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Relationship Between Lignin Degradation and Production of Reduced Oxygen Species by *Phanerochaete chrysosporium*

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The relationship between the production of reduced oxygen species, hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$), and hydroxyl radical (·OH), and the oxidation of synthetic lignin to CO_2 was studied in whole cultures of the white-rot fungus *Phanerochaete chrysosporium* Burds. The kinetics of the synthesis of H_2O_2 coincided with the appearance of the ligninolytic system; also, H_2O_2 production was markedly enhanced by growth under 100% O_2, mimicking the increase in ligninolytic activity characteristic of cultures grown under elevated oxygen tension. Lignin degradation by whole cultures was inhibited by a specific H_2O_2 scavenger, catalase, implying a role for H_2O_2 in the degradative process. Superoxide dismutase also inhibited lignin degradation, suggesting that $O_2^{\cdot-}$ is also involved in the breakdown of lignin. The production of ·OH was assayed in whole cultures by a benzoate decarboxylation assay. Neither the kinetics of ·OH synthesis nor the final activity of its producing system obtained under 100% O_2 correlated with that of the lignin-degrading system. However, lignin degradation was inhibited by compounds which react with ·OH. It is concluded that H_2O_2, and perhaps $O_2^{\cdot-}$, are involved in lignin degradation; because these species are relatively unreactive per se, their role must be indirect. Conclusions about a role for ·OH in ligninolysis could not be reached.

Lignin is degraded in nature primarily by the white-rot basidiomycetes. These organisms oxidize lignin completely to CO_2 by an as yet unknown mechanism. Lignin is a recalcitrant substrate: it is heterogeneous, hydrophobic, and optically inactive, and it contains nonlabile carbon-carbon and ether linkages, which are arranged in a random fashion (1). Hall (9) suggested that the nonspecific oxidation of lignin is mediated by a nonenzymatic agent, namely "activated" oxygen. This term describes those partially reduced or excited species derived from ground-state molecular oxygen, including hydrogen peroxide (H_2O_2), superoxide radical ($O_2^{\cdot-}$), hydroxyl radical (·OH), and singlet oxygen ($^1O_2$), as shown in Fig. 1. Of these species, ·OH is by far the most reactive.

Hydroxyl radical has been detected in certain biological systems, where it acts as a powerful oxidizing agent. In mammals, cell-mediated immunity may be based in part on the ability of macrophages and polymorphonuclear leukocytes to produce ·OH, which is lethal to microbial invaders (26). The brown-rot wood-decaying fungi may effect the oxidative depolymerization of cellulose by the excretion of H_2O_2, which is presumed to react with ambient Fe(II) to create ·OH (or an equally reactive oxidizing agent) (10, 18). These organisms, although unable to fully break down lignin, do catalyze a limited oxidative alteration of the substrate (14).

Several investigators have attempted to correlate the generation of active oxygen species with the degradation of lignin by the white-rot fungi. The rate of lignin degradation by whole cultures of *Phanerochaete chrysosporium* Burds. has been observed to increase when assayed under high oxygen concentration (3), implying a role for molecular oxygen or its products in the actual decomposition of lignin. Although $^1O_2$ has been implicated in lignin degradation by *P. chrysosporium* (24), subsequent work has discredited $^1O_2$ as a ligninolytic agent (16, 19). Amer and Drew (2) reported the detection of extracellular $O_2^{\cdot-}$ in ligninolytic cultures of the white-rot fungus *Coriolus versicolor* and have suggested that it is involved in the initial depolymerization of lignin. Forney et al. (7) and Kutsuki and Gold (20) have presented indirect evidence for the involvement of ·OH in lignin degradation by *P. chrysosporium*.

