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Decolorization of Several Polymeric Dyes by the Lignin-Degrading Basidiomycete *Phanerochaete chrysosporium*

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The polymeric dyes Poly B-411, Poly R-481, and Poly Y-606 were examined as possible alternatives to the radiolabeled lignin previously used as a substrate in lignin biodegradation assays. Like lignin degradation, the decolorization of these dyes by the white rot basidiomycete *Phanerochaete chrysosporium* occurred during secondary metabolism, was suppressed in cultures grown in the presence of high levels of nitrogen, and was strongly dependent on the oxygen concentration in the cultures. A variety of inhibitors of lignin degradation, including thiourea, azide, and 4′-*O*-methylisoeugenol, also inhibited dye decolorization. A pleiotropic mutant of *P. chrysosporium*, 104-2, lacking phenol oxidase and ligninolytic activity was also not able to decolorize the polymeric dyes, whereas a phenotypic revertant strain, 424-2, regained this capacity. All of these results suggest that the ligninolytic degradation activity of the fungus was responsible for the decolorization of these dyes.

A variety of ^{14}C-radiolabeled and unlabeled substrates have been used to measure ligninolytic activity in *Phanerochaete chrysosporium* and other white rot fungi. Radiolabeled substrates such as synthetic ^{14}C-dehydropolymerizates (13), ^{14}C-natural plant lignins (15), ^{14}C-polyguaiacol (4), and ^{14}C-dimeric (10, 17) and -monomeric (10, 16) model compounds have been utilized in studies of the physiology and genetics of lignin degradation. Assays with these substrates, however, are relatively slow and measure, at least in the case of the polymers, the result of a relatively large number of reactions. In addition, these substrates are not commercially available, and their synthesis requires considerable labor and expertise. Unlabeled dimeric compounds have also been used to elucidate the individual reactions involved in lignin degradation. However, assays with these substrates, although chemically elegant, are cumbersome. They minimally require gas chromatographic analysis and are not suitable for routine assays. Lignin degradation can be conveniently divided into depolymerization reactions, which are extracellular, and the further metabolism of fragments released from the polymer, which is likely to be intracellular. Since the localization of the metabolism of dimeric compounds has not been rigorously examined in previous studies, these compounds may not in all cases be suitable models for lignin depolymerization. The production of ethylene from α-keto-γ-methylthiobutyric acid, a measure of fungal ·OH radical production (8, 14), has been proposed as a measure of ligninolytic activity (8). However, only indirect evidence (14) exists suggesting that fungal ligninolytic activity actually catalyzes this reaction. Finally, none of the assays described above readily lends itself to simple selection processes for the isolation of ligninolytic mutants.

In this report, we describe the decolorization of three polymeric dyes by *P. chrysosporium*. These substrates are readily soluble and stable in the absence of the fungus, are inexpensive, are obtained commercially in high purity, and possess high extinction coefficients and low toxicity toward *P. chrysosporium*. The evidence presented here indicates that these dyes serve as substrates of the fungal lignin degradation system (LDS) and also that they have value for the determination of the onset of secondary metabolism in this organism. Further, these dyes can be used in simple, rapid, and quantitative spectrophotometric assays, which can easily be modified for the selection of lignin degradation mutants of this organism.

MATERIALS AND METHODS

Culture methods. Cultures of *P. chrysosporium* wild-type (ME-446) and mutant strains were maintained on slants as previously described (9). Erlenmeyer flasks (250 ml) containing 25 ml of medium were inoculated with ~5 × 10^7 conidia and incubated at 37°C in high humidity to reduce evaporation. The medium, containing 2% glucose and 1.2 mM $(NH_4)_2$ tartrate and buffered with 20 mM sodium 2,2-dimethylsuccinate, pH 4.5, was as previously described (13). Cultures, which were 6 days old and described as O_2

grown, were allowed to grow for 3 days in air and subsequently were fitted with ports and periodically purged with O_2 as described previously (12, 17). For the time dependence experiments, flasks were purged with O_2 after inoculation and at 2-day intervals thereafter.

