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Nutritional Regulation of Lignin Degradation by *Phanerochaete chrysosporium*

THOMAS W. JEFFRIES,* SUKI CHOI, AND T. KENT KIRK

Forest Products Laboratory, Forest Service, U. S. Department of Agriculture, Madison, Wisconsin 53705

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Previous studies have shown that a lignin-degrading system appears in cultures of the white rot fungus *Phanerochaete chrysosporium* in response to nitrogen starvation, apparently as part of secondary metabolism. We examined the influence of limiting carbohydrate, sulfur, or phosphorus and the effect of varying the concentrations of four trace metals, Ca^{2+}, and Mg^{2+}. Limitation of carbohydrate or sulfur but not limitation of phosphorus triggered ligninolytic activity. When only carbohydrate was limiting, supplementary carbohydrate caused a transient repression of activity. In carbohydrate-limited cultures, ligninolytic activity appeared when the supplied carbohydrate was depleted, and this activity was associated with a decrease in mycelial dry weight. The amount of lignin degraded depended on the amount of carbohydrate provided, which determined the amount of mycelium produced during primary growth. Carbohydrate-limited cultures synthesized only small amounts of the secondary metabolite veratryl alcohol compared with nitrogen-limited cultures. L-Glutamate sharply repressed ligninolytic activity in carbohydrate-starved cultures, but NH_4^+ did not. Ligninolytic activity was also triggered in cultures supplied with 37 μM sulfur as the only limiting nutrient. The balance of trace metals, Mg^{2+}, and Ca^{2+} was important for lignin degradation.

Previous studies have shown that the ligninolytic system of *Phanerochaete chrysosporium* is synthesized in the absence of lignin and in response to nitrogen starvation (10) and that a supplemental growth substrate is required for lignin degradation (12, 13, 16). Primary growth ceases when nitrogen is depleted, and ligninolytic activity subsequently appears after a lag period (10). Therefore, we consider lignin degradation to be a secondary metabolic event (5; P. Fenn and T. K. Kirk, Arch. Microbiol., in press) and have fortuitously discovered that the de novo synthesis of a typical secondary metabolite, veratryl alcohol, begins simultaneously with the onset of ligninolytic activity in nitrogen-starved cultures of this fungus (Fenn and Kirk, in press).

The secondary metabolic state in fungi is perhaps triggered more often by carbohydrate or phosphorus limitation than by nitrogen starvation (3, 4). Therefore, the objective of this study was to examine the effects of carbohydrate and phosphorus limitations, as well as limitation by the fourth major nutrient, sulfur, on the appearance of ligninolytic activity. In addition, because many secondary metabolic events are strongly influenced by trace metal nutrition (21), we also examined this nutritional factor in nitrogen-starved cultures. Our results showed that carbohydrate and sulfur limitations but not phosphorus limitation can initiate ligninolytic activity and that the balance of trace metals influences this activity significantly.

MATERIALS AND METHODS

Lignin. Synthetic ring [*U*-^{14}C]lignin (dehydrogenative polymersate of coniferyl alcohol; specific activity, 10^6 dpm/mg) was prepared and handled as described previously (11, 13). Ligninolytic activity was assayed by collecting $^{14}CO_2$ from cultures to which this synthetic lignin was added (10).

Fungus. *P. chrysosporium* Burds (ME-446) was obtained from the Center for Forest Mycology Research, Forest Products Laboratory, U. S. Department of Agriculture, Madison, Wis., and was maintained at room temperature on 2% malt agar slants. Inocula were prepared as previously described (10, 13).