The argument for the involvement of partially reduced oxygen species, H_2O_2, $O_2^{\cdot-}$, and ·OH, in lignin degradation can be based on their detection in whole, ligninolytic cultures and on the inhibition of lignin degradation by the addi-

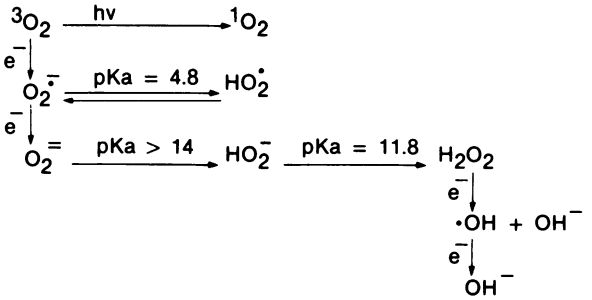

FIG. 1. The chemistry of active oxygen.

tion of scavengers of these species. Further evidence can be obtained by the investigation of the temporal and regulatory phenomena associated with their synthesis. The objectives of this study were therefore (i) to determine the kinetics of the production of partially reduced oxygen species by whole cultures of *P. chrysosporium*, (ii) to describe the effect of high oxygen concentrations on the synthesis of these oxygen species, and (iii) to assess the effect of their removal from cultures on ligninolytic activity.

MATERIALS AND METHODS

Organism. *P. chrysosporium* Burds. ME-446 (ATCC 34541; maintained at the Forest Products Laboratory, Madison, Wis.) was grown in a defined liquid medium (17) limiting in nitrogen and containing 1.0% glucose as the carbon source. This medium was buffered with polyacrylic acid, 10 mM in carboxyl groups, pH 4.2. The organism was grown at 39°C in shallow stationary cultures, i.e., 10 ml of fluid in a 125-ml Erlenmeyer flask (13, 17). Cultures were equilibrated with either air or pure oxygen immediately after inoculation and flushed with the corresponding gas every 3 days thereafter (13, 17).

Reagents. Synthetic ring-labeled [^{14}C]lignin (specific activity, 9×10^5 dpm/mg), was prepared as described previously (13, 15). [*U*-^{14}C]glucose (specific activity, 10.9 mCi/mmol) and [7-^{14}C]benzoic acid (specific activity, 50.2 mCi/mmol) were obtained from ICN Pharmaceuticals, Irvine, Calif.

Cycloheximide, cytochrome *c* (type III), bovine serum albumin (fraction V), and *o*-dianisidine were purchased from Sigma Chemical Co., St. Louis, Mo. *N*,*N*'-dimethyl-4-nitrosoaniline (DMNA) was obtained from Aldrich Chemical Co., Milwaukee, Wis.

Catalase (specific activity, 3,100 U/mg) was purchased from Worthington Biochemicals, Freehold, N.J. Aqueous solutions of 20 to 30 mg were dialyzed against distilled water (three 1,000-ml changes at 2 h each at 4°C) before use to remove ammonium salts. Peroxidase (type II; specific activity, 152 U/mg) was obtained from Sigma Chemical Co. Superoxide dismutase (SOD; specific activity, 300 U/mg) was purchased from Calbiochem, Richmond, Calif. With the exception of catalase, all enzymes were supplied in essentially salt-free form.

Assays for lignin degradation and reduced oxygen species. All assays were conducted on whole cultures of *P. chrysosporium*, i.e., in a heterogeneous system consisting of intact mycelial mats plus surrounding growth medium. The displacement or fragmentation of the mycelia during growth and assay reactions was scrupulously avoided, for such disruptions interfere with ligninolytic activity. Centrifugation was carried out at 10°C in a Sorvall model RC-5 centrifuge fitted with a type SE-12 rotor. Optical measurements were performed in a Gilford model 250 spectrophotometer.

Lignin degradation, monitored by the release of $^{14}CO_2$ from a labeled substrate, was measured as described previously (13, 15, 17). Glucose oxidation was measured in the same fashion.

Hydrogen peroxide production was quantitated via the peroxidase-dependent oxidation of *o*-dianisidine. To each culture was added 0.5 ml of an aqueous solution containing 3.1 μmol of *o*-dianisidine and 0.3 mg of peroxidase. This solution also contained 0.25 μmol of cycloheximide to prevent further protein synthesis (5). Cultures were flushed with 100% O_2 for 15 min and then incubated at 39°C. Sets of six replicate cultures were removed after 2, 4, and 6 h of incubation, chilled on ice for 10 to 15 min, and centrifuged at 12,000 × *g* for 10 min. The absorbance of the supernatant was read at 500 nm against a water blank. An increase in absorbance was observed only in the presence of exogenous peroxidase (i.e., no endogenous peroxidase was detected).