For assays on petri dishes, the same medium was used except 1.5% agar and either 1% glucose for the minimal plates or 4% sorbose for the induction of colonies (9) were added. The plates were incubated at 28°C.

Decolorization assay. Dye was added to the liquid medium as an aqueous solution to a final concentration of 0.02% with gentle swirling. Directly after its addition and at the indicated intervals, 0.1 ml of the extracellular culture medium was removed and diluted 10-fold with water. The wavelengths in nanometers used for the absorbance ratios of Poly B-411 (Poly B), Poly R-481 (Poly R), and Poly Y-606 (Poly Y) were A_{593}/A_{483}, A_{513}/A_{362}, and A_{430}/A_{392}, respectively. Visible absorption spectra and routine absorbance measurements of the dyes were determined on a Cary 15 spectrophotometer.

Chemicals. Poly B, Poly R, and Poly Y, synthesized as previously described (5), and thiourea were obtained from Sigma Chemical Co. 4′-*O*-Methylisoeugenol was synthesized as reported earlier (7). All other chemicals were reagent grade.

RESULTS

The chemical structure and visible spectrum of each of the three polymeric dyes used in this study are shown in Fig. 1. Because fungal adsorption, as well as fungal transformation, reduces the intensity of the dyes in solution, it was necessary to measure soluble dye absorbance at two wavelengths. The determination of dye (Poly R) adsorption to the mycelium is shown in Fig. 2. In this experiment, cultures were inactivated with sodium azide before the addition of Poly R. The absorbance at both wavelengths decreased significantly, whereas the absorbance ratio (A_{513}/A_{362}) remained almost constant (Fig. 2). This was predictable because adsorption leads to a proportional decrease in absorbance at all wavelengths, resulting in a minimal change in absorbance ratios. Similar results were obtained with Poly B and Poly Y. The two wavelengths indicated were chosen to produce the greatest change in the absorbance ratio as the dyes were degraded. As the change in the absorbance ratio during dye degradation indicates, decolorization did not occur equally at all wavelengths. This led to a change in color from blue to light brown for Poly B, from red to orange for Poly R, and from yellow to light green-yellow for Poly Y.

Since the LDS in the fungus is expressed as a secondary metabolic process (10, 11, 13), the exhaustion of certain nutrients from the medium leads to a cessation of growth and derepression of the LDS (11, 13). The effect of nutrient nitrogen concentration on the fungal decolorization of the polymeric dyes is shown in Fig. 3.

