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Involvement of a New Enzyme, Glyoxal Oxidase, in Extracellular H_2O_2 Production by *Phanerochaete chrysosporium*

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The importance of extracellular H_2O_2 in lignin degradation has become increasingly apparent with the recent discovery of H_2O_2-requiring ligninases produced by white-rot fungi. Here we describe a new H_2O_2-producing activity of *Phanerochaete chrysosporium* that involves extracellular oxidases able to use simple aldehyde, α-hydroxycarbonyl, or α-dicarbonyl compounds as substrates. The activity is expressed during secondary metabolism, when the ligninases are also expressed. Analytical isoelectric focusing of the extracellular proteins, followed by activity staining, indicated that minor proteins with broad substrate specificities are responsible for the oxidase activity. Two of the oxidase substrates, glyoxal and methylglyoxal, were also identified, as their quinoxaline derivatives, in the culture fluid as secondary metabolites. The significance of these findings is discussed with respect to lignin degradation and other proposed systems for H_2O_2 production in *P. chrysosporium*.

The white-rot wood-metabolizing basidiomycetes appear to be major degraders of lignin, and, of these, *Phanerochaete chrysosporium* is the most widely studied species. The ligninolytic system of this organism is expressed in defined media in response to nutrient carbon, nitrogen, or sulfur limitation (19, 23, 25, 38). Other factors, such as O_2 partial pressure, agitation, metal ion balance, and pH, are also important (2, 19, 25, 30). Under ligninolytic conditions, *P. chrysosporium* produces extracellular H_2O_2 (10) and a number of H_2O_2-requiring peroxidases (ligninases) that oxidize and partially depolymerize lignin or lignin model compounds (14, 31, 39, 41, 42).

Several schemes have been proposed for the production of extracellular H_2O_2 in ligninolytic cultures. These involve either intracellular enzymes, including glucose-1-oxidase (20), glucose-2-oxidase (9), and fatty acyl-coenzyme A oxidase (16), or extracellular Mn^{2+}-dependent peroxidases that reduce O_2 to H_2O_2 by using NADH, NADPH, or glutathione as an electron source (1, 13, 34, 35).

Here we describe a new H_2O_2-producing activity, which we term glyoxal oxidase, from *P. chrysosporium*. Importantly, this activity, as well as two of its many substrates, glyoxal and methylglyoxal, are found in the extracellular fluid of ligninolytic cultures. Extracellular production of H_2O_2 seems particularly relevant to the lignin biodegradation process because the ligninases are extracellular and the organism has strong intracellular catalase activity (15).

MATERIALS AND METHODS

Organism and culture conditions. *P. chrysosporium* strain BKM-F-1767 (= ATCC 24725) was grown at 39°C in shallow stationary cultures as previously described (24), except that 0.01 M *trans*-aconitic acid (pH 4.3) was used as the growth buffer instead of 2,2-dimethylsuccinic acid. The stoppered cultures were flushed with 100% O_2 after 2 days of growth under air.

Enzyme assays. H_2O_2-producing activity was determined by using a modified peroxidase-coupled assay with phenol red as the peroxidase substrate (36). The reaction mixture contained 50 mM Na^+ 2,2-dimethylsuccinate (pH 6.0), 10 mM oxidase substrate, 0.01% phenol red, 10 μg of horseradish peroxidase (type II; Sigma Chemical Co., St. Louis, Mo.), and up to 300 μl of culture fluid in a total reaction volume of 1 ml. The reaction was stopped by adding 50 μl of 2 N NaOH, and then the preparation was assayed at 610 nm against an appropriate blank. For determining the pH optimum for oxidase activity, the buffer contained 25 mM malate, 25 mM 2,2-dimethylsuccinate, and 25 mM 3-(*N*-morpholino)propanesulfonic acid, and the pH was adjusted with NaOH. In this case, the reactions were stopped with 100 μl of 2 N NaOH. The reactions were shown to be not limited by peroxidase activity.

Ligninase activity was assayed by measuring the increase in absorbance at 310 nm due to H_2O_2-dependent oxidation of veratryl alcohol to veratrylaldehyde (42). The assay mixture contained 25 mM Na^+ tartrate (pH 3.0), 2 mM veratryl alcohol, and 50 to 325 μl of culture fluid in a total reaction volume of 0.5 ml; the reaction was intiated with 0.4 mM H_2O_2.

Preparation of crude extracellular protein. For isoelectric focusing, for determining substrate specificity, and for determining the pH optimum for oxidase activity, a concentrated and dialyzed preparation of crude extracellular protein was used. Culture fluid from 300 5-day-old cultures (2.6 liters) was collected by filtration through glass wool, and the protein was concentrated to 250 ml with a Minitan concentrator (Millipore Corp., Bedford, Mass.) equipped with a 10,000-dalton cutoff membrane. The extracellular polysaccharide associated with the preparation was precipitated by adding 1/3 volume of cold acetone at a near-freezing temperature and was separated by filtration through glass wool. The clear filtrate was then dialyzed (10,000-dalton cutoff) against 4 liters of water and then 4 liters of 1 mM Na^+ 2,2-dimethylsuccinate (pH 6) before being further concentrated to 5 ml (1.75 mg of protein per ml) by ultrafiltration (type PM10; Amicon Corp., Lexington, Mass.). The protein prep-

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aration for electrophoresis was further concentrated to 6 mg of protein per ml. The protein content was determined with Coomassie blue (3), using bovine serum albumin (Sigma) as the standard.