The assay for ·OH was based on the decarboxylation of benzoate, with release of CO_2 (26). Whole cultures were each spiked with 25,000 dpm of [7-^{14}C]benzoic acid in 0.5 ml of water, which also contained 0.35 μmol (unlabeled) of sodium benzoate and 0.25 μmol of cycloheximide. Six replicate cultures were included in each experiment. Cultures were flushed with 100% O_2 for 15 min and then incubated at 39°C. At 2, 4, and 6 h, the gas phases of the cultures were flushed into an ethanolamine-containing scintillation fluid to trap $^{14}CO_2$ (17). $^{14}CO_2$ evolution was linear over the 6-h time course.

Inhibition studies. To determine the effect of scavengers of the reduced oxygen species on the rate of lignin degradation, 6-day-old oxygen-grown ligninolytic cultures were incubated with synthetic lignin or glucose (plus cycloheximide) as described above, with the addition of an appropriate amount of the scavenger in aqueous solution, in a total volume not exceeding 1 ml. The compounds tested were catalase, which is specific for H_2O_2; SOD, which is specific for $O_2^{\cdot-}$; cytochrome *c*, which also reacts with $O_2^{\cdot-}$; and DMNA, salicylate, and benzoate, which scavenge ·OH. Each experiment

included six replicate cultures. The cultures were then flushed with 100% O_2 for 15 min and incubated at 39°C. Radioactive CO_2 release was monitored as described above; cultures were flushed at 2.5, 5.0, and 7.5 h.

RESULTS

The growth characteristics of *P. chrysosporium* in stationary culture in this medium have been described elsewhere (11, 13, 17). Under conditions of nitrogen limitation, growth, as measured by DNA increase, is linear for 1 to 1.5 days. Growth ceases within 2.5 days, although glucose oxidation (17) and mycelial weight gain (due to polysaccharide synthesis [17, 21]) continue for several days thereafter. Lignin degradation (to CO_2) occurs during the secondary metabolic phase: it is initiated on day 4 and reaches a maximal rate on days 5 to 6. The ligninolytic rate remains fairly constant for several days thereafter.

Assay of H_2O_2. Ligninolytic cultures, whether grown under air or under oxygen, produced extracellular H_2O_2 (Fig. 2). The activity of the H_2O_2-generating system produced under 100% O_2 was substantially higher than that produced under air; indeed, the latter was barely detectable. In either case, little or no H_2O_2 production was observed during primary growth. A large increase in H_2O_2 production was detected on day 3 in oxygen-grown cultures. A maximal rate of H_2O_2 release was attained by day 4; this rate held constant through day 6. The addition of catalase to ligninolytic cultures resulted in decreased rates of lignin degradation (Table 1). This inhibition of lignin oxidation could be distinguished from any effect on general metabolism, as represented by glucose oxidation. Furthermore, this inhibition was distinct from the previously acknowledged suppressive effect of exogenous nutrient nitrogen (5, 11, 13, 17), as demonstrated by the inclusion of inert protein controls.

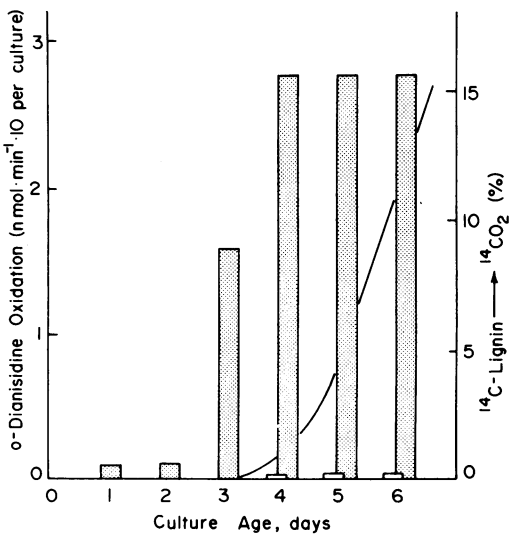

FIG. 2. Kinetics of H_2O_2 production (*o*-dianisidine oxidation by peroxidase) by whole cultures. Cultures were grown under either air (open bars) or 100% O_2 (solid bars) and assayed under 100% O_2. Values represent the mean of six replicate cultures ± 6% standard deviation. Oxidation of synthetic [ring-*U*-^{14}C]lignins to $^{14}CO_2$ in similar cultures (under O_2) is shown by the solid line.

