

## Manganese Peroxidase-Dependent Oxidation of Glyoxylic and Oxalic Acids Synthesized by *Ceriporiopsis subvermispora* Produces Extracellular Hydrogen Peroxide

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The ligninolytic system of the basidiomycete *Ceriporiopsis subvermispora* is composed of manganese peroxidase (MnP) and laccase. In this work the source of extracellular hydrogen peroxide required for MnP activity was investigated. Our attention was focused on the possibility that hydrogen peroxide might be generated by MnP itself through the oxidation of organic acids secreted by the fungus. Both oxalate and glyoxylate were found in the extracellular fluid of *C. subvermispora* cultures grown in chemically defined media, where MnP is also secreted. The *in vivo* oxidation of oxalate was measured; <sup>14</sup>CO<sub>2</sub> evolution was monitored after addition of exogenous [<sup>14</sup>C]oxalate to cultures at constant specific activity. In standard cultures, evolution of CO<sub>2</sub> from oxalate was maximal at day 6, although the MnP titers were highest at day 12, the oxalate concentration was maximal (2.5 mM) at day 10, and the glyoxylate concentration was maximal (0.24 mM) at day 5. However, in cultures containing low nitrogen levels, in which the pH is more stable, a better correlation between MnP titers and mineralization of oxalate was observed. Both MnP activity and oxidation of [<sup>14</sup>C]oxalate were negligible in cultures lacking Mn(II). *In vitro* assays confirmed that Mn(II)-dependent oxidation of [<sup>14</sup>C]oxalate by MnP occurs and that this reaction is stimulated by glyoxylate at the concentrations found in cultures. In addition, both organic acids supported phenol red oxidation by MnP without added hydrogen peroxide, and glyoxylate was more reactive than oxalate in this reaction. Based on these results, a model is proposed for the extracellular production of hydrogen peroxide by *C. subvermispora*.

White rot fungi are the most efficient ligninolytic microorganisms in nature. They bring about lignin decay through an oxidative process that is thought to involve enzymes such as lignin peroxidase (LiP), manganese-dependent peroxidase (MnP), and laccase, all of which have broad substrate specificities (22). LiP attacks both phenolic and nonphenolic aromatic residues, and the latter give rise to cation radicals that fragment spontaneously (17). MnP catalyzes the oxidation of Mn(II) to Mn(III), which in turn can oxidize phenolic substrates (10). Laccase abstracts one electron from phenolic compounds, although in the presence of primary substrates it can also oxidize nonphenolic aromatic compounds as well as Mn(II) (3, 7). Both LiP and MnP are able to depolymerize synthetic lignin *in vitro* (13, 38).

Due to the participation of peroxidase in lignin breakdown, the extracellular production of hydrogen peroxide by white rot fungi is essential to the process. Several oxidases have been proposed to be enzymes which accomplish this task; these oxidases include, among others, pyranose oxidase (9), methanol oxidase (28), aryl alcohol oxidase (11), and glyoxal oxidase (GLOX) (18, 19). The fact that GLOX is secreted by *Phanerochaete chrysosporium* and is activated by LiP and its corresponding aromatic substrate (26) strongly suggests that GLOX plays a key role in regulation as well as production of extracellular hydrogen peroxide by *Phanerochaete chrysosporium*, the most-studied ligninolytic basidiomycete.

In recent years we have studied the ligninolytic system of another basidiomycete, *Ceriporiopsis subvermispora*, which is

characterized by its selective decay of lignin when it is grown on wood (1). *C. subvermispora* secretes several isoenzymes of MnP and laccase, and the isoelectric points of these isoenzymes vary with the composition of the growth medium (27, 33). The strategy of this organism for dealing with the catabolism of nonphenolic lignin structures could involve MnP-mediated peroxidation of lipids (4). In addition to lacking LiP, *C. subvermispora* also differs from *Phanerochaete chrysosporium* in that it does not produce GLOX (31), which raises uncertainty concerning the mechanism utilized by this fungus to produce hydrogen peroxide.