Identification and quantitation of metabolites. Most of the identified substrates for the oxidase activity can form bis derivatives with 2,4-dinitrophenylhydrazine because they are either α-hydroxycarbonyl or α-dicarbonyl compounds. These derivatives precipitate from acidic aqueous solutions and therefore can easily be separated from excess reagent and chromatographed. This derivatization procedure is a modification of the procedure described by Dagley et al. (7), and the chromatography procedure used was that of Byrne (4). To 1 ml of culture fluid was added 1 ml of 0.2% 2,4-dinitrophenylhydrazine in 2 N HCl. This solution was heated at 100°C for 30 min and then cooled on ice. The turbid mixture was then centrifuged, and the supernatant was removed. The precipitate was then taken up in approximately 0.3 ml of methylene chloride, and small samples, together with authentic derivatives, were applied to thin-layer silica plates (Silica Gel 60 F-254; thickness, 0.2 mm; EM Science, Gibbstown, N.J.). The plates were developed in benzene-tetrahydrofuran (98:2 or 93:7), which gave R_f values similar to those reported by Byrne (4). The identities of the compounds were further verified by spraying the plates with ethanolamine and observing the color change (4). This method is not totally specific because glycolaldehyde gives the same bis derivative as glyoxal; glyceraldehyde, dihydroxyacetone, and acetol all give the same bis derivative as methylglyoxal (37).

Glyoxal can easily be identified and quantitated spectrophotometrically, even in the presence of glycolaldehyde, as the derivative formed with 2,3-diaminophenazine in a 4.3 N acetic acid solution (8). Four reagents were used for color development. Reagent A consisted of 21 mg of 2,3-diaminophenazine in 100 ml of 8.5 N acetic acid, reagent B was a 0.02% solution of KNO_2 which was chilled before use, reagent C was a 50% solution of hypophosphorus acid, and reagent D was glacial acetic acid. A 1-ml sample of culture fluid was heated with 1 ml of reagent A for 10 min in steam and then cooled to 4°C in an ice bath. Then 1 ml of reagent B and 0.5 ml of reagent C were added, and the solution was heated in steam for an additional 20 min. The volume was then brought to 4 ml with reagent D. The derivative of glyoxal absorbs strongly at 600 nm, and the smaller contribution of the methylglyoxal derivative at this wavelength could be corrected for by subtracting the amount of methylglyoxal determined at 715 nm (8). However, this method was used here primarily for qualitative identification of glyoxal (as distinct from glycolaldehyde).

The method used for quantitation of glyoxal and methylglyoxal was based on the selective derivatization of α-dicarbonyl compounds with *o*-phenylenediamine (32). The corresponding derivatives (e.g., quinoxaline from glyoxal and 2-methylquinoxaline from methylglyoxal) were quantitated by high-performance liquid chromatography (HPLC). For HPLC, 0.1 volume of a 0.5% solution of *o*-phenylenediamine was added to ultrafiltered (Amicon YM10 filters) culture fluid obtained from 10 cultures, and the condensation reaction was allowed to develop at room temperature for at least 2 h in the dark. Then 100-μl injection samples were monitored at 310 nm with a Gilson HPLC system equipped with a type RP8 (C8) column (0.46 by 30 cm; Spectra-Physics, San Jose, Calif.). An isocratic solvent system of water-methanol (55:45) at a flow rate of 1 ml/min was used. Standard solutions of glyoxal and methylglyoxal were derivatized and used for quantitation. For identification of the metabolites by gas chromatography-mass spectrometry, the derivatization procedure described above was used with 10 ml of filtered culture fluid. The reaction solution was then extracted with 3 ml of methylene chloride, and the concentrated organic extract was analyzed with a model 4510 gas chromatograph-mass spectrometer (Finnigan MAT, San Jose, Calif.) equipped with a 60-m type SP2340 fused-silica capillary column (film thickness, 0.20 μm; Supelco, Inc., Bellefonte, Pa.). Electron impact mass spectra were obtained at 70 eV.

Electrophoresis. Isoelectric focusing gels with a thickness of 0.1 mm and a pH range of pH 3 to 6 (Serva Fine Biochemicals, Inc., Garden City Park, N.Y.) were used to separate the protein components of concentrated culture fluids. The gels were 4 cm long by 6 cm wide and were typically run with limiting settings of 2.5 W and 4 mA. We used an anode solution that contained 25 mM L-aspartic acid and 25 mM L-glutamic acid and a cathode solution that contained 2 M ethylene diamine, 25 mM L-arginine, and 25 mM L-lysine. Samples (0.5 μl; 6 mg of protein per ml) were applied directly to the surfaces of the gels, and the electrophoresed gels were stained for protein with Serva Violet 49 (Serva Fine Biochemicals).