Assay of $O_2^{\cdot-}$. Technical problems associated with the use of whole cultures prevented the development of a satisfactory assay for $O_2^{\cdot-}$. Cytochrome *c*, a nonspecific $O_2^{\cdot-}$ scavenger which has been used as the basis for assay (23), became bound to the mycelia, precluding quantitation of the amount reacted with $O_2^{\cdot-}$. Nitrobluetetrazolium, another nonspecific scavenger used to assay $O_2^{\cdot-}$ (23), was insoluble in this system. These difficulties prohibited the measurement of $O_2^{\cdot-}$. It was possible, however, to demonstrate the possible involvement of $O_2^{\cdot-}$ in lignin degradation by indirect means, i.e., by the use of scavengers of $O_2^{\cdot-}$. The addition of either cytochrome *c* or SOD to ligninolytic cultures markedly decreased the rate of lignin degradation (Table 2). Again, this inhibition could be distinguished from an effect on general oxidative metabolism or from that ascribable to nutrient nitrogen addition.

Assay of ·OH. Ligninolytic cultures produced ·OH whether grown under air or oxygen (Fig. 3). The rate of ·OH evolution in mature oxygen-grown cultures was slightly less than that in air-grown cultures. In both cases, ·OH was pro-

TABLE 1. Effect of scavengers of H_2O_2 on fungal lignin degradation

Experimental condition	Rate of CO_2 evolution (% of control)[a]	
	Synthetic lignin	Glucose
No additions	(100)[b]	(100)[c]
Catalase system		
Controls		
Bovine serum albumin[d]	87	NA[e]
Denatured catalase[d,f]	70	NA
Native catalase[d]	6	122

[a] Standard deviations were ±10% of the values shown.
[b] Rate of $^{14}CO_2$ evolution = 0.23% of total ^{14}C applied per culture per hour.
[c] Rate of $^{14}CO_2$ evolution = 0.05% of total ^{14}C applied per culture per hour.
[d] Added at 1 mg per culture.
[e] NA, Not assayed.
[f] Incubated at 100°C for 5 min.

TABLE 2. Effect of scavengers of O_2^- on fungal lignin degradation

Experimental condition	Rate of CO_2 evolution (% of control)[a]	
	Synthetic lignin	Glucose
No additions	(100)[b]	(100)[c]
Nonenzymatic scavenging system		
Controls		
Bovine serum albumin[d]	50	170
Dithiothreitol[e]	60	NA[f]
Native cytochrome c_{red}[d,g]	45	NA
Denatured cytochrome c_{ox}[d,h]	55	NA
Native cytochrome c_{ox}[d]	13	69
Enzymatic scavenging system		
Control (Denatured SOD[h,i])	58	NA
Native SOD[i]	21	94

[a] Standard deviations were ±10% of the values shown.
[b] Rate of $^{14}CO_2$ evolution = 0.23% of total ^{14}C applied per culture per hour.
[c] Rate of $^{14}CO_2$ evolution = 0.05% of total ^{14}C applied per culture per hour.
[d] Added at 1.23 mg per culture.
[e] Added at 0.5 mg per culture (0.1 mM final concentration).
[f] NA, Not assayed.
[g] Reduced with 0.1 mM dithiothreitol.
[h] Incubated at 100°C for 2 min.
[i] Added at 5 μg per culture.

duced during the primary growth phase and peaked on day 4. Throughout this preligninolytic period, the activity of the ·OH-generating system was substantially higher in oxygen-grown cultures. However, after day 4, ·OH production dropped to a constant value similar to that in air-grown cultures. Lignin degradation was inhibited by the addition of any of three ·OH scavengers, i.e., DMNA, salicylate, or benzoate (Table 3). Again, this effect was distinct from any inhibition of general oxidative metabolism; indeed, salicylate and benzoate stimulated glucose oxidation.

