect because of chelation of Mn. Mn was found to precipitate in all treatments, with or without the addition of malonate. α-Keto-γ-methiolbutyric acid cleavage to ethylene, an indication of ·OH production, was observed as early as 3 days of incubation in all treatments.

Manganese appears to play an important role in biological oxidation as well as in lignin biodegradation (9). It has been shown to take part in the mineralization of synthetic lignins by white rot fungi, both as an active mediator for manganese peroxidase and as a regulator of Mn peroxidase, lignin peroxidase, and laccase production (1, 20–22). Perez and Jeffries (21) studied the effect of manganese and various organic chelators on the distribution of degradation products in the growth medium and on depolymerization and mineralization of synthetic [^{14}C]lignin (DHP) by *Phanerochaete chrysosporium*. In the presence of high levels of manganese [720 μM Mn(II) or Mn(III)] and malonate as a chelator, Mn peroxidase production was found to be stimulated, while lignin peroxidase production was inhibited. They also found that mineralization of DHP decreases in the presence of high levels of manganese (720 μM). Cui and Dolphin (9) found that malonic acid is a most efficient ligand for stabilizing Mn(III) in aqueous solution and that the complex formed functions as a diffusible oxidant in lignin model compound oxidations. Blanchette (4) observed black areas in wood decayed by several white rot fungi, which contained high concentrations of manganese (24 to 51 times higher than in areas surrounding the delignified zones).

The objective of the present study was to examine the ligninolytic system of the white rot fungus *Pleurotus ostreatus* and to compare it with that of *P. chrysosporium*. *P. ostreatus* presents a particularly interesting system because it lacks lignin peroxidase (12, 18, 25, 26) but may possess Mn peroxidase. The effects of manganese (at concentrations ranging from 0 to 4.5 mM) and malonate on the degradation and modification of a [^{14}C]lignin preparation of cotton stalks by *P. ostreatus*, were studied.

MATERIALS AND METHODS

Preparation ^{14}C-labeled cotton-lignin. [^{14}C]lignin lignocellulose was prepared according to the method described by Crawford and Crawford (7), with some modifications (14). Freshly cut, 40-cm-long cotton branches were provided with aqueous L-[U-^{14}C]phenylalanine (Amersham International, Buckinghamshire, England) as a precursor for lignin biosynthesis. The bark was then peeled off the branches, and the latter were extracted as described by Crawford (8) and by Colberg and Young (6). The resultant lignocellulose exhibited a specific activity of 22×10^3 dpm/mg.

Solid-state fermentation. *P. ostreatus* (5) was grown in a chemically defined medium, as described previously (15). Manganese-deficient inoculum was prepared by twice transferring homogenized hyphae into Mn-deficient liquid medium. Four initial manganese concentrations were used as follows: manganese was deleted from the medium (Mn-deficient medium) or 73 or 730 μM or 4.5 mM $MnSO_4$ was added to the defined medium. Corresponding treatments were amended with 10 mM malonate. The media were inoculated, poured onto perlite particles, and incubated at 30°C. Perlite (agricultural grade) was purchased from Agrical (Habonim, Israel) and was used as the solid support for the SSF. Prior to the addition of growth media, the solid particles were washed of impurities with 5% nitric acid and then with distilled water until the pH of the washing water was 5.0. A typical preparation consisted of 1.5 g of dry perlite in a 40-ml flat-bottom tube, to which 6.0 ml of the inoculated, chemically defined medium was added.