A new procedure for activity staining the gels for H_2O_2-producing enzymes was developed because the common method involving peroxidative oxidation of diaminobenzidine and its subsequent precipitation (5) did not work in this study due to interference by several of the oxidase substrates. The new method involved coupling the peroxidase-phenol red reaction to the production of H_2O_2 and binding the resulting oxidized phenol red to nitrocellulose paper. In this procedure, nitrocellulose paper (pore size, 0.45 μm; Schleicher & Schuell, Inc., Keene, N.H.) was incubated in a horseradish peroxidase solution (0.4 mg/ml of water) for at least 30 min and then briefly immersed in an oxidase assay solution (50 mM Na^+ 2,2-dimethylsuccinate, pH 6.0, 10 mM oxidase substrate, 0.01% phenol red, 10 μg of horseradish peroxidase per ml). The treated nitrocellulose paper was then applied to the surface of a freshly electrophoresed gel and gently pressed so that the paper adhered to the gel. The gel was then immersed in the oxidase assay solution and gently agitated to develop the orange activity stain. The nitrocellulose activity imprint could be stored in pH 6 Na^+ 2,2-dimethylsuccinate buffer supplemented with 0.01% phenol red and 0.02% sodium azide.

Chemicals. 2,3-Diaminophenazine was synthesized by using the method of Steigmann (40) and was purified by using the method of Fischer and Hepp (11). All other chemicals were commercial products.

RESULTS

Our rationale was that extracellular H_2O_2-producing enzymes are probably present in ligninolytic cultures and that their substrate(s) should be glucose or metabolites easily derived from glucose. Under the given growth conditions, the cultures became limited for nutrient nitrogen after approximately 1 day of growth, and the ligninase activity became maximal on approximately day 5. Therefore, we sought extracellular H_2O_2-producing oxidases by using culture fluid from day 5 cultures as an enzyme source with various potential substrates. Activity was not observed with glucose, but was seen with several C_1 to C_3 compounds. After discovering this activity, we determined its substrate specificity, pH optimum, and the time course of its appear-

TABLE 1. Oxidase activities of crude extracellular protein

Compound	Activity (%)[a]
Methylglyoxal (CH_3COCHO)	100
Glycolaldehyde (CH_2OHCHO)	69
Acetaldehyde	61
Formaldehyde	54
Glyoxal (CHOCHO)	45
Glyoxylic acid ($CHOCO_2H$)	27
Dihydroxyacetone	26
DL-Glyceraldehyde	25
Acetol (CH_3COCH_2OH)	4
D-Cellobiose	<2
D-Glucose	<2
D-Galactose	<2
D-Xylose	<2
Glycerol	<2
Hydroxypyruvic acid	<2
Pyruvic acid	<2
Ethylene glycol	<2
Oxalic acid	<2
Methanol	<2

[a] H_2O_2 production by a concentrated and dialyzed preparation of day 5 culture fluid was determined with a peroxidase-coupled assay at pH 6. The activity with methylglyoxal was arbitrarily set at 100%; this reflects an activity of 2.5 nmol/min per ml of reaction solution.

ance in cultures. We then showed by isoelectric focusing that the activity is associated with at least two proteins. Finally, we identified and quantitated two of the oxidase substrates, glyoxal and methylglyoxal, in the culture fluid.

Substrate specificity. A number of simple aldehyde, α-hydroxycarbonyl, and α-dicarbonyl compounds were found to be substrates for extracellular H_2O_2-producing oxidases (Table 1). The highest activity was observed with methylglyoxal. No activity was observed with the sugars glucose, xylose, galactose, and cellobiose.

pH optimum. Figure 1 shows the pH optimum of the oxidase activity in the crude protein preparation from 5-day-old cultures with methylglyoxal as the substrate. Activity was observed over a broad pH range, with maximum activity at pH 6. The pH optimum with glyoxal was essentially the same.

Time course of oxidase expression. The extracellular oxidase activity (with methylglyoxal as the substrate) and ligninase activity were monitored over a 7-day period (Fig. 2). Both of these activities are secondary metabolic events, which were expressed in this study in response to nitrogen limitation. The maximum oxidase activity of 6 nmol of H_2O_2 produced per min per ml of culture fluid was observed on day 4.

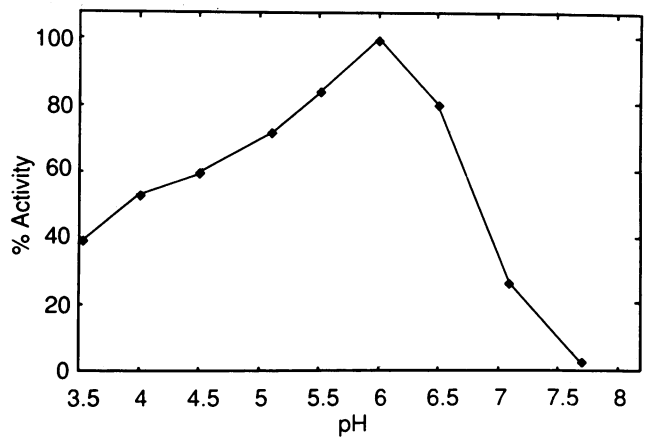

FIG. 1. pH optimum of oxidase activity. The pH optimum of oxidase activity in day 5 culture fluid was determined with methylglyoxal as the substrate.