Our preliminary work failed to reveal the presence of H<sub>2</sub>O<sub>2</sub>-generating oxidase activities in the extracellular fluid of *C. subvermispora* cultures when substrates reported to work with other fungal oxidases were used. We therefore explored the prospect that hydrogen peroxide could arise from reactions involving free radicals derived from the oxidation of organic acids by MnP. It is known that white rot fungi secrete acids (8), and the decomposition of these acids by MnP has been documented. Indeed, it has recently been reported that the formate radical and superoxide are detected in reaction mixtures containing oxalate, Mn(II), and MnP from *Phanerochaete chrysosporium* (20, 24). Mn(II) oxidation by superoxide gives rise to hydrogen peroxide and Mn(III) (2), which can further accelerate MnP-catalyzed reactions. Thus, oxalate can support phenol red oxidation by MnP in the absence of exogenous H<sub>2</sub>O<sub>2</sub> and in the presence of dioxygen (24). A similar oxidative mechanism has been described for glyoxylate (25).

Here we report the identification of both glyoxylate and oxalate in cultures of *C. subvermispora* and provide evidence suggesting that the oxidation of these compounds by MnP may provide a physiological source of extracellular hydrogen peroxide for this fungus.

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## MATERIALS AND METHODS

**Fungus and cultivation.** *C. subvermispora* FP-105752 was obtained from the Center for Forest Mycology Research of the Forest Products Laboratory, Madison, Wis. The fungus was maintained on agar slants of potato dextrose agar (Difco). Liquid cultures of *C. subvermispora* were grown at 30°C with orbital shaking (180 rpm) in 125-ml Erlenmeyer flasks containing 30 ml of minimal salts medium supplemented with 1% glucose as the carbon source (31). When indicated below, either the concentration of ammonium tartrate was lowered from 10 to 1 mM or MnSO<sub>4</sub> was omitted from the medium. Since in the presence of a low nitrogen concentration or in the absence of Mn(II) the amount of fungal biomass decreases by about one-half, the results related to oxalate and glyoxylate concentration, oxalate mineralization, and MnP titers are corrected below with respect to the dry biomass obtained in standard medium.

**Extracellular H<sub>2</sub>O<sub>2</sub>-generating oxidase activity.** Aliquots were withdrawn from cultures every 2 days, and they were clarified by centrifugation for 15 min at 9,000 × g. The supernatant was assayed for oxidase activity as follows. A 100-μl portion of sample was added to 100 μl of a reaction mixture containing 50 mM sodium succinate (pH 5.0) and 10 mM substrate. The resulting mixtures were incubated at 30°C for 30 min and then filtered through polysulfone membrane filters (Ultrafree-MC; nominal molecular weight limit [NMWL], 10,000; Millipore). The H<sub>2</sub>O<sub>2</sub> present in the filtrate was determined by the method of Bernt and Bergmeyer (6), modified as follow. Culture filtrate (100 μl) was added to 0.4 ml of a chromogenic solution containing 0.3 mM *o*-dianisidine and 6.25 U of horseradish peroxidase (HRP) per ml in 50 mM sodium succinate (pH 5.6). After a 15-min incubation at room temperature, the reactions were stopped by adding 0.3 ml of 6 N HCl. A<sub>520</sub> values were obtained by using a blank in which the sample was replaced by uninoculated culture medium.

**Determination of oxalate.** Aliquots (0.5 ml) withdrawn from cultures were filtered through polysulfone membrane filters (Ultrafree-MC; NMWL, 10,000; Millipore) and were analyzed by ion exclusion high-performance liquid chromatography (HPLC) with a model SCL-6A system controller (Shimadzu) equipped with a type RT 300-6.5 Polyspher OAHY prepacked column (Merck), a model LC-6A pump, a model SPD-6A detector, and a Chromatopac model C-R3A recorder. Compounds were eluted with 0.01 N H<sub>2</sub>SO<sub>4</sub> pumped at a flow rate of 0.4 ml/min, and A<sub>260</sub> values were determined.

**Determination of glyoxylate.** Previously described procedures (21) for the detection of glyoxylate were adapted as follows. Culture aliquots (0.5 ml) were reacted with 0.5 ml of 1 mM 2,4-dinitrophenylhydrazine in 0.32 N HCl for 1 h at room temperature. The hydrazone derivatives were analyzed by HPLC by using the instrument described above equipped with a mBondapak C<sub>18</sub> column. The mobile phase was 10 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 6.8) in 15% methanol and was pumped at a flow rate of 1 ml/min, and A<sub>260</sub> values were recorded.