[^{14}C]lignin utilization. To study radioactive compound utilization during SSF on a synthetic medium, radioactive substrate (21×10^4 dpm; 10 ± 0.1 mg) was added to each of five replicates of 1.5 g of perlite in 20-ml polyethylene cups, before sterilization. Each polyethylene cup was then sterilized, inoculated as described above, sealed in a 300-ml biometer flask (2) with two gas-tight caps, and incubated at 30°C. [^{14}C]lignin mixed, as described above, with 1.5 g of perlite in a plastic cup was autoclaved and then incubated with all media for 30 days to serve as a control. Evolved $^{14}CO_2$ in each flask was dissolved in a NaOH trap daily (15), and the flask was then flushed for 1 min with moistened, sterile atmospheric air. Sequential extractions were performed on the degraded product, after removal of the plastic cup contents (SSF products) into a 50-ml conical plastic tube. Distilled water (15 ml) was added to each sample, which was then ground and soaked for 5 h. Aliquots (2 ml) of

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the solutions were taken for counting in Hionic-Fluor (Packard Instruments, Downers Grove, Ill.) scintillation liquid. Lysing enzymes (from *Trichoderma harzianum* [Sigma, St. Louis Mo.]) were added to the water, which was then incubated for an additional 12 h. Aliquots (2 ml) of the solutions were taken for scintillation counting as above. The remainder was extracted with 23 ml of a chloroform-methanol-water (1:0.5:0.4) solution. Aliquots (1 ml) of the solutions were taken for scintillation counting.

Soluble manganese in the growth medium. The concentration of soluble manganese ions was determined by washing the growth media with 2 volumes of double-distilled water, acidifying the washing water with 5% nitric acid, and analysis in an inductively coupled plasma-atomic emission spectrometer (Spectroflame; Spectro Analytical Instruments, Kleve, Germany).

Ethylene generation in vivo. The cleavage of α-keto-γ-methiolbutyric acid (KTBA) to ethylene was measured by using a modification of the method described by Pryor and Tang (23), in which the production of ethylene was studied in vivo. Triplicates of the perlite-mycelia bulk were dispersed in 6 ml of a water solution containing 0.8 mM KTBA. Air samples (1 ml) were taken after 30 and 120 min to determine the concentration of evolved ethylene per hour per culture by gas chromatography (with a gas chromatograph, model 3300; Varian, Palo Alto, Calif.).

RESULTS

Effect of *P. ostreatus* on soluble Mn concentration. Growth of *P. ostreatus* was not visibly affected by the absence of manganese. In the range of concentrations tested, only 4.5 mM Mn had a growth-inhibiting effect on the fungus, whereby preferential growth was toward the upper part of the glass tube. Perlite particles in the Mn-deficient treatment remained white throughout the experiment, whereas yellow deposits appeared in the 73 μM treatment, and brown to dark-brown deposits appeared at the higher manganese concentrations (730 μM and 4.5 mM). The appearance of deposits was delayed in the malonate-amended treatments, and at the highest manganese concentration, pink was observed at the edges of the growing hyphae. The brown deposit was identified as MnO_2 by two diagnostic test: by generation of gas bubbles in the presence of H_2O_2 (21) and by a manganese electron demand test (3). Throughout the experiment, the pH-s of all treatments remained between 5 and 5.5, with a slight tendency toward high pH-s at the higher manganese concentrations.

The SSF media were washed with double-distilled water and soluble manganese concentrations were analyzed. In the Mn-deficient treatment, the manganese concentration was found to be below 8 μM; this concentration is at the lowest limit of detection (Fig. 1B). Addition of 73 μM Mn(II) to the growth medium resulted in less than 20 μM Mn that was detectable after 6 days of SSF. Addition of 730 μM Mn(II) to the growth medium resulted in 250 μM after 6 days. Similar patterns of decreasing soluble manganese concentrations were detected in the corresponding treatments amended with malonate. However, a different trend was observed in the treatment with 4.5 mM Mn: an immediate spontaneous precipitation of manganese occurred, with only 3.6 mM detectable at inoculation (Fig 1A). Later precipitation was markedly attenuated, with a concentration of 2.4 mM after 6 days and 1.7 mM after 12 days of SSF. These observations are in agreement with the visual observation of growth inhibition at this manganese concentration. Upon addition of