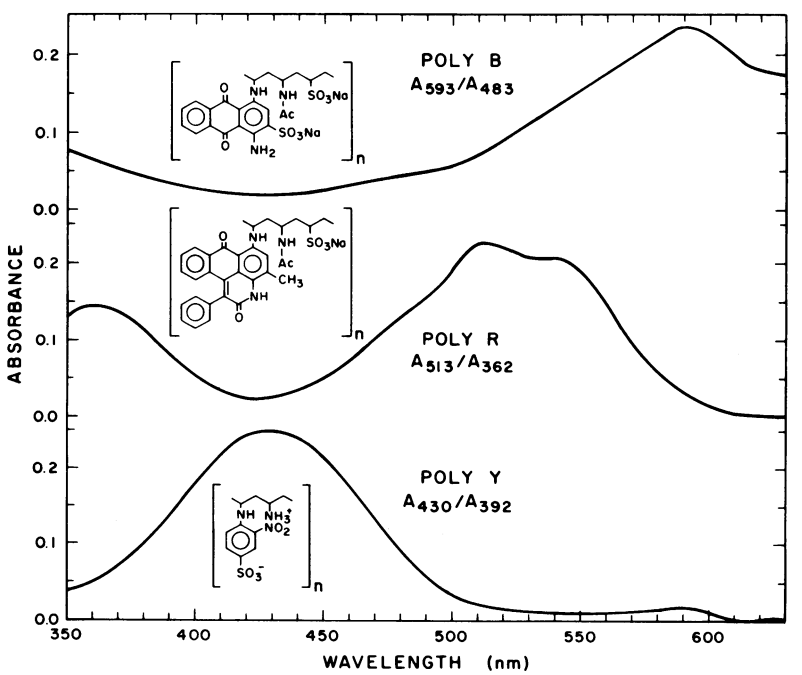

FIG. 1. Chemical structure and visible spectra of the polymeric dyes Poly B, Poly R, and Poly Y. The spectra of the dyes (0.002%) in sodium 2,2-dimethylsuccinate, pH 4.5, were taken with a Cary 15 spectrophotometer.

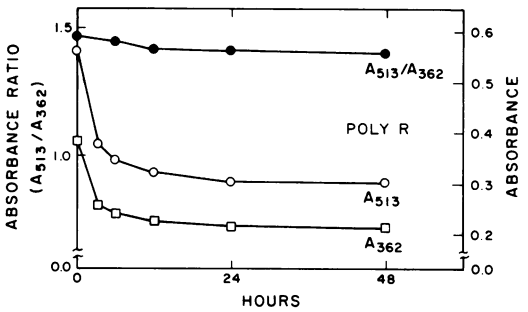

FIG. 2. A_{513} (○), A_{362} (□), and A_{513}/A_{362} ratio (●) of Poly R in culture filtrates. Cultures were 100% O_2 grown, after which they were inactivated with N_3^-, and the dye was added as described in the text. Aliquots (0.1 ml) of medium were removed at the times indicated and diluted 10-fold with water.

Cells grown in high-N [12 mM $(NH_4)_2$ tartrate] medium were less than 5% as effective as cells grown in low-N [1.2 mM $(NH_4)_2$ tartrate] medium at decolorizing all three dyes. The repressive effect of high nitrogen on the decolorization of these dyes was similar to that described for the effect of nitrogen on the metabolism of ^{14}C-lignin to $^{14}CO_2$ (13).

The temporal dependence of the decolorization of Poly B and Poly R by cultures of *P. chrysosporium* ME-466 is shown in Fig. 4. Decolorization started after an initial lag of approximately 3 days and continued through the 14-day course of the experiment. Growth of the organism in stationary cultures as measured by dry weight (data not shown) was essentially complete after 2 days, confirming earlier experimentation (13, 17). Poly B decolorization reached a peak at about day 8 and then declined. Ligninolytic activity in this organism peaks at approximately day 6 (11). Of note, Poly R decolorization, like the fungal decolorization of Kraft E_1 effluent (6), remained optimal over a longer period of time. The temporal dependence of Poly Y decolorization was not examined because of the significantly slower rate of decolorization for this dye.

Several studies have described the dependence of lignin (2, 13) and model compound metabolism (16, 17) on the oxygen concentration of the fungal cultures. The effect of the oxygen concentration of the cultures on Poly B and Poly Y decolorization is shown in Fig. 5. With the dye Poly B, the fastest rate of decolorization (Δ absorbance ratio per hour = 0.62) occurred in cultures purged with 100% O_2, whereas the slowest rate of decolorization (Δ absorbance ratio per hour = 0.16) occurred in cultures purged with air. Intermediate rates were observed in cultures grown under oxygen and purged with air after the dye addition and in cultures grown in air and purged with oxygen after the dye addition. Essentially identical results were obtained with Poly R (data not shown). Similar, but more pronounced, results were observed when Poly Y was used. The latter was not appreciably decolorized in cultures under an air atmosphere. Cultures grown in O_2 and purged with air after the addition of the dye showed an intermediate rate of decolorization. The activation by 100% O_2 ($O_2 \rightarrow O_2$ versus $O_2 \rightarrow$ air) of the dye-degrading system(s) was approximately twofold with Poly B and threefold with Poly Y. In addition, O_2-grown cultures continued to degrade Poly Y long after they were transferred to an air atmosphere, suggesting that the induced condition is permanent.