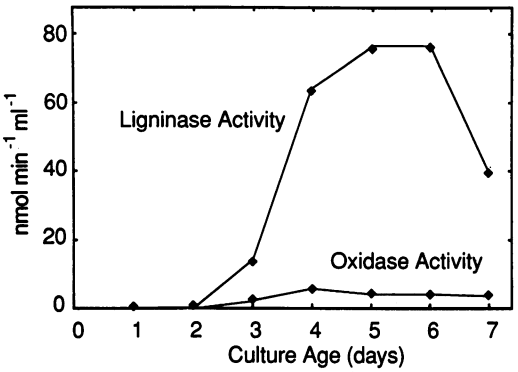

FIG. 2. Ligninase and oxidase activities in cultures. Ligninase activity was monitored by its H_2O_2-dependent oxidation of veratryl alcohol. H_2O_2 production by the new oxidase, with methylglyoxal as the substrate, was monitored with a peroxidase-coupled assay. Cultures became limited for nitrogen before day 2, and maximal glyoxal oxidase activity (6 nmol of H_2O_2 per min per ml of culture) was observed on day 4.

Identification of oxidase substrates in culture fluid. The majority of the substrates for the oxidase activity in Table 1 formed bis-2,4-dinitrophenylhydrazone derivatives under the conditions described in Materials and Methods. These derivatives could be easily separated from excess reagent and chromatographed by thin-layer chromatography. Despite the ambiguity of the resulting identifications (i.e., a number of compounds could form the same bis derivative, as indicated in Materials and Methods), this method quickly narrowed the search for substrates in the culture fluid in one comparatively easy experiment. By its use, glyoxal (or glycolaldehyde or both) was detected in 3-day-old cultures but not in 2-day-old cultures. (Note that although glucose in the culture was also derivatized by 2,4-dinitrophenylhydrazine, formation of its derivative did not prohibit use of this method.)

With the search for substrates in culture fluid narrowed to two compounds, glyoxal and glycolaldehyde, we used a more selective and quantitative method for the detection of glyoxal, based on its derivatization with 2,3-diaminophenazine. Accordingly, the spectrum of a derivatized 3-day-old culture was compared with that of authentic glyoxal in uninoculated medium (Fig. 3). The results indicated that there were significant amounts of glyoxal in cultures. Other α-dicarbonyl compounds can be similarly derivatized; however, their absorbance maxima are different from the 600-nm maximum observed for glyoxal (8). The disproportionate absorbance of the derivatized culture fluid at longer wavelengths of the spectrum, with respect to the derivatized authentic glyoxal solution (Fig. 3), suggested the possibility that other α-dicarbonyl compounds were also present in cultures. To test this possibility, a modification of the method described by Moree-Testa and Saint-Jalm (32) was used to identify and quantitate the compounds as the corresponding quinoxaline derivatives by HPLC. Figure 4 shows a representative chromatogram of a derivatized 4-day-old culture, with peaks corresponding to glyoxal and methylglyoxal. Gas chromatography-mass spectrometry confirmed the identities of these metabolites (Fig. 5).

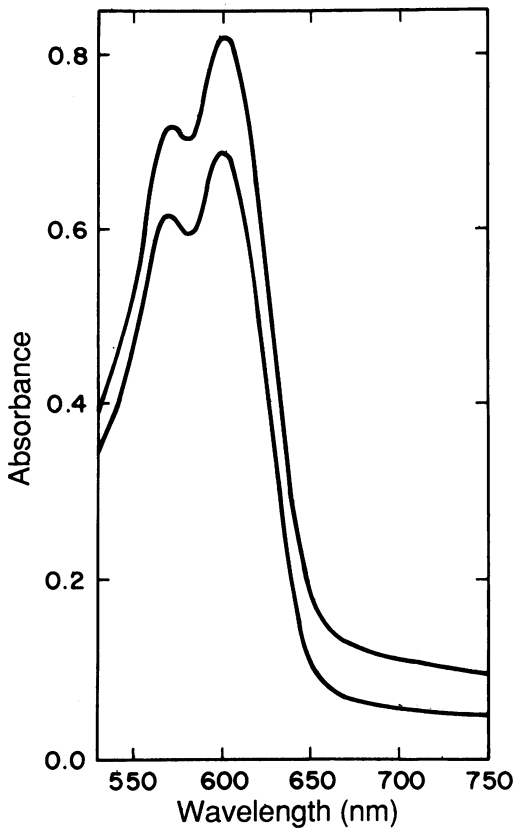

FIG. 3. Colorimetric detection of glyoxal in culture fluid. Culture fluid from 3-day-old cultures was derivatized with 2,3-diaminophenazine as described in Materials and Methods, and the spectrum was recorded (upper spectrum) against uninoculated medium similarly treated. The lower spectrum is the spectrum of derivatized authentic glyoxal (0.11 mM) in uninoculated medium.

Time course of glyoxal and methylglyoxal formation. The metabolites glyoxal and methylglyoxal were quantitated in culture fluid over a 7-day period by HPLC of the quinoxaline derivatives (Fig. 6). As observed with the oxidase activity, the production of these metabolites was a secondary metabolic event.

Identification of oxidases by isoelectric focusing. Crude extracellular protein from 5-day-old cultures was isoelectrically focused, and the gels were analyzed for protein and activity staining patterns. Figure 7 shows a gel stained for protein aligned with an imprint stained for H_2O_2-producing oxidase activity, with methylglyoxal as the substrate. The results indicate that there were at least two proteins in the culture fluid with activity toward methylglyoxal. Similar bands of activity were obtained at the same positions when glyoxal was used as the oxidase substrate.