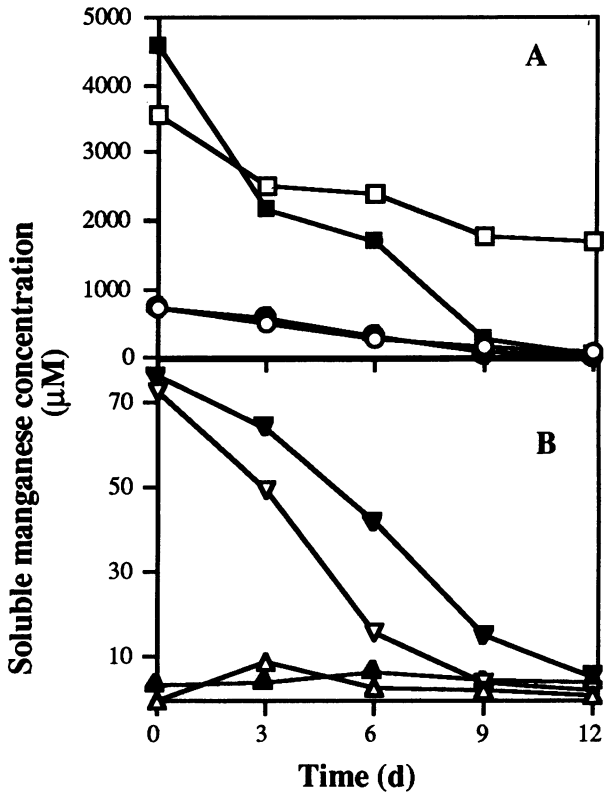

FIG. 1. Mn concentration in *P. ostreatus* growth medium during solid-state fermentation on perlite particles. □ 4.5 mM Mn(II); ■, 4.5 mM Mn (II) plus malonate; ○, 730 μM Mn(II); ●, 730 μM Mn(II) plus malonate; ▽ medium; 73 μM Mn (II); ▼, 73 μM Mn(II) plus malonate; △, Mn-deficient medium; ▲, Mn-deficient plus malonate medium High (A) and low (B) ranges of manganese treatments are shown.

4.5 mM Mn(II) with malonate, all of the manganese remained in solution, as evidenced by the washing procedure. A rapid rate of precipitation was then observed, and manganese concentrations similar to those in the lower concentration treatments were reached. The medium on day 6 contained 1.65 mM Mn; however it contained only 42 μM Mn on day 12. After 12 days, soluble manganese concentrations decreased to less than 70 μM in all treatments except for that with the highest manganese concentration not supplemented with malonate.

Effect of Mn concentration on [^{14}C]lignin degradation. The effects of adding various concentrations of Mn(II) ions to the growth medium of *P. ostreatus* on lignin degradation were monitored. *P. ostreatus* was grown under SSF conditions, with the Mn concentrations described, with malonate added to corresponding treatments, and ^{14}C-labeled cotton lignin as the substrate (14). Under conditions of Mn deficiency (Fig. 2A), mineralization was first detected on day 6 and reached 11% of the total [^{14}C]lignin after 27 days of SSF. In the treatment amended with 73 μM Mn (Fig. 2B), mineralization was detected earlier, reaching 14% on day 27. Mineralization in the treatment containing 730 μM Mn (Fig. 2C) was higher, reaching 16% on day 27. The rate of mineralization from day 5 was 0.63% per day in the 73 μM treatment, 0.68% per day in the 730 μM treatment, and only 0.48% per day in the Mn-deficient treatment. In the 4.5 mM Mn treatment (Fig. 2D), mineralization was slower for the first 13 days (only