Culture conditions. The experimental culture conditions used have been described previously (13). Three to five replicates of the organism were grown in 125-ml Erlenmeyer flasks fitted with ports to permit flushing the cultures with gases and to enable the trapping of evolved $^{14}CO_2$. Unless otherwise noted, stationary cultures were flushed with a mixture containing 60% O_2 and 40% N_2 at the time of inoculation and every 1 to 3 days thereafter. This O_2 concentration gave maximum rates of lignin metabolism under the conditions used here (1). Unless otherwise noted, each

flask contained 10 ml of medium having the following composition (per 1,000 ml of distilled water): KH_2PO_4, 0.2 g; $CaCl_2$, 0.01 g; $MgCl_2 \cdot 6\,H_2O$, 0.044 g; mineral salts solution, 1.0 ml; and vitamin solution, 0.5 ml. Nitrogen and sulfur were supplied as NH_4NO_3, L-asparagine, and Na_2SO_4 at various concentrations. Normal levels of each (per 1,000 ml) were 0.05 g of NH_4NO_3, 0.1 g of L-asparagine·H_2O, and 28.8 mg of Na_2SO_4. This provided approximately 0.36 mg of nitrogen (2.58 mM) and 65 μg of sulfur (200 μM) per 10-ml culture. The mineral salts solution was reformulated from a solution used previously (13) in order to replace sulfate with chloride salts. This solution contained the following (per 1,000 ml of distilled water): nitrilotriacetate, 1.5 g; $MnCl_2 \cdot 4H_2O$, 0.48 g; $FeCl_3 \cdot 6H_2O$, 97 mg; $CoCl_2$, 84 mg; $ZnCl_2$, 47 mg; $CuCl_2 \cdot 2H_2O$, 7 mg; $AlCl_3 \cdot 6H_2O$, 10 mg; H_3BO_4, 10 mg; and $Na_2MoO_4 \cdot 2H_2O$, 10 mg. The vitamin solution used has been described previously (13). Cultures were buffered with 0.01 M sodium 2,2-dimethylsuccinate (pH 4.5). Either glucose or cellobiose was used as the carbohydrate source. All constituents were prepared as 10- to 100-fold-concentrated stock solutions, sterilized by filtration through a membrane filter (pore size, 0.45 μm), and stored at 3°C until used.

Analytical procedures. Veratryl alcohol concentrations were determined by gas chromatography as described elsewhere (Fenn and Kirk, in press). Mycelial dry weights were determined either after centrifugation, washing, and drying in tared centrifuge tubes or after filtration and drying on tared filter paper disks. Glucose and cellobiose concentrations were determined by the method of Nelson (18). Total nitrogen concentrations were determined by the micro-Kjeldahl procedure. Lignin degradation was assayed by the release of $^{14}CO_2$ from synthetic ring [*U*-^{14}C]lignin (dehydrogenative polymersate), and degradation was expressed as the cumulative amount of radioactivity released per culture with time.

RESULTS

Carbohydrate limitation. Restricting the carbohydrate source led to the appearance of ligninolytic activity earlier than in nitrogen-starved cultures, and the amount of carbohydrate provided was important. The experiment shown in Fig. 1 was designed to examine the relationships between carbohydrate and nitrogen as nutritional factors in the appearance of ligninolytic activity. When cultures were grown with limiting carbohydrate (4.4 mM cellobiose) and limiting nitrogen (total concentrations, 2.6 mM), ligninolytic activity appeared after day 3 of incubation. When excess nitrogen (7.7 mM) was supplied with a limiting amount of carbohydrate, activity appeared even earlier (after day 2). A similar nitrogen effect was observed with 8.8 mM cellobiose. The onset of ligninolytic activity occurred earlier with 4.4 mM cellobiose than with 8.8 mM cellobiose, but degradation was incomplete in both cases. Increasing the amount of carbohydrate either at the time of

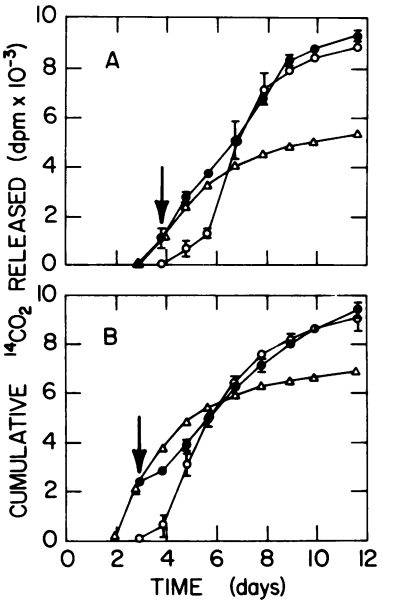

FIG. 1. *Effects of excess nitrogen and supplemental carbohydrate on ligninolytic activity. Cultures received 32,000 dpm of synthetic [^{14}C]lignin, either 4.4 mM (△ and ●) or 8.8 mM (○) cellobiose, and either 2.6 mM (A) or 8.3 mM (B) nitrogen at zero time. On day 3 or 4 (arrows), 4.4 mM additional cellobiose was added to some of the flasks* (●).