DISCUSSION

Although it has been known for some time that wood-rotting fungi can produce extracellular H_2O_2 (27), the importance of H_2O_2 in lignin degradation became apparent only recently. Faison and Kirk (10) showed that addition of catalase to cultures strongly suppresses lignin degradation and noted a temporal correlation between production of extracellular H_2O_2 and lignin degradation to CO_2 in cultures of *P. chrysosporium*. A role for the H_2O_2 was established soon thereafter with the discovery, in the same organism, of H_2O_2-requiring peroxidases (ligninases) that modify lignin and lignin model compounds (14, 41). Recent investigations have shown that these enzymes oxidize aromatic nuclei to reactive cation radicals that undergo subsequent reactions that depend largely on the structure of the substrate (17, 22, 26, 43).

The origin of the extracellular H_2O_2 has been the subject of several investigations. It has been suggested that extracellular peroxidases play a role. These enzymes, which are studied primarily for their ability to oxidize Mn^{2+} to Mn^{3+}, also produce H_2O_2 when they oxidize glutathione, dithiothreitol, dihydroxymaleic acid, NADH, or NADPH (1, 13, 34, 35). Kuwahara et al. (28, 29) reported that intact cultures of *P. chrysosporium* can produce extracellular NAD(H) and NADP(H). However, how the organism may generate the reduced coenzyme(s) extracellularly is not known.

After detecting glyoxal oxidase activity in cultures, we soon discovered that the activity was not due to a peroxidase. This became apparent when purified fractions showed an obligate requirement for exogenous peroxidase to oxidize phenol red in a coupled-enzyme assay. Accordingly, all assays described here for detecting glyoxal oxidase included non-rate-limiting amounts of horseradish peroxidase. Further study showed specifically that purified oxidase preparations (manuscript in preparation) had neither ligninase activ-

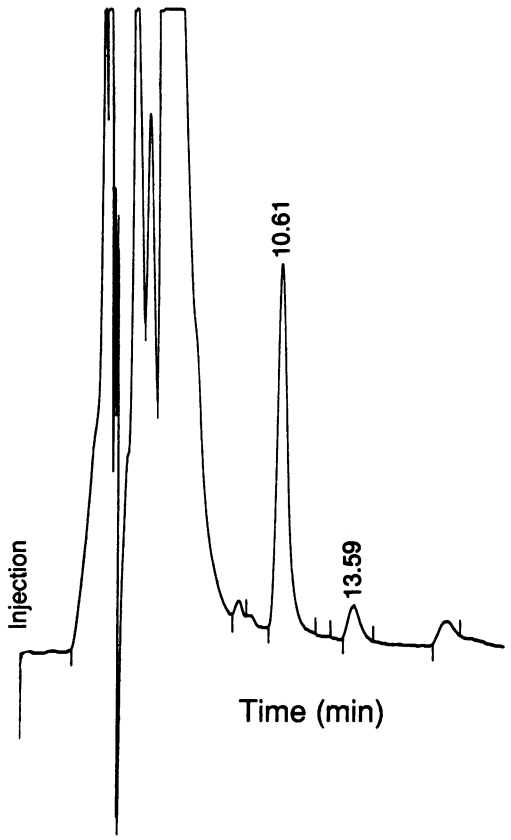

FIG. 4. HPLC chromatogram of quinoxaline derivatives. Glyoxal and methylglyoxal were quantitated by reverse-phase HPLC of the corresponding quinoxaline derivatives at 310 nm. A chromatogram obtained with derivatized 4-day-old cultures is shown. The glyoxal and methylglyoxal derivatives had retention times of 10.61 and 13.59 min, respectively.

ity (under the assay conditions described here) nor Mn-peroxidase activity when the assay conditions described by Paszczyński et al. were used (35). The Mn-peroxidase assay of these authors includes a peroxidase substrate (e.g., 2,6-dimethoxyphenol), manganese sulfate, H_2O_2, and enzyme in pH 5.0 tartrate buffer.

Possible involvement of intracellular enzymes in H_2O_2 production has also been studied. Forney et al. (12) showed a temporal correlation between the ligninolytic activity of *P. chrysosporium* and intracellular glucose oxidase activity. Since then, two different glucose oxidases in *P. chrysosporium* have been described. Kelley and Reddy (20) reported the isolation of intracellular glucose-1-oxidase. These authors showed that nutritional parameters that affect lignin degradation have a similar effect on glucose oxidase activity and suggested that their enzyme is the primary source of peroxide in ligninolytic cultures (21). Similarly, Eriksson et al. (9) partially characterized an intracellular glucose-2-oxidase from nonligninolytic shake cultures and indicated that it is synthesized also under ligninolytic conditions. However, it has not been demonstrated that the action of these intracellular H_2O_2-producing glucose oxidases actually results in extracellular H_2O_2. In this respect, it is also notable that *P. chrysosporium* has strong intracellular catalase activity (15; unpublished data).

Greene and Gould (16) suggested that intracellular fatty acyl-coenzyme A oxidase may be an important source of extracellular H_2O_2. These authors observed increased H_2O_2 production and O_2 consumption in the presence of stearoyl-coenzyme A with mycelia permeabilized with the detergent Triton X-100. However, the specific activity that they reported was about 3 orders of magnitude less than the glucose oxidase activity reported by Kelley and Reddy (21).