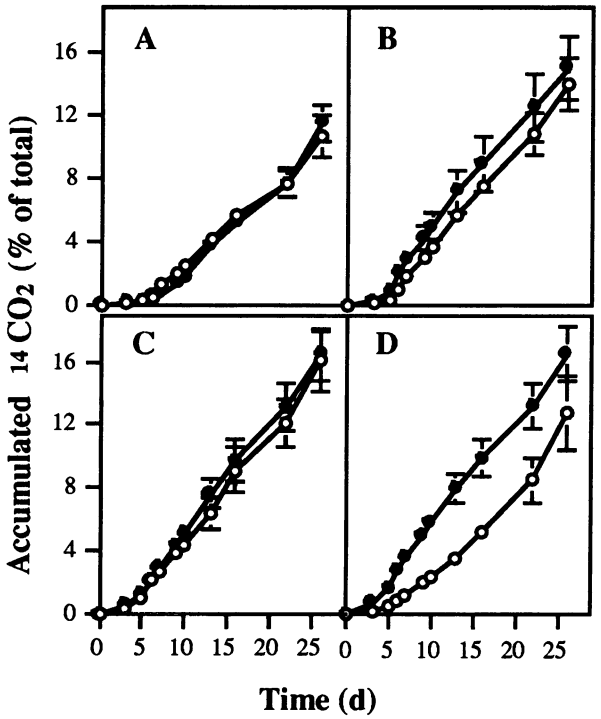

FIG. 2. Mineralization of [^{14}C]lignin during solid-state fermentation by *P. ostreatus* on defined medium. Graphs plot mineralization with no addition of Mn (A), 73 μM Mn (B), 730 μM Mn (C), and 4.5 mM Mn (D) in the absence (○) or presence (●) of 10 mM malonic acid. Bars represent standard errors.

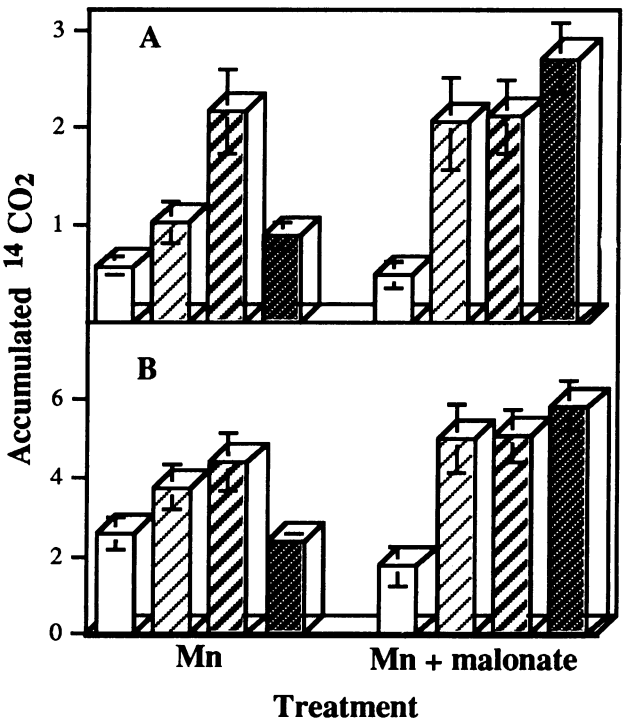

FIG. 3. Mineralization of [^{14}C]lignin on days 6 (A) and 10 (b). □ Mn-deficient medium; □ 73 μM Mn; □ 730 μM Mn; □ 4.5 mM Mn.

0.39% per day). However, the mineralization rate increased from day 13 on, to 0.69% per day, reaching a total accumulation of 13% ± 2.4% of the total [^{14}C]lignin as $^{14}CO_2$ on day 27. This phenomenon was probably due to the inhibition of fungal growth described earlier rather than to specific inhibition of lignin degradation. Addition of malonate to the synthetic medium in the Mn-deficient treatment did not change the mineralization rate or timing (Fig. 2A). In the 73 μM Mn treatment, mineralization patterns in the absence and presence of malonate were identical from day 9 on, reaching 15% mineralization on day 27. In the 4.5 mM Mn treatment, the effect of malonate was substantial, resulting in faster degradation. Again, this was probably the result of better fungal growth. Indeed, the pattern of mineralization in this treatment was similar to that at the lower (730 μM Mn) manganese concentration.