inoculation or during the active phase of degradation significantly increased the total $^{14}CO_2$ released. The effect of supplemental carbohydrate varied with the amount of nitrogen present. When nitrogen was limiting, increasing the amount of carbohydrate during lignin metabolism increased the amount of $^{14}CO_2$ released; however, when excess nitrogen was provided, the supplemental carbohydrate resulted in a transient interruption of lignin degradation. Higher concentrations of carbohydrate supplied at the time of inoculation delayed the appearance but increased the extent of ligninolytic activity in cultures containing excess nitrogen.

Cultures in which ligninolytic activity appeared on day 2 in response to carbohydrate starvation contained only trace amounts of the secondary metabolite veratryl alcohol (15).

When other nutrients were present in excess amounts mycelial dry weight decreased as soon as extracellular carbohydrate was depleted, and the appearance of ligninolytic activity was associated with this event (Fig. 2 and 3). The mycelial dry weight, which was proportional to the amount of carbohydrate supplied originally if nitrogen was present in excess, increased linearly until the carbohydrate was exhausted. A rapid decrease in mycelial weight and an in-

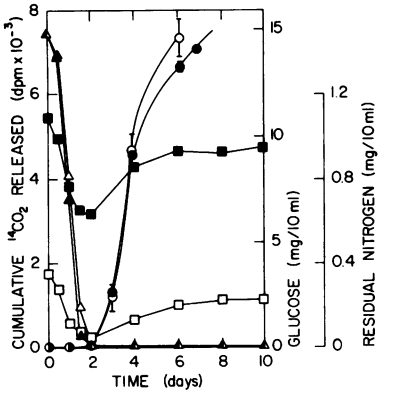

FIG. 2. *Course of lignin degradation as related to $^{14}CO_2$ released (○ and ●), residual nitrogen (□ and ■), and carbohydrate (△ and ▲). Each flask received 10 ml of medium containing 15 mg of glucose, either 0.36 mg (open symbols) or 1.08 mg (closed symbols) of nitrogen, and 48,300 dpm of synthetic [^{14}C]lignin at zero time.*

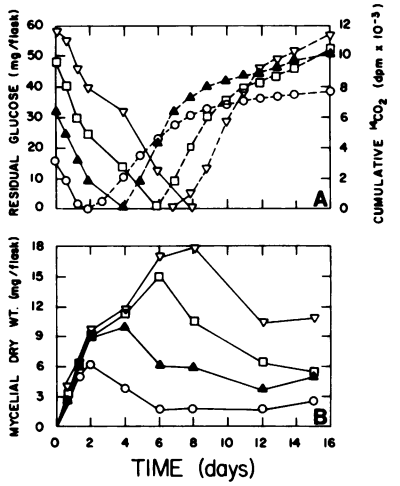

FIG. 3. *Course of ligninolytic activity after depletion of glucose when the carbohydrate/nitrogen ratio was constant. Each culture received 10 ml of basal medium containing varying amounts of glucose and nitrogen and 54,600 dpm of synthetic [^{14}C]lignin at zero time. Initial concentrations were as follows (per 10 ml): 16.2 mg of glucose and 0.3 mg of nitrogen (○), 32.6 mg of glucose and 0.6 mg of nitrogen (▲), 48.6 mg of glucose and 0.9 mg of nitrogen (□), and 58.4 mg of glucose and 1.2 mg of nitrogen (▽). (A) Residual glucose (solid lines) and $^{14}CO_2$ released (dashed lines). (B) Mycelial dry weight.*

crease in extracellular nitrogen occurred immediately thereafter (Fig. 2).