The magnitude of the glyoxal oxidase activity in cultures reported here (6 nmol/min per ml of culture) compares favorably with that of glucose oxidase reported by Kelly and Reddy (4.5 nmol/min per ml of culture; calculation based on 45 U obtained from the mycelia grown in 10 liters of culture fluid). (Note that such a comparison is meant only to serve as

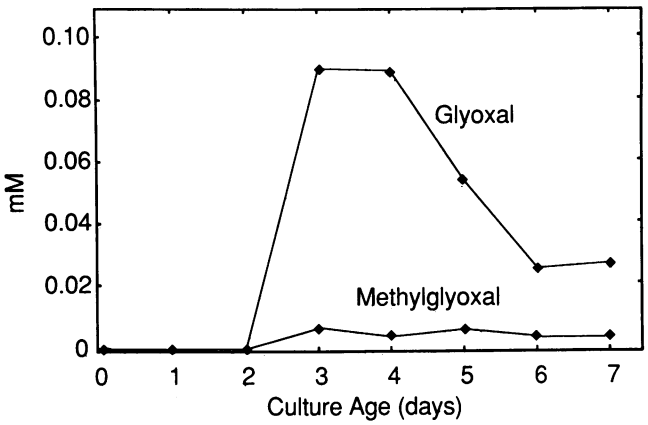

FIG. 6. Extracellular glyoxal and methylglyoxal. The substrates, glyoxal and methylglyoxal, were quantitated in culture fluid by HPLC analyses of the corresponding quinoxaline derivatives.

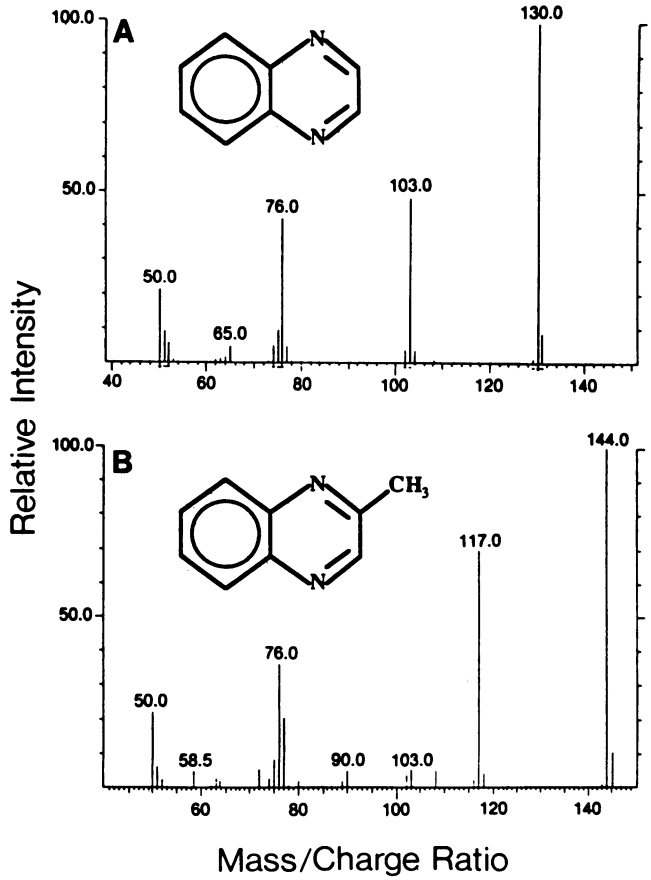

FIG. 5. Gas chromatography-mass spectrometry analysis of quinoxaline derivatives. Culture fluid from 3-day-old cultures was derivatized with *o*-phenylenediamine for detection of glyoxal and methylglyoxal as the corresponding derivatives quinoxaline (A) and 2-methylquinoxaline (B), respectively.

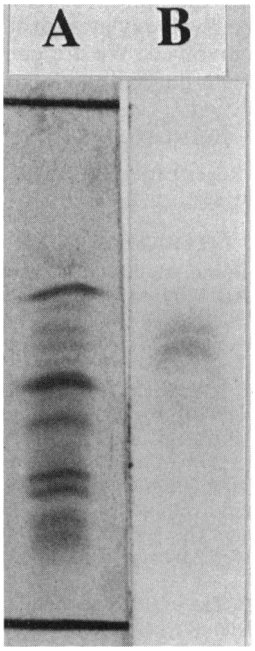

FIG. 7. Isoelectric focusing and activity staining. Extracellular protein from day 5 cultures was concentrated to 6 mg/ml and isoelectrically focused from pH 6 (top) to pH 3 (bottom). Lane A was stained for protein, and lane B was stained for H_2O_2-producing activity with methylglyoxal as the substrate. A similar activity stain pattern was obtained with glyoxal as the substrate.

a rough index of the relative amounts of activity since factors such as differences in culture conditions, enzyme recovery, culture age, etc. are not taken into account). The glyoxal oxidase is active within a broad pH range, with maximum activity at pH 6. The extracellular peroxidases of *P. chrysosporium* have activity pH optima that vary from pH 5.0 (18) to approximately 3.0 (42). The pH optimum of culture media for lignin degradation to CO_2 is ~pH 4.5 (25). Glyoxal oxidase activity is approximately 50% of its maximum at pH 4.5.

The oxidase activity of our crude protein preparations with dihydroxyacetone as the substrate resembles that of another extracellular oxidase, galactose oxidase, from the white-rot fungus *Polyporus circinatus* (44). (Note that *Dactylium dendroides*, a deuteromycete, has been misnamed as *Polyporus circinatus* in related studies [33], and therefore the source of the galactose oxidase was possibly *D. dendroides*.) However, our enzyme preparation did not oxidize galactose.