DISCUSSION

The data presented above are consistent with a role for partially reduced oxygen species in lignin degradation by *P. chrysosporium*. Specifically, these results indicate that H_2O_2 is of primary importance in the process. Hydrogen peroxide was detected by the use of a specific scavenging system, i.e., the peroxidase-dependent oxidation of *o*-dianisidine. The production of this species increased dramatically after the cessation of active growth; this increase occurred slightly before the onset of lignin degradation (Fig. 2). Forney et al. (7) cited a correlation between intracellular H_2O_2 production and lignin degradation in this organism. However, the work presented here provides the first evidence for extracellular H_2O_2 production by whole ligninolytic cultures. Microscopic examination of these resting cultures verified cell wall integrity; the appearance of extracellular H_2O_2 could therefore not be ascribed to autolysis. This observation is consistent with a report by Forney et al. (6) which identified "periplasmic" structures as the site of intracellular H_2O_2 production in this organism. Cultures grown under oxygen produced substantially more H_2O_2 than did those grown under air. In these respects, the synthesis of H_2O_2 mimics that of the ligninolytic system (13). The addition of catalase, a specific scavenger of H_2O_2, to ligninolytic cultures strongly depressed the rate of lignin oxidation, confirming the role of H_2O_2 in fungal lignin degradation. A limited inhibitory effect of catalase on lignin degradation has recently been noted by Kutsuki and Gold (20). In their work, catalase and radioactive lignin were added to nitrogen-starved cultures, and total $^{14}CO_2$ release was measured 3 days later. It is unlikely that the catalase remained active or stable over such a long incubation with the cultures; in fact, the apparent inhibition of ligninolysis may have reflected instead the well-documented (5) repressive effect of added nutrient nitrogen. Cata-

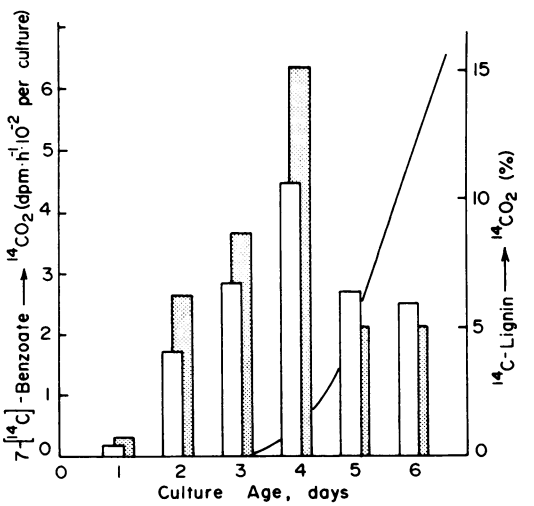

FIG. 3. Kinetics of ·OH production (benzoate decarboxylation) by whole cultures. Cultures were grown under either air (open bars) or 100% O_2 (solid bars) and assayed under O_2. Values represent the mean of six replicate cultures ± 10% standard deviation. Oxidation of synthetic [ring-*U*-^{14}C]lignins to $^{14}CO_2$ in similar cultures (under O_2) is shown by the solid line.

TABLE 3. Effect of scavengers of ·OH on fungal lignin degradation

Addition (μM)	Rate of CO_2 evolution (% of control)[a]	
	Synthetic lignin	Glucose
None	(100)[b]	(100)[c]
DMNA		
5	48	NA[d]
10	43	NA
50	13	54
Salicylate[e]		
50	66	140
250	30	145
500	17	180
Benzoate[e]		
5	89	157
50	71	172
100	68	200
500	65	239

[a] Standard deviations were ±10% of the values shown.
[b] Rate of $^{14}CO_2$ evolution = 0.23% of total ^{14}C applied per culture per hour.
[c] Rate of $^{14}CO_2$ evolution = 0.05% of total ^{14}C applied per culture per hour.
[d] NA, Not assayed.
[e] Sodium salt.

lase is generally prepared by precipitation with ammonium salts. It is not clear that the authors took steps to minimize nitrogen contamination of their enzyme. Also, the authors reported cumulative CO_2 release, which is a poor indicator of the immediate effect of H_2O_2 removal on lignin degradation. Finally, their work lacked proper controls, e.g., inactivated catalase or inert protein. In contrast, the work presented here reflects the effect of the enzyme alone during a short-term rate experiment in the absence of excess nutrient nitrogen and with appropriate controls.