**In vivo <sup>14</sup>C<sub>2</sub>O<sub>4</sub> evolution from [<sup>14</sup>C]oxalic acid.** To determine the rate of in vivo decarboxylation of oxalic acid, the concentration of oxalate in culture fluid was determined by HPLC as described above, and [<sup>14</sup>C]oxalate (10<sup>4</sup> dpm/mmol; Sigma) was added to the cultures to a final specific activity of 17,000 cpm/mmol. The cotton plugs of the flasks were then replaced by gas-tight rubber stoppers. After an additional 8 h of incubation at 30°C, the flasks were flushed with air for 10 min to trap the <sup>14</sup>CO<sub>2</sub> in an ethanolamine-containing scintillation fluid (32, 34).

**Assays for MnP activity and in vitro oxidation of [<sup>14</sup>C] oxalic acid.** MnP was routinely assayed with vanillylacetone as the substrate (29). The assay to measure in vitro oxidation of [<sup>14</sup>C]oxalate was conducted in gas-tight 50-ml tubes containing 1-ml reaction mixtures that consisted of 0.04 U of MnP, 1 mM [<sup>14</sup>C]oxalate (6.0 × 10<sup>4</sup> cpm/mmol), 0.1 mM MnSO<sub>4</sub>, and 50 mM sodium succinate (pH 5.0). Incubations were at 30°C, and the kinetics of <sup>14</sup>CO<sub>2</sub> evolution was followed by trapping the <sup>14</sup>CO<sub>2</sub> released as indicated above. Where mentioned below, anaerobic experiments were conducted by purging the reaction mixtures for 10 min with nitrogen. In vitro oxidation of glyoxylate by MnP was assayed in open tubes curtaining 0.04 U of the enzyme, 0.25 mM glyoxylate, 0.1 mM MnSO<sub>4</sub>, and 50 mM sodium succinate (pH 5.0) in 1-ml reaction mixtures. Formate generated in this reaction was quantitated by using formate dehydrogenase (15). Phenol red oxidation was assayed in 10-ml mixtures containing 0.4 U of MnP, 1 mM oxalate or 0.25 mM glyoxylate, 0.03 mM phenol red, 0.1 mM MnSO<sub>4</sub>, and 50 mM sodium succinate (pH 5.0) (24, 25, 30). Aliquots (0.7 ml) were removed at the times indicated below, and 50 μl of 5 N NaOH was added to halt the reaction. A<sub>510</sub> values were determined.

**Other methods.** When indicated below, Mn(III) acetate (Aldrich Chemical Co.) was added from a 2 mM stock solution freshly prepared in 96% methanol. MnP was fractionated as reported previously, except that the preparative isoelectric focusing step was omitted (36). Mycelial dry weight was determined as previously described (32).

## RESULTS

**Search for an extracellular H<sub>2</sub>O<sub>2</sub>-generating oxidase activity.** The presence of laccase in *C. subvermispora* culture fluid is problematic for the detection of peroxide in HRP-coupled assays. We therefore assayed for peroxide generation in reaction solutions after removal of protein by ultrafiltration. Ali-

quots of the culture fluid were withdrawn at various times during the growth period and were assayed as described above by using the following substrates: methylglyoxal, glyoxal, formaldehyde, glycolaldehyde, dihydroxyacetone, methanol, ethanol, anisyl alcohol, oxalic acid, glucose, and galactose. After repeated attempts with samples from different cultures, none of these compounds was able to promote the generation of the hydrogen peroxide required by HRP in the coupled assay.