To our knowledge, this is the first report of either glyoxal or methylglyoxal production by a basidiomycete. In other microbial systems, the latter compound has been shown to have strong growth-regulatory properties (for a review of methylglyoxal metabolism, see reference 6). Whether the extracellular methylglyoxal in *P. chrysosporium* has such a role has not been determined yet. Relatively little information exists on the biological importance of glyoxal.

In conclusion, the temporal relationship among the glyoxal oxidase, the production of oxidase substrates (glyoxal and methylglyoxal), and ligninase activity and the fact that these activities are all extracellular lead us to suggest that the glyoxal oxidase system is important in H_2O_2 production in ligninolytic cultures. The broad specificity of the crude oxidase activity suggests further that substrates other than glyoxal and methylglyoxal could be involved in H_2O_2 production. Furthermore, sequential extracellular oxidations (e.g., glyoxal→glyoxylate→oxalate, yielding 2 mole of H_2O_2) might be involved. We are currently investigating these possibilities with purified oxidase preparations.

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LITERATURE CITED

1. **Asada, Y., M. Miyabe, M. Kikkawa, and M. Kuwahara.** 1986. Oxidation of NADH by a peroxidase of a lignin-degrading basidiomycete, *Phanerochaete chrysosporium*, and its involvement in the degradation of a lignin model compound. Agric. Biol. Chem. **50**:525–529.
2. **Bar-lev, S. S., and T. K. Kirk.** 1981. Effects of molecular oxygen on lignin degradation by *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun. **99**:373–378.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
4. **Byrne, G. A.** 1965. The separation of 2,4-dinitrophenylhydrazones by thin layer chromatography. J. Chromatogr. **20**:528–540.
5. **Cohen, H. J.** 1972. The use of diaminobenzidine for spectrophotometric and acrylamide gel detection of sulfite oxidase and its applicability to hydrogen peroxide-generating enzymes. Anal. Biochem. **53**:208–222.
6. **Cooper, R. A.** 1984. Metabolism of methylglyoxal in microorganisms. Annu. Rev. Microbiol. **38**:49–68.
7. **Dagley, S., P. W. Trudgill, and A. G. Callely.** 1961. Synthesis of cell constituents from glycine by a *Pseudomonas*. Biochem. J. **81**:623–631.
8. **Dechary, J. M., E. Kun, and H. C. Pitot.** 1954. Spectrophotometric determination of ethanedial. Anal. Chem. **26**:449–452.
9. **Eriksson, K.-E., B. Pettersson, J. Volc, and V. Musilek.** 1986. Formation and partial characterization of glucose-2-oxidase, a H_2O_2 producing enzyme in *Phanerochaete chrysosporium*. Appl. Microbiol. Biotechnol. **23**:257–262.
10. **Faison, B. D., and T. K. Kirk.** 1983. Relationship between lignin degradation and production of reduced oxygen species by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **46**: 1140–1145.
11. **Fischer, O., and E. Hepp.** 1889. Oxydation des orthophenylendiamins. Ber. Dtsh. Chem. Ges. **22**:355–359.
12. **Forney, L. J., C. A. Reddy, M. Tien, and S. D. Aust.** 1982. The involvement of hydroxyl radical derived from hydrogen peroxide in lignin degradation by the white rot fungus *Phanerochaete chrysosporium*. J. Biol. Chem. **257**:11455–11462.
13. **Glenn, J. K., and M. H. Gold.** 1985. Purification and characterization of an extracellular Mn(II)-dependent peroxidase from the lignin-degrading basidiomycete, *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **242**:329–341.
14. **Glenn, J. K., M. A. Morgan, M. B. Mayfield, M. Kuwahara, and M. H. Gold.** 1983. An extracellular H_2O_2-requiring enzyme preparation involved in lignin biodegradation by the white rot basidiomycete *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun. **114**:1077–1083.
15. **Greene, R. V., and J. M. Gould.** 1983. Substrate-induced H_2O_2 production in mycelia from the lignin-degrading fungus *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun. **117**:275–281.
16. **Greene, R. V., and J. M. Gould.** 1984. Fatty acyl-coenzyme A oxidase activity and H_2O_2 production in *Phanerochaete chrysosporium* mycelia. Biochem. Biophys. Res. Commun. **118**: 437–443.
17. **Hammel, K. E., M. Tien, B. Kalyanaraman, and T. K. Kirk.** 1985. Mechanism of oxidative C_α-C_β-cleavage of a lignin model dimer by *Phanerochaete chrysosporium* ligninase. J. Biol. Chem. **260**:8348–8353.
18. **Huynh, V.-B., and R. L. Crawford.** 1985. Novel extracellular enzymes (ligninases) of *Phanerochaete chrysosporium*. FEMS Microbiol. Lett. **28**:119–123.
19. **Jeffries, T. W., S. Choi, and T. K. Kirk.** 1981. Nutritional regulation of lignin degradation by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **42**:290–296.
20. **Kelley, R. L., and C. A. Reddy.** 1986. Purification and characterization of glucose oxidase from ligninolytic cultures of *Phanerochaete chrysosporium*. J. Bacteriol. **166**:269–274.
21. **Kelley, R. L., and C. A. Reddy.** 1986. Identification of glucose oxidase activity as the primary source of hydrogen peroxide in ligninolytic cultures of *Phanerochaete chrysosporium*. Arch. Microbiol. **144**:248–253.
22. **Kersten, P. J., M. Tien, B. Kalyanaraman, and T. K. Kirk.** 1985. The ligninase of *Phanerochaete chrysosporium* generates cation radicals from methoxybenzenes. J. Biol. Chem. **260**:2609–2612.
23. **Keyser, P., T. K. Kirk, and J. G. Zeikus.** 1978. Ligninolytic enzyme system of *Phanerochaete chrysosporium*: synthesized in the absence of lignin in response to nitrogen starvation. J. Bacteriol. **135**:790–797.
24. **Kirk, T. K., S. Croan, M. Tien, K. E. Murtagh, and R. L. Farrell.** 1986. Production of multiple ligninases by *Phanerochaete chrysosporium*: effect of selected growth conditions and use of a mutant strain. Enzyme Microb. Technol. **8**:27–32.
25. **Kirk, T. K., E. Schultz, W. J. Connors, L. F. Lorenz, and J. G. Zeikus.** 1978. Influence of culture parameters on lignin metabolism by *Phanerochaete chrysosporium*. Arch. Microbiol. **117**:277–285.
26. **Kirk, T. K., M. Tien, P. J. Kersten, M. D. Mozuch, and B. Kalyanaraman.** 1986. Ligninase of *Phanerochaete chrysosporium*: mechanism of its degradation of the non-phenolic arylglycerol β-aryl ether substructure of lignin. Biochem. J. **236**:279–287.
27. **Koenigs, J. W.** 1972. Production of extracellular hydrogen peroxide and peroxidase by wood-rotting fungi. Phytopathology **67**:100–110.