We calculated the ratio of carbohydrate to nitrogen which led to simultaneous depletion of both nutrients. We estimated that, under luxury conditions, the production of 1 mg (dry weight) of mycelium required 2.2 mg of carbohydrate, 54 μg of nitrogen, and 0.5 μg of sulfur. A series of cultures containing increasing but balanced amounts of carbon and nitrogen and excess sulfur were then examined. In all cases, initial growth was linear, and ligninolytic activity did not appear until the carbohydrate was exhausted. Mycelial dry weight also decreased immediately after carbohydrate depletion (Fig. 3). The nitrogen content of the mycelium was essentially constant at 4.2% of the dry weight during growth on the balanced media. Only about 70% of the available nitrogen was present in the mycelium, suggesting either that nitrate was not utilized or that a portion of the assimilated nitrogen was excreted as extracellular enzymes.

In nitrogen-limited cultures containing excess carbohydrate, the addition of nutrient nitrogen represses ligninolytic activity (10), and glutamate is the most repressive of the several nitrogen compounds which have been examined (Fenn and Kirk, in press). Hence, we examined the effect of glutamate on lignin degradation in carbohydrate-starved cultures. After onset of activity in response to carbohydrate starvation, the addition of glutamate suppressed activity markedly. Ammonium chloride added at the same concentration did not affect ligninolytic activity; α-ketoglutaric acid added alone or with NH_4Cl was stimulatory, but this compound did not reverse the suppression by glutamate (Fig. 4).

Phosphorus or sulfur limitation. The early onset of ligninolytic activity was not triggered by phosphorus limitation. In cultures containing a low level of nitrogen (2.6 mM) and excess cellobiose (8.8 mM), limiting phosphorus (0.147 mM; 10% of basal level) delayed the appearance of ligninolytic activity by 1 day and reduced the rate of such activity to approximately 50% of the rate in the controls. In these cultures, lignin degradation appeared as nitrogen became limiting. At lower phosphorus concentrations, growth was restricted severely.

The effects of sulfur concentrations ranging from 17 to 77 μM were examined in cultures that contained excess glucose (56 mM) and low (2.6 mM) or high (7.7 mM) nitrogen levels and were grown under 60% oxygen. We observed no effect of sulfur concentration at the lower nitrogen concentration because nitrogen became limiting before sulfur did. However, in the cultures containing 7.7 mM nitrogen, the earliest onset was observed in cultures receiving less (37 μM) rather than more (77 μM) sulfur. When the sulfur supply was decreased too far below this level (e.g., to 17 μM), the onset was delayed, and ligninolytic activity was restricted because of

poor growth. Figure 5 shows the effects of 20 and 200 μM sulfur in the presence of excess carbohydrate and high or low nitrogen levels. Although all cultures degraded lignin to some extent beginning on day 4 or 5 (the usual time for onset of ligninolytic activity under nitrogen-limited conditions), the sulfur-limited, nitrogen-rich cultures became ligninolytic between days 7 and 8; the sulfur-rich, nitrogen-rich cultures did not. Additional experiments showed an apparent interaction between sulfur concentration and the oxygen atmosphere. The effect of sulfur limitation was demonstrated most readily in cultures growing under 40% O_2–60% N_2. The effects of sulfur limitation were also apparent in the cell yields. With 2.6 mM nitrogen, there was little difference between the dry weights of mycelia obtained with 20 and 200 μM sulfur (11 versus 15 mg); however, with 7.7 mM nitrogen, the lower sulfur concentration restricted growth (11 versus 28 mg).