**Identification of organic acids.** Glyoxylate and oxalate were identified and quantitated by HPLC of the extracellular fluids of *C. subvermispora* cultures grown in both the standard medium (containing 10 mM ammonium tartrate) and in low-nitrogen medium (containing 1 mM ammonium tartrate). Figure 1 shows examples of HPLC profiles obtained with cultures grown in the standard medium. Glyoxylate reached its maximal concentration of 0.24 mM on day 5, whereas the concentration of oxalate was maximal on day 10, and the oxalate levels were almost 10-fold higher than the levels of glyoxylate (Fig. 2). In cultures containing a low concentration of nitrogen, glyoxylate reached its maximal concentration of 0.025 mM on day 6, whereas the maximum oxalate concentration was 1.5 mM on days 8 and 16. No other carboxylic acids were identified in either early or late cultures in standard salt medium.

**MnP titers and mineralization of oxalate.** The in vitro oxidation of both oxalate and glyoxylate that is catalyzed by MnP from *Phanerochaete chrysosporium* (20, 24, 25) suggested that a corresponding enzymatic activity might be responsible for the metabolism of these organic acids in vivo in *C. subvermispora*. To test this hypothesis, MnP titers were determined throughout the growth of *C. subvermispora* in standard medium with and without Mn(II) and in low-nitrogen medium (Fig. 3a). In standard medium, the level of MnP activity increased continually up to about 1.0 U/ml on day 12. The MnP activity was lower in cultures in low-nitrogen medium and was virtually nil in cultures lacking Mn(II) (31, 34).

[<sup>14</sup>C]oxalate was added to parallel cultures, and <sup>14</sup>CO<sub>2</sub> evolution was determined as a measure of oxalate mineralization in vivo (Fig. 3b). To facilitate interpretation of the data, the specific activity of oxalate at the time of addition was kept constant (calculations were based on the oxalate concentrations in the cultures). In standard medium cultures, the oxidation of oxalate was most active between days 4 and 10, with the maximum activity at day 6. This profile does not correspond to MnP titers (Fig. 3a) or to oxalate concentrations (Fig. 2) but, significantly, correlates better with glyoxylate concentrations (Fig. 2). In low-nitrogen cultures, the level of mineralization of oxalate was low in early cultures, although throughout late cultures the profile roughly mirrored the MnP titers (Fig. 3a) and oxalate concentrations (Fig. 2). In medium lacking Mn(II), the <sup>14</sup>CO<sub>2</sub> evolution from labeled oxalate was negligible. Interpretation of these results must take into consideration the fact that MnP titers were measured in a standard buffered reaction mixture and that the pH profiles for standard and low-nitrogen cultures are not the same (34). These pH changes may have important implications for the activity of MnP in culture (see below).

**In vitro oxidation of [<sup>14</sup>C]oxalate and glyoxylate by MnP.** Day 6 cultures grown in standard medium were of particular interest because of the dynamic changes occurring the oxalate concentration was increasing, the glyoxylate concentration was near the maximal value and beginning to decrease, oxalate oxidation was maximal, and MnP activity was at early onset. To explore the possible connection between MnP activity and oxalate and glyoxylate metabolism in culture, in vitro experiments were performed with the approximate physiological concentrations of these organic acids in day 6 cultures (1 mM for