28. **Kuwahara, M., and Y. Asada.** 1983. Metabolism of pyridine coenzymes in *Phanerochaete chrysosporium*, p. 164–175. *In* T. Higuchi, H.-M. Chang, and T. K. Kirk (ed.), Recent advances in lignin biodegradation research. Uni Publisher, Tokyo.
29. **Kuwahara, M., Y. Ishida, Y. Miyagawa, and C. Kawakami.** 1984. Production of extracellular NAD and NADP by a lignin-degrading fungus, *Phanerochaete chrysosporium*. J. Ferment. Technol. **62**:237–242.
30. **Leatham, G. F.** 1986. The ligninolytic activities of *Lentinus edodes* and *Phanerochaete chrysosporium*. Appl. Microbiol. Biotechnol. **24**:51–58.
31. **Leisola, M., F. Meussdoerffer, R. Waldner, and A. Fiechter.** 1985. Production and identification of extracellular oxidases of *Phanerochaete chrysosporium*. J. Biotechnol. **2**:379–382.
32. **Moree-Testa, P., and Y. Saint-Jalm.** 1981. Determination of α-dicarbonyl compounds in cigarette smoke. J. Chromatogr. **217**:197–208.
33. **Nobles, M. K., and C. Madhosingh.** 1963. *Dactylium dendroides* (Bull.) Fr. misnamed as *Polyporus circinatus* Fr. Biochem. Biophys. Res. Commun. **12**:146–147.
34. **Paszcyński, A., V.-B. Huynh, and R. Crawford.** 1985. Enzymatic activities of an extracellular, manganese-dependent peroxidase from *Phanerochaete chrysosporium*. FEMS Microbiol. Lett. **29**:37–41.
35. **Paszczyński, A., V.-B. Huynh, and R. Crawford.** 1986. Comparison of ligninase-I and peroxidase-M2 from the white-rot fungus *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **244**: 750–765.
36. **Pick, E., and Y. Keisari.** 1980. A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. J. Immunol. Methods **38**:161–170.
37. **Reich, H., and B. K. Samuels.** 1956. The reaction of simple ketols with 2,4-dinitrophenylhydrazine. J. Org. Chem. **21**:68–71.
38. **Reid, I. D.** 1979. The influence of nutrient balance on lignin degradation by the white-rot fungus *Phanerochaete chrysosporium*. Can. J. Bot. **57**:2050–2058.
39. **Renganathan, V., K. Miki, and M. H. Gold.** 1985. Multiple molecular forms of diarylpropane oxygenase, an H_2O_2-requiring, lignin-degrading enzyme from *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. **241**:304–314.
40. **Steigmann, A.** 1946. Phenazinotriazol as desensitiser and reagent. Br. J. Photogr. **93**:256–257.
41. **Tien, M., and T. K. Kirk.** 1983. Lignin-degrading enzyme from the hymenomycete *Phanerochaete chrysosporium* Burds. Science **221**:661–663.
42. **Tien, M., and T. K. Kirk.** 1984. Lignin-degrading enzyme from *Phanerochaete chrysosporium*: purification, characterization, and catalytic properties of a unique H_2O_2-requiring oxygenase. Proc. Natl. Acad. Sci. USA **81**:2280–2284.
43. **Umezawa, T., S. Kawai, S. Yokota, and T. Higuchi.** 1986. Aromatic ring cleavage of various β-0-4-lignin model dimers by *Phanerochaete chrysosporium*. Wood Res. **73**:8–17.
44. **Zancan, G. T., and D. Amaral.** 1970. New substrate for galactose oxidase. Biochim. Biophys. Acta **198**:146–147.