Figure 3A and B focuses on events occurring on days 6 and 10, respectively. The effects of both Mn and malonate on mineralization were most prominent during the first 10 days of SSF. During this period, Mn was still available in a soluble form (Fig. 1). The treatment amended with 730 μM Mn and no malonate exhibited the highest accumulation of $^{14}CO_2$ on day 6, constituting 2.1% of the total. It was four times higher than under Mn deficiency, and twice as high as that in the 73 μM Mn medium; however, it was identical to accumulated $^{14}CO_2$ detected in both the 73 and the 730 μM Mn treatments with added malonate. The highest mineralization values on day 10 ranged from 4.4 to 5.8% of the total in the treatment amended with 730 μM Mn and those amended with Mn and malonate (Fig. 3B). Low values, ranging from 1.8 to 2.6, were recorded under Mn deficiency. Sterile controls were run in parallel; in the absence of malonate, Mn precipitated for the first 3 days to a concentration similar to those of the respective inoculated treatments. However, no further precipitation was observed after the third day. The precipitation of Mn was not a result of chemical oxidation, as demonstrated by a negative reaction with H_2O_2 and the lack of brown deposits.

Effect of Mn concentration on [^{14}C]lignin modification. SSF of *P. ostreatus* on chemically defined medium with ^{14}C-labeled cotton lignin exhibits a constant rate of mineralization for 60 days, reaching over 50% mineralization in that time (15). In the present work, however, SSF was stopped after 27 days, at about 15% mineralization, to study the nature of the degradation process intermediates. More information about the effect of manganese on the process of lignin degradation by *P. ostreatus* was revealed via several extraction procedures. Similar results were obtained for all of the controls, and their averages are presented in Table 1. The total radioactivity released from the control was 10%, compared with 45 to 60% from the biodegraded lignin.

Water-soluble ^{14}C in the control amounted to 5% of the total. Significant modifications of the labeled lignin were evidenced by the accumulation of water solubles in all treatments. In the Mn-deficient and low-Mn (73 μM initial concentration) treatments, about 20% of the total [^{14}C]lignin was dissolved in water after 27 days of growth. A significantly larger proportion of the total amount (32.1%) was water soluble in the 730 μM Mn treatment. In the 4.5 mM Mn treatment, the water solubles constituted only 15% of the total.

Addition of malonate to the synthetic medium in the Mn-deficient treatment resulted in a small increase in water solubles. In both the 73 and 730 μM Mn treatments, addition of malonate to the medium resulted in the highest accumu-

TABLE 1. Distribution of [^{14}C]lignin modification and degradation products

Medium and initial concn of Mn (μM)	% Total initial radioactivity in the following extraction step[a]:			Mineralization (%)[e]
	Water solubles[b]	Lysing enzymes[c]	Wet chloroform[d]	
Uninoculated control	5.0 ± 0.5	0.4 ± 0.3	4.6 ± 2.1	
Synthetic medium				
0	19.6 ± 1.9	3.5 ± 0.6	12.2 ± 2.6	10.7 ± 1.3
73	21.7 ± 1.6	3.4 ± 0.2	3.6 ± 2.9	14.0 ± 1.7
730	32.1 ± 6.4	4.5 ± 0.8	4.4 ± 3.5	16.1 ± 1.9
4.5×10^3	15.1 ± 2.9	2.3 ± 0.6	19.1 ± 7.8	12.8 ± 2.4
Synthetic medium + 10 mM malonate				
0	24.5 ± 2.2	3.2 ± 0.2	8.8 ± 4.1	11.6 ± 1.2
73	33.4 ± 2.3	3.7 ± 0.2	8.1 ± 2.4	15.1 ± 2.0
730	33.4 ± 3.4	4.4 ± 0.4	1.4 ± 0.7	16.4 ± 1.7
4.5×10^3	28.3 ± 2.5	4.4 ± 0.6	ND[f]	16.5 ± 1.7