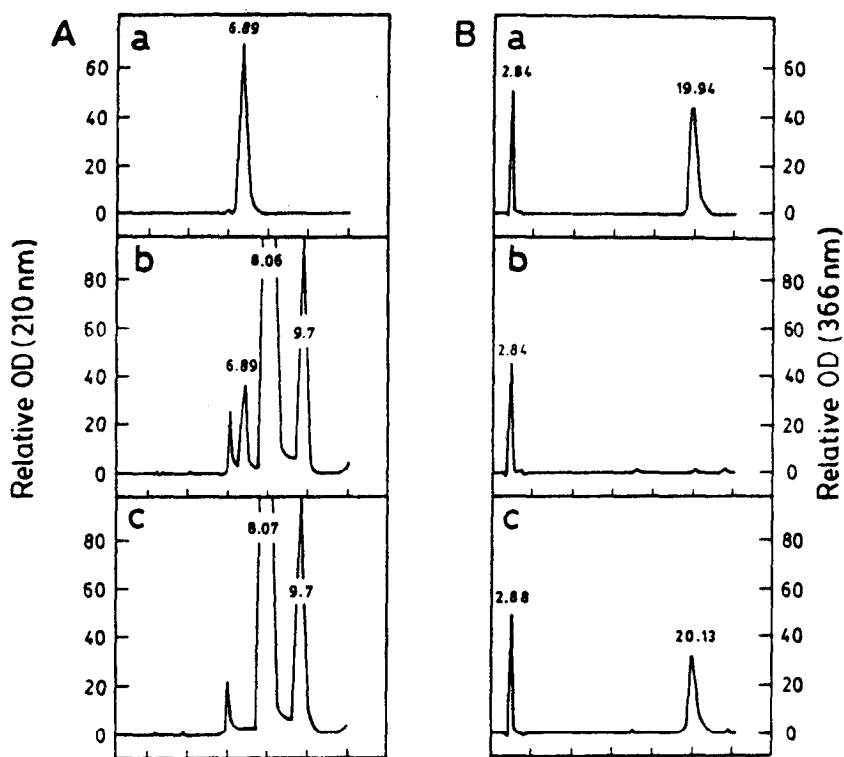


FIG. 1. Identification of oxalate and glyoxylate in the extracellular fluid of *C. subvermispora* cultures. (A) Underivatized samples were analyzed by ion exclusion HPLC, and elution profiles were determined for oxalate (a), extracellular fluid from day 6 cultures (b), and culture fluid after treatment with oxalate oxidase (c). Major peaks of the profile (8.06 and 9.7 min) were also detected with uninoculated cultures. (B) Samples were derivatized with 2,4-dinitrophenylhydrazine for reverse-phase HPLC analysis of glyoxylate (a), uninoculated growth medium (b), and extracellular fluid of day 9 cultures (c). OD (210 nm), optical density at 210 nm; OD (366 nm), optical density at 366 nm.

oxalate and 0.25 mM for glyoxylate). We determined that MnP from *C. subvermispora* catalyzes the oxidation of both glyoxylate and oxalate in reactions requiring Mn(II) (Fig. 4). The extent of the mineralization of oxalate was determined by measuring the evolution of  $^{14}\text{CO}_2$  from [ $^{14}\text{C}$ ]oxalate, while the extent of the oxidation of glyoxylate was determined by monitoring the appearance of formate (see above). As shown in Fig. 4a, oxidation of oxalate exhibited a lag which was shortened when glyoxylate was simultaneously added to the incubation mixture. Trace amounts of exogenous hydrogen peroxide or Mn(III) acetate greatly stimulated the reaction. The level of mineralization of labeled oxalate by MnP in the absence of hydrogen peroxide decreased to about 5 to 10% when the reaction was conducted under nitrogen (see above), or in the presence of 0.5 mM glutathione or 0.5 mM nitroblue tetrazolium (data not shown). Figure 4b shows that glyoxylate is more susceptible than oxalate to oxidation by MnP. In this case, trace amounts of Mn(III) acetate, but not of hydrogen peroxide, increased the rate of the reaction, while the addition of oxalate inhibited the oxidation of glyoxylate.

**Oxalate- and glyoxylate-supported phenol red oxidation by MnP.** A useful assay for monitoring MnP activity employs phenol red as the substrate; the formation of oxidized phenol red is monitored at 610 nm. This assay has been used to characterize the oxidations of MnP from *Phanerochaete chrysosporium* with glyoxylate and oxalate (24, 25). As shown in Fig. 5, both organic acids support oxidation of phenol red with the MnP of *C. subvermispora* at physiological concentrations for the organic acids. Similar to the results in Fig. 4, glyoxylate is

more reactive than oxalate even at a fourfold-lower concentration. These reactions have an absolute requirement for Mn(II).

## DISCUSSION

Extracellular hydrogen peroxide is required in ligninolysis as a cosubstrate of LiP and MnP. Peroxide-generating oxidases have been identified from wood decay fungi but not from

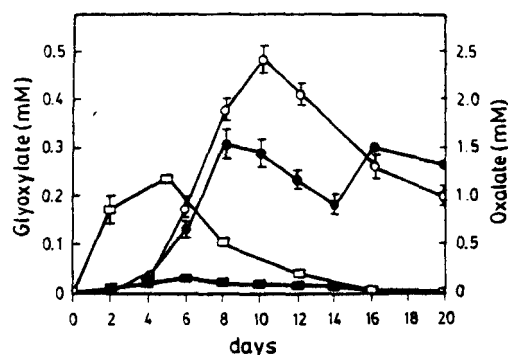


FIG. 2. Time course for oxalate and glyoxylate concentrations in extracellular fluid of *C. subvermispora*. Cultures grown in defined medium containing ammonium tartrate at an initial concentration of 10 mM (open symbols) or 1 mM (solid symbols) were analyzed to determine their oxalate (circles) and glyoxylate (squares) concentrations.