Inorganic nutrients. We examined the inorganic nutrients Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, and Zn^{2+} (all as chlorides) and MoO_4^{2-} (as the sodium salt) for their effects on lignin degradation by using a three-factor composite design (2) (Fig. 6). Mg^{2+} exerted a strong influence on both growth and lignin degradation, and the basal level was optimal. Mn^{2+} was inhibitory to lignin degradation but not growth at 10 to 100 times the basal concentration, and the highest rate of degradation was obtained when this nutrient was deleted from the medium. Ca^{2+} had little effect alone, but either Ca^{2+} or Mg^{2+} could alleviate the inhibitory effect of Mn^{2+} (Fig. 6). When Fe^{2+}, Zn^{2+}, and MoO_4^{2-} were examined in a similar manner, Fe^{2+} and Zn^{2+} concentrations appeared to be optimal at the basal levels. MoO_4^{2-} had no apparent effect over the concentration range tested (0.001 to 100 times the basal level). These three elements did exhibit some interactive effects, and the initial rate of lignin degradation was about 30% higher when Zn^{2+}, Fe^{2+}, and MoO_4^{2-} were present at about 1/10 their basal levels.

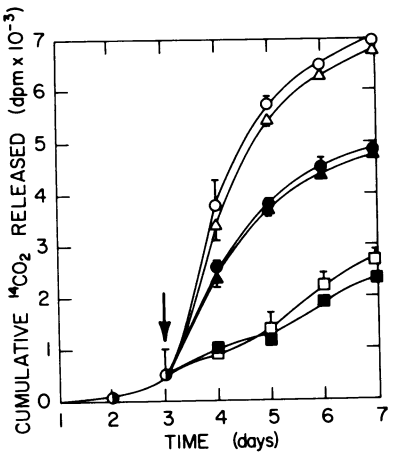

FIG. 4. *Repression of ligninolytic activity by glutamate in carbon-limited cultures. All cultures initially contained 8.33 mM glucose, 5.14 mM nitrogen, and 50,100 dpm of synthetic [^{14}C]lignin. On day 3 (arrow) an additional 28.6 μmol (final concentration, 2.6 mM) of either L-glutamic acid (■), α-ketoglutaric acid plus glutamic acid (□)), NH_4Cl (▲), α-ketoglutaric acid (○), or α-ketoglutaric acid plus NH_4Cl (△) in 1.0 ml was added to each 10-ml culture. Controls (●) received 1.0 ml of sterile distilled water.*

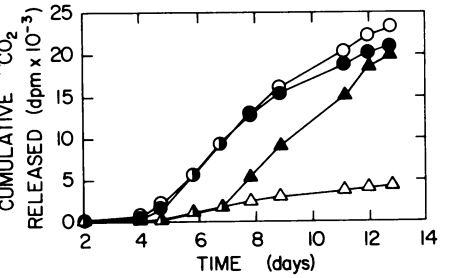

FIG. 5. *Initiation of ligninolytic activity in response to sulfur starvation. Each culture contained 56 mM glucose and 51,200 dpm of synthetic [^{14}C]-lignin plus either 20 μM (closed symbols) or 200 μM (open symbols) sulfur and either 2.57 mM (○ and ●) or 7.7 mM (△ and ▲) nitrogen.*

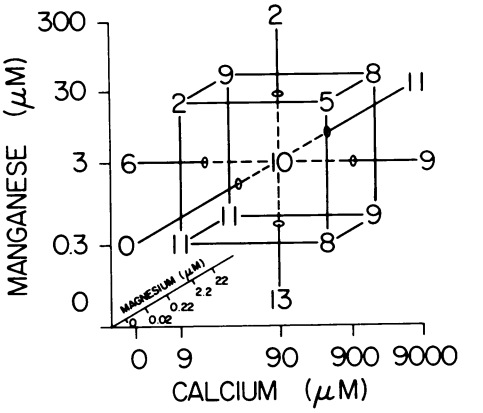

FIG. 6. *Rates of lignin degradation with different concentrations of divalent cations. Calcium, magnesium, and manganese (all as chloride salts) were added at five different concentrations each to a total of 15 different experimental media. All flasks received 56 mM glucose, 2.57 mM nitrogen, and 56,750 dpm of synthetic [^{14}C]lignin. Cultures were flushed with 100% oxygen. Numerical values indicate initial rates of lignin degradation in percent per day, as determined by the collection of $^{14}CO_2$. Each point was from triplicate cultures; standard deviations were all less than 10% of the values shown.*