[a] Data are expressed as percentages of total initial radioactivity in each substrate (210,000 dpm) after 27 days of SSF.
[b] ^{14}C-labeled water solubles after homogenization and 5 h of shaking in distilled water.
[c] Additional water-soluble ^{14}C-labeled compounds dissolved by incubation with lysing enzymes for 12 h.
[d] ^{14}C-labeled compounds dissolved in chloroform-methanol-water (1:0.5:0.4).
[e] Percent $^{14}CO_2$ accumulated.
[f] ND, under detection level.

lation of water soluble ^{14}C-labeled compounds. A high accumulation of water solubles was also detected in the 4.5 mM Mn treatment supplemented with malonate, relative to that of the corresponding manganese-only treatment.

Lysing enzymes were added to the water to dissolve cell-bound or additional intracellular labeled compounds originating from the [^{14}C]lignin. Treating the uninoculated control in this manner resulted in the detection of only traces of radioactivity. Additional labeled compounds comprised 3 to 5% of the total radioactivity in most treatments but only 2.3% in the 4.5 mM Mn treatment.

The next extraction step was designed to measure the accumulation of chloroform-soluble [^{14}C]lignin compounds during the 27 days of fungal growth. The control contained 4.6% of the total at this point in the extraction sequence. In the treatments showing lower degradation capacity, a higher accumulation of chloroform-soluble labeled products was observed. The highest amount (19% of the total) was detected in the 4.5 mM Mn treatment; however, there was wide variability, again reflecting irregular fungal growth. Addition of malonate resulted in a lower accumulation of chloroform-soluble labeled products as manganese concentrations were raised, decreasing from 8% of the total ^{14}C dissolved in the Mn-deficient treatment to nondetectable levels in the 4.5 mM Mn treatment.

Cleavage of KTBA to ethylene was studied in vivo. Activity was found in all treatments as early as 3 days after inoculation (data not shown). Headspace ethylene reached 0.01 μl per tube per h or more in all treatments, with wide variations and no significant differences among treatments at specific time points. In general, lower activity was observed in the absence of malonate. The lowest activity was observed in the 4.5 mM Mn treatment during the first 9 days.

DISCUSSION

The chemical nature and abundance of manganese constitute the basis for its involvement in lignin biodegradation. Oxidation and cleavage of the hydrophobic regions of the lignin molecule may be affected by manganese ions in high oxidation states, such as Mn(III). Mn(III) can serve as a mediator for free-radical production (27) or as an indigenous oxidant.

Complete metabolism of the lignin macromolecule is evidenced by its mineralization to CO_2, whereas modifications and primary breakdown steps result in different products and intermediates. Soluble oligomeric fragments have been observed during lignin degradation by *Trametes versicolor*, resulting not only from direct lignin breakdown but also from the cleavage of lignin carbohydrate bonds (24). Perez and Jeffries (21) observed partial lignin depolymerization by *P. chrysosporium* at high manganese levels (720 μM). However, further depolymerization to smaller compounds was more efficient when lower levels of manganese were used.

Mineralization and modification of native [^{14}C]lignin by *P. ostreatus* occurred in synthetic media with no added manganese. Addition of malonate to the Mn-deficient medium only slightly inhibited lignin mineralization for the first 2 weeks, whereas increased accumulation of labeled water solubles was detected on day 27. However, the addition of 730 μM Mn to the growth medium resulted in the highest mineralization, as well as the highest accumulation of water-soluble ^{14}C-labeled products. Total lignin modification in this treatment summed to 57% of the labeled lignin. The observations are contrary to those made for *P. chrysosporium* (21), in which a higher mineralization rate was achieved in the absence of manganese.