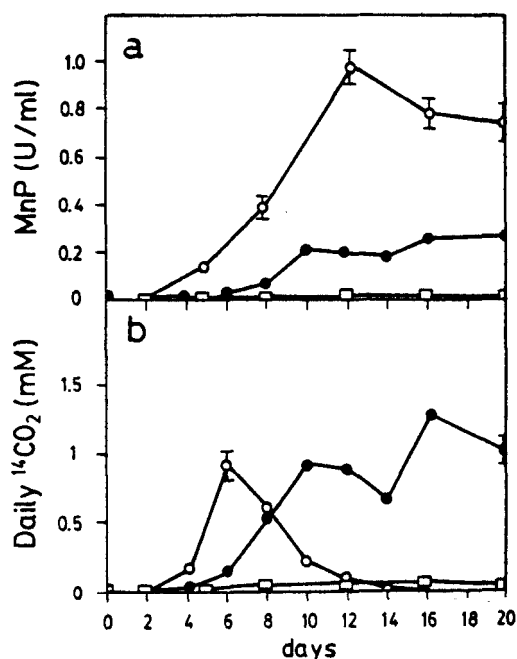


FIG. 3. Time course for MnP titers and oxalate mineralization in cultures. Cultures grown in defined medium containing 10 mM ammonium tartrate (○), defined medium containing 1 mM ammonium tartrate (●), or defined medium containing 10 mM ammonium tartrate but lacking Mn(II) (□) were analyzed to determine MnP activity (a) and [ $^{14}\text{C}$ ]oxalate mineralization (b).

*C. subvermispora* (31, 41; this study) and may require new detection strategies to uncover. Based on the results presented here, we propose an alternative pathway for the generation of hydrogen peroxide by *C. subvermispora* involving MnP, organic acids, and  $\text{O}_2$  as a terminal electron acceptor (Fig. 6). The evidence supporting this scheme includes: (i) the detection of two metabolites secreted by the fungus (i.e., glyoxylate and oxalate) which are known to be oxidized in vitro by MnP with the concomitant production of  $\text{H}_2\text{O}_2$  and Mn(III) (20, 24, 35); (ii) the correlation observed in cultures containing low nitrogen concentrations between MnP titers and mineralization of [ $^{14}\text{C}$ ]oxalate; and (iii) the ability of the organic acids mentioned above to support oxidation of phenol red by MnP in the absence of externally added hydrogen peroxide.

Organic acids play an important role in MnP-catalyzed reactions; they both facilitate the release of Mn(III) from the active site of the enzyme (37) and stabilize this species by chelation (24, 37, 39). It is not surprising then that compounds such as oxalate (5, 8, 23, 24, 39), malonate (39), and glyoxylate (this study) are secreted by fungi producing MnP. The fact that only glyoxylate and oxalate were found in *C. subvermispora* cultures does not rule out the possibility that other metabolites may also contribute to hydrogen peroxide production. It is conceivable that the carbon source for growth and energy may influence both the identity and the concentration of organic acids secreted by the fungus.

The mineralization of oxalate in cultures provided a useful marker with which to compare other culture parameters. The lack of mineralization in cultures without Mn(II), when MnP is not produced (Fig. 3), gave an indication that MnP could be involved in the oxidation of oxalate. However, the rate of oxalate oxidation in standard cultures (containing high concentrations of nitrogen), with Mn(II) present, decreases after day 6 when both MnP activity and oxalate concentration are still