DISCUSSION

Our findings are in accord with the view that ligninolytic activity is an expression of secondary metabolism in *P. chrysosporium*. This activity is triggered not only by nitrogen limitation (10), but also by carbohydrate limitation and sulfur limitation. Synthesis of the secondary metabolite veratryl alcohol correlated with the occurrence of ligninolytic activity in both carbohydrate- and nitrogen-starved cultures, but it was only weak in the former, perhaps because of a lack of carbohydrate needed for synthesis. Previous studies have shown that the addition of veratryl alcohol to non-ligninolytic cultures before the normal appearance of activity does not induce activity. This metabolite does not seem to have a direct role in regulating lignin degradation, despite a structural similarity between lignin units and the alcohol. Carbon catabolite repression does not seem to be involved in the regulation of lignin metabolism in *P. chrysosporium* (Fenn and Kirk, in press), although it does regulate basidiocarp formation (6).

Our results show that ligninolytic activity is triggered faster in response to carbohydrate depletion from the medium than in response to nitrogen depletion. After extracellular nitrogen is depleted, there is a 1- to 2-day lag period during which substantial changes in intracellular amino acids and protein concentrations occur before establishment of an equilibrium associated with an apparently stable maintenance metabolism (Fenn and Kirk, in press). Ligninolytic activity begins as this physiological equilibrium is reached. A decrease in mycelial dry weight does not occur until later, when carbohydrate is depleted (10, 13). As shown here, cultures depleted of carbohydrate immediately begin to decrease in weight, and lignin degradation is initiated concomitantly. The wood-destroying basidiomycetes are remarkably well adapted to nitrogen-deficient environments and are able to recycle and conserve this nutrient, which is normally present in very low concentrations in their woody substrates (17). In previous nitrogen limitation experiments, ligninolytic activity was correlated with an increased rate of protein turnover (Fenn and Kirk, in press). This finding and the results reported here suggest that nitrogen starvation and carbohydrate starvation prevent further primary growth and lead to the onset of secondary metabolism. The triggering mechanisms relating carbohydrate or nitrogen starvation to mycelial weight loss, protein turnover, and the onset of ligninolytic activity may or may not be the same. This question bears further investigation.

The relationship between lignin degradation and growth substrate metabolism is unclear (5). In our experiments, cellular materials evidently provided the carbon and energy required for lignin degradation in carbohydrate-starved cultures. The amount of lignin degradation depended upon the amount of mycelium present. This observation was in agreement with the previous finding that the extent of lignin degradation depends on the amount of growth substrate supplied (7, 8, 12, 16, 19) and suggested that lignin, like other recalcitrant molecules, is actually cometabolized. However, cometabolism has been defined as non-energy-yielding oxidation of a nongrowth substrate during growth on a utilizable carbon and energy source (9). This restricted definition cannot be applied here because lignin degradation and primary growth do not occur simultaneously.

We can ask whether lignin metabolism can provide carbon and energy for maintenance (secondary) metabolism. That is, can lignin provide sufficient carbon and energy to prevent a loss of dry weight in carbohydrate-limited cultures? We have found that the carbon from lignin can be incorporated, with scrambling, into veratryl alcohol, indicating that the carbon enters central metabolism, as expected (unpublished data). Thus, it seems likely that lignin degradation can provide some carbon, and it probably can also provide energy. On the medium used here but under conditions of nitrogen limitation, cultures of *P. chrysosporium* use approximately 0.3 mg of glucose per mg (dry weight) of mycelium per day for maintenance metabolism (10). When synthetic ring [*U*-^{14}C]lignin is supplied to such cultures in saturating amounts, it is metabolized to CO_2 at a maximum rate of 0.01 mg/mg (dry weight) of mycelium per day (13; unpublished data). Even assuming that only one-half of the lignin metabolized is completely oxidized to CO_2, it is unlikely that the synthetic lignin supports maintenance metabolism. However, other studies in our laboratory have shown that when gravimetric analysis is used to follow the degradation of a natural lignin from *Alnus rubra* (red alder), lignin is removed at a maximum rate of 0.18 mg/mg (dry weight) of mycelium per day (22). To our knowledge, this is the maximum rate reported for lignin biodegradation, and even this rate is probably marginal for supporting maintenance metabolism. Moreover, the yield of metabolic energy from lignin is not yet known, and it may be significantly less than that from glucose.