Our evidence for a role of extracellular H_2O_2 in lignin degradation is consistent with the recent discovery of a lignin-degrading enzyme that requires H_2O_2 for activity (27). The enzyme, isolated from the extracellular fluid of ligninolytic cultures of *P. chrysosporium* (grown as described here), catalyzes, in the presence of added H_2O_2, an important oxidative cleavage reaction in lignin substructure model compounds and in lignin itself. It partially depolymerizes lignin (27). By itself, H_2O_2 is not reactive; its antimicrobial (26) and cellulolytic (10, 18) activities result from its conversion to a more reduced species.

Our results here suggest that the previously reported (3, 17) stimulation of the synthesis of the ligninolytic system by molecular oxygen may be linked to the effect of oxygen on H_2O_2 production. Exposure to high oxygen levels causes an increase in the levels of partially reduced oxygen species, including H_2O_2, in other biological systems (8, 28). The mechanism underlying this stimulatory effect is poorly understood.

The inhibition of lignin degradation by SOD, a specific scavenger of $O_2^{\cdot-}$, suggests that $O_2^{\cdot-}$ is involved in lignin degradation. However, like H_2O_2, it is a weak oxidant and cannot be considered a ligninolytic agent per se. The assertion by Amer and Drew (2) that $O_2^{\cdot-}$ plays a role in lignin depolymerization was not supported by evidence that their $O_2^{\cdot-}$-producing cultures were ligninolytic. Thus, our results with SOD provide the first indication of a role for $O_2^{\cdot-}$ in ligninolysis.

Forney et al. (7) demonstrated ·OH in whole cultures of *P. chrysosporium* and suggested that it is a primary agent in fungal lignin degradation. This conclusion was based on the generation of ethylene from α-keto-γ-methylthiobutyric acid by whole cultures, an activity ascribed to ·OH (12). However, in their work, the appearance of this activity preceded that of the lignin-degrading system. It has been reported elsewhere (25) that the generation of ethylene from methional (3-methiolpropionaldehyde) is not a specific assay for ·OH. Because α-keto-γ-methylthiobutyric acid is closely related to methional, the α-keto-γ-methylthiobutyric acid assay may not be specific for ·OH either. Our assay for ·OH is based on the decarboxylation of benzoate: it is specific for ·OH and does not detect H_2O_2, $O_2^{\cdot-}$, or $^1O_2$ (22). A likely criticism of the assay is that the observed decarboxylation may be catalyzed by an enzyme. *P. chrysosporium* has indeed been shown to produce a decarboxylase that is active against aromatic acids, including vanillate, throughout its growth phase (4). However, this enzyme is not active against benzoate (4). Thus, the activity we observed in whole cultures presumably can be ascribed to ·OH.

According to our assay, ·OH is produced throughout the growth phase and appears before the onset of ligninolysis (Fig. 3). Moreover, idiophasic ·OH synthesis is immune to stimulation by molecular oxygen. Interpretation of the inhibitory effects of benzoate, salicylate, and DMNA is hampered by the non-specificity of these compounds as scavengers of ·OH. However, the kinetic and regulatory data obtained with the decarboxylation assay seem inconsistent with a major role for ·OH in lignin degradation. The kinetic data presented here indicate that ·OH production from H_2O_2 cannot be a rate-limiting step in lignin degradation.

In conclusion, our data indicate that extracellular H_2O_2, and possibly $O_2^{\cdot-}$, play a role in

lignin degradation. Because neither of these species per se degrades lignin, their role must be indirect, as in the H_2O_2-dependent lignin-degrading enzyme mentioned above (27). Further research is needed to clarify a possible role for ·OH in ligninolysis.

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