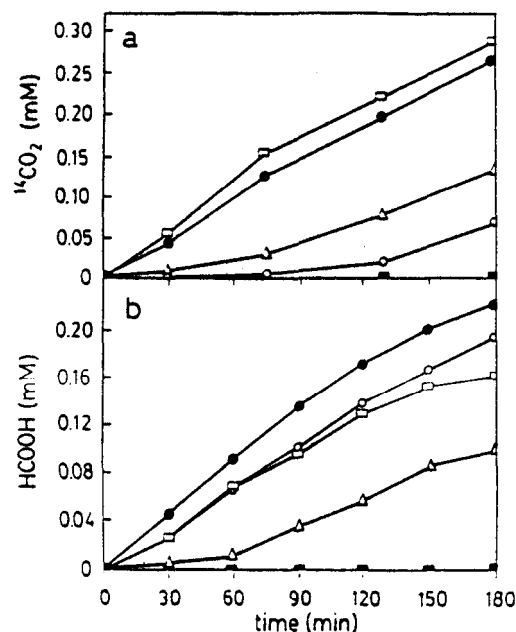


FIG. 4. In vitro oxidation of oxalate and glyoxylate by MnP. Oxalate oxidation was followed by determining  $^{14}\text{CO}_2$  evolution from [ $^{14}\text{C}$ ]oxalate (a), and glyoxylate oxidation was followed by determining formate production (b). Reaction mixtures contained 1 mM oxalate (a) or 0.25 mM glyoxylate (b) as individual substrates (○) and in combination (△). Reaction mixtures containing the individual substrates plus 5  $\mu\text{M}$  hydrogen peroxide (□) or 10  $\mu\text{M}$  Mn(III) acetate (●) and reaction mixtures containing the individual substrate but lacking Mn(II) (■) were also examined.

increasing. This apparent contradiction might be explained if MnP activity in cultures is inhibited by a steady increase in the pH of the medium (34). This effect is not observed in in vitro assays of MnP in which the preparations are buffered with 100 mM sodium tartrate (pH 5.0). Therefore, the apparent lack of correlation among oxalate oxidation, MnP titers, and oxalate concentration might be explained if the MnP is increasingly inactive due to pH effects in culture.

Interestingly, the parameter that correlates best with the oxidation of oxalate in standard cultures (Fig. 3b) is the concentration of glyoxylate (Fig. 2). This is particularly relevant because glyoxylate also stimulates the oxidation of oxalate by MnP in vitro (Fig. 4), and furthermore, glyoxylate stimulates the oxidation of phenol red at physiological levels of glyoxylate

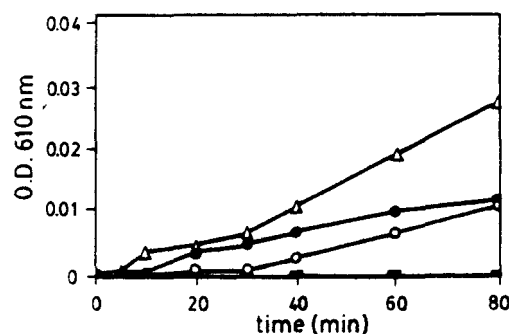


FIG. 5. Organic acid-supported oxidation of phenol red by MnP from *C. subvermispora*. Assays were conducted with oxalate (○), glyoxylate (●), and oxalate plus glyoxylate (△). No oxidation was observed in reaction mixtures lacking Mn(II) (■). O.D. 610 nm, optical density at 610 nm.

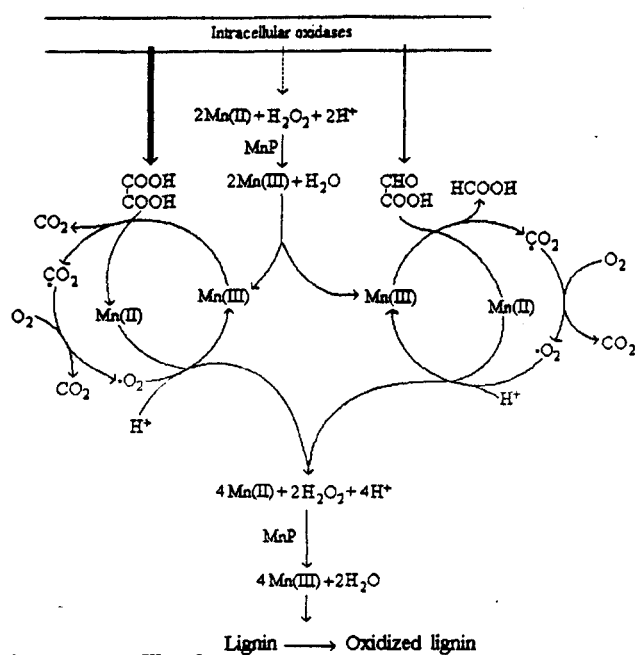


FIG. 6. Proposed scheme for MnP-dependent extracellular oxidation of organic acids in cultures of *C. subvermispora*.