L-Glutamate, a strong repressor of ligninolytic activity in nitrogen-starved cultures (Fenn and Kirk, in press), was quite effective with carbohydrate-limited cultures. Repression by glutamate might have been expected to reflect car-

bohydrate repression and not nitrogen repression; the deamination product α-ketoglutarate is a good carbon source for *P. chrysosporium* growth (unpublished data), and experiments have shown that the addition of carbohydrate to carbohydrate-starved ligninolytic cultures transiently represses activity when nitrogen is present in excess. However, the addition of α-ketoglutarate actually stimulated ligninolytic activity, so that one would expect a similar stimulation if glutamic acid were deaminated and catabolized. Therefore, the repression observed when glutamate was added cannot be attributed to the carbon content of this compound. These results provide further evidence of a more central role for glutamate metabolism in regulating secondary metabolism, including lignin degradation, in *P. chrysosporium* (Fenn and Kirk, in press); they also raise the possibility that carbohydrate limitation and nitrogen limitation trigger lignin degradation by similar physiological mechanisms. Stimulation of respiration is obtained when NH_4^+ is added to nitrogen-starved cultures oxidizing [^{14}C]glucose or [^{14}C]succinate to $^{14}CO_2$ (Fenn and Kirk, in press) and is presumed to occur via an increase in the respiratory machinery. Added NH_4^+ represses lignin oxidation in such cultures (10). In our experiments, under carbohydrate-limited conditions, added NH_4^+ did not repress ligninolytic activity. Taken together, these facts indicate that NH_4^+ is not a direct repressor, but rather that this ion could be converted to a repressive species.

The triggering of ligninolytic activity by sulfur limitation was unexpected. To our knowledge, secondary metabolism has not been reported to be initiated in response to limitation by this element, and indeed, Reid (19) concluded that lignin degradation is not triggered by low sulfur concentrations in *P. chrysosporium*. However, Reid did note that excess nitrogen represses ligninolytic activity much less when the sulfur concentration is low (50 μM) than when it is high (250 μM). Also, the low concentration of sulfur used by Reid (50 μM) was higher than the concentration (~20 μM) which triggered ligninolytic activity in our experiments when nitrogen and carbohydrate were present in excess. In addition, Reid used 100% O_2, whereas we found that 40 to 60% O_2 was necessary for clear expression of the sulfur effect. We do not understand the interaction between sulfur and O_2 which we found, but it may simply reflect a more rapid depletion of sulfur at 40 to 60% O_2 than at 100% O_2. Also, because SO_4^{2-} requires reduction before incorporation into amino acids, the lower O_2 concentration could facilitate this activity.

Inorganic phosphate concentrations that permit replicative (primary) growth are inhibitory to various secondary metabolic functions in bacteria and fungi (21). However, we conclude on the basis of previous results from this laboratory (14), from Reid (19), and from the present study that lignin degradation by *P. chrysosporium* is not initiated by phosphate limitation, nor is it inhibited by concentrations that permit vegetative growth.

Weinberg (21) summarized the powerful and often critical influences of trace metals on secondary metabolism in various procaryotes and eucaryotes. He found that Zn^{2+} and Fe^{2+} are usually most influential. Our results indicated that the concentrations of the trace metals, Mg^{2+}, and Ca^{2+} present in our basal medium were apparently about optimum for lignin degradation, although some improvement in the initial rate could be achieved by limiting Zn^{2+}, Fe^{2+}, and Mo^{6+} simultaneously. In our experiments the only metal which exerted a significant influence on lignin degradation was Mn^{2+}, which at 10 times the basal medium concentration was strongly inhibitory. Increasing the concentrations of all of the basal medium ingredients 10 times was not inhibitory (13), and Ca^{2+} or Mg^{2+} countered the effect of Mn^{2+}. Thus, our results show that the balance of these metals is critical. It is worth noting that biosynthesis of the secondary metabolite malformin in *Aspergillus niger* is also inhibited strongly by Mn^{2+} (20).

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