The degradation of lignin by *P. chrysosporium* was studied in a liquid culture flushed daily with pure oxygen, with DHP as the substrate (21). Under these conditions, rapid mineralization at low Mn concentrations was shown on day 5, with a rate reaching over 10% per day, up to a total mineralization of 40% on day 10. At the higher Mn concentration (720 μM), the highest mineralization rate was detected after 7 days (also about 10% per day); however, it reached a plateau at less than 40% on day 14. *P. chrysosporium* degrades lignin faster than *P. ostreatus* during SSF on cotton stalks (14). Mineralization of labeled lignocellulose by the former was shown to be 1% per day, reaching a plateau at 12% after 18 days, whereas *P. ostreatus* was shown to degrade the same substrate much more slowly (0.3% per day from days 9 to 60), resulting in mineralization of 17% of the total radiolabeled substrate.

Earlier studies (10) have shown that Mn(III) is a ligninolytic agent but requires a suitable chelator. Malonic acid was chosen in the present work since the malonate-Mn(III) complex has been reported to oxidize free phenolic lignin

substructures (16) and lignin model compounds (9) and to stabilize Mn(III) in the liquid growth medium of *P. chrysosporium* (21). Enhanced degradation was observed with the addition of malonate to medium containing a low concentration of manganese (73 μM). However, this effect was not apparent when malonate was added to medium containing 730 μM Mn. This suggests the possibility of intrinsic production of a Mn chelator by *P. ostreatus* which may be induced by the presence of manganese ions in the medium. This may also be the cause of the increased lignin mineralization rate at the high manganese concentration from day 13 on.

Kern (17) has shown that MnO_2 can serve as a manganese reservoir for *P. chrysosporium*. Perez and Jeffries (21) found that only soluble Mn ions can stimulate Mn peroxidase production or inhibit lignin peroxidase production by this fungus. Therefore, it may be assumed that a higher rate of lignin modification was prevented by the rapid precipitation of Mn(II).

MnO_2 precipitated rapidly in most of the manganese-containing media, indicating an extracellular oxidizing activity specific to manganese or high extracellular oxidizing conditions. The precipitation of Mn(II) from the growth medium of *P. ostreatus* probably occurred via its oxidation to higher oxidative forms. These forms precipitate on the perlite particles and were not dissolved by the addition of water. This is in agreement with previous reports, suggesting the oxidation of Mn(II) to MnO_2 by *P. chrysosporium* (16, 21). Extracellular production of H_2O_2 by *Pleurotus eryngii* has been reported elsewhere (11) and may explain the observed oxidation of manganese. The growth inhibition observed in the presence of 4.5 mM Mn was reversed by the addition of malonic acid. This could have been the result of either stabilization of the oxidized form of manganese in the medium until its deposition on nuclei or oxidation of the organic acid itself, thereby protecting the fungus.

The addition of KTBA to ligninolytic cultures of *P. ostreatus* and *P. chrysosporium* results in cleavage of this substrate and release of ethylene to the headspace atmosphere (13, 14, 19). It has also been shown to accompany the lignin degradation phase in both fungi. Cleavage of KTBA to ethylene has been recently shown to be a suitable detector of the presence of ·OH, although cleavage could result from metallic free radicals as well (28). In the present work, similar amounts of ethylene were detected in the absence and presence of manganese in the medium. Moreover, at the highest manganese concentration, without malonate, ethylene production decreased. Ethylene production seems to appear simultaneously with the onset of extracellular lignin breakdown and hence can serve as an indicator for the beginning of the ligninolytic phase. Nevertheless, the presence of specific free radicals in the ligninolytic cultures needs to be proven via more specific assays, together with an identification and characterization of free-radical production mechanisms or ligninolytic enzymes in *P. ostreatus*.

Manganese can be found in one of seven positive oxidation states, some of which are potentially strong oxidants (3). A manganese-dependent system may also exist in *P. ostreatus* and may be responsible for the enhancement of the macromolecule's breakdown to water-soluble oligomers. Nevertheless, this work shows that significant lignin degradation and mineralization by *P. ostreatus* also occur under manganese deficiency.

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