and oxalate (Fig. 5). Another noteworthy observation is that there is a short burst of activity, before the addition of exogenous peroxide, when MnP is assayed in fresh culture samples with vanillylacetone as the substrate (data not shown). This activity is greatest between days 4 and 10 with standard cultures, a time when oxalate oxidation is most active and glyoxylate concentrations are highest.

In contrast to the results obtained with standard medium, the pH of cultures containing a low level of nitrogen (1 mM ammonium tartrate) remains stable (34). In this medium, the level of  $^{14}\text{CO}_2$  evolution from [ $^{14}\text{C}$ ]oxalate was initially lower than that in standard cultures (Fig. 3b), as might be expected based on the lower titers of MnP (Fig. 3a). However, mineralization proceeded at a high rate beyond day 12, when virtually no mineralization of oxalate takes place in cultures in standard medium. This oxidation profile for low-nitrogen cultures is in reasonable agreement with the MnP and oxalate concentration levels detected. In low-nitrogen cultures, the concentrations of glyoxylate are considerably lower than those in standard cultures, although the highest levels occur at approximately the same time. Why glyoxylate concentration profiles differ from oxalate concentration profiles in both low-nitrogen and standard media is not known. An answer will require, at least in part, an understanding of the metabolic pathways leading to the production of these metabolites in *C. subvermispora*.

Oxidation of carboxylic acids by peroxidase in the presence of Mn(II) was described several decades ago (16). This finding was confirmed later with a wide variety of substrates (40). Oxidation of glyoxylate by Mn(III) leads to the formation of formic acid plus formate radical, which reacts with dioxygen to produce superoxide plus carbon dioxide. Superoxide oxidation of Mn(II) generates Mn(III) plus  $\text{H}_2\text{O}_2$  (1, 25). In turn, oxidation of oxalate by Mn(III) produces carbon dioxide plus formate radical, which undergoes similar chemical reactions, with the formation of Mn(III) plus  $\text{H}_2\text{O}_2$  (20, 35).

In our scheme, we propose that trace amounts of Mn(III)

can be amplified by the action of MnP in the presence of organic acids and oxygen (Fig. 6). The initiating Mn(III) can be produced by MnP by using hydrogen peroxide as an oxidant, and this peroxide might originate from the mycelium. Alternatively, slow autoxidation of an extracellular fungal metabolite may spark the initial formation of peroxide. In early cultures, the Mn(III) generated should oxidize glyoxylate since it appears earlier than oxalate (Fig. 2) and is more reactive (Fig. 4). MnP oxidized by the peroxide generated in this reaction should give rise to more Mn(III), which could react with oxalate as its concentration increases in the cultures, thus producing an amplifying effect. Alternatively, Mn(III) could react directly with lignin, if present, or with any glyoxylate still remaining in the medium.

We do not expect that this model represents the only source of extracellular hydrogen peroxide in this fungus nor that it is unique to *C. subvermispora*. Indeed, Guillén et al. (12) have recently proposed that quinone redox cycling in *Pleurotus eryngii* leads to the production of an extracellular superoxide anion radical, a system that may also operate in other fungi, including *C. subvermispora*. Moreover, fungi with GLOX (e.g., *Phanerochaete chrysosporium*) may also exhibit the reactions shown in Fig. 6 because all of the components necessary appear to be present; glyoxal is produced in *Phanerochaete chrysosporium* cultures (18), and glyoxylate and oxalate are reaction products formed from glyoxal with GLOX (14). The relative contributions of multiple sources of peroxide in culture are difficult to measure, especially if the processes are metabolically connected and interdependent.

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