The effect of various lignin biodegradation inhibitors (8, 14) on the fungal decolorization of

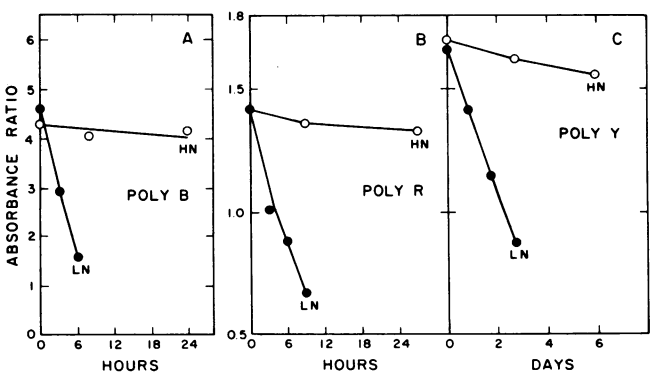

FIG. 3. Effect of nutrient nitrogen on dye decolorization. The dyes were added to duplicate 6-day-old cultures previously grown in the presence of 1.2 mM $(NH_4)_2$ tartrate (low N, LN) (●) or 12 mM $(NH_4)_2$ tartrate (high N, HN) (○). As indicated, samples were removed and diluted, and the absorbance ratios were measured as described in the text.

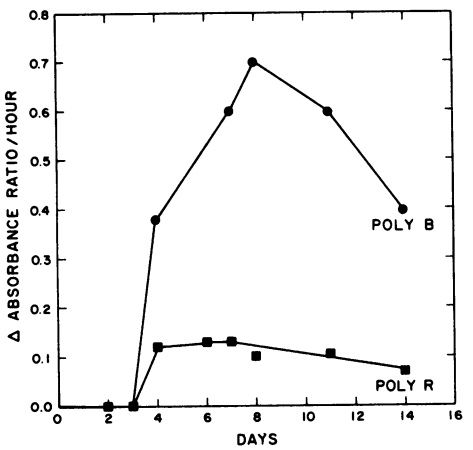

FIG. 4. Dependence of dye decolorization on culture age. To measure dye decolorization, Poly B (●) or Poly R (■) was added to individual flasks of O_2-grown cultures on days 2, 3, 4, 6, etc., as indicated, and the initial rate of decolorization was determined as described in the text.

Poly B, Poly R, and Poly Y is shown in Table 1. Sodium azide (1 mM) and thiourea (5 mM), potent scavengers of hydroxyl radicals (1), effectively inhibited dye decolorization. This was consistent with previous results obtained for lignin biodegradation (8, 14). Urea (5 mM), which served as a control for thiourea, was not an inhibitor. Sufficient preincubation with urea, however, resulted in repression related to the repression of secondary metabolism. Cyanide acted as a temporary inhibitor, producing 80 to 95% inhibition for ~6 h with Poly B and Poly R, and then rapidly lost effectiveness. Chemically synthesized lignin (synthetic ^{14}C-dehydropolymerizate) (10) and 4′-*O*-methylisoeugenol probably acted as competitive inhibitors. These two agents have been previously shown to inhibit lignin biodegradation and the production of ethylene from methional by this organism (14). In contrast, mannitol, another (although less effective) hydroxyl radical scavenger (1), had no effect on dye decolorization at a concentration of 0.1 M.

The decolorization of Poly B, Poly R, and Poly Y by the wild-type and two mutant strains of *P. chrysosporium* is shown in Fig. 6. The lignin degradation mutant 104-2 (10) was unable to decolorize any of the dyes, whereas a phenotypic revertant strain, 424-2, which partially regained the capacity to degrade lignin (10) also partially regained the capacity to degrade the dyes. Similar results were obtained with the wild-type and mutant strains on solid medium. With solid medium containing colony-inducing agents and Poly B or Poly R, the colonies produced a clear halo in the colored background, allowing the possible visual selection of new mutants of lignin biodegradation.

DISCUSSION

In this paper we have examined the degradation of three polymeric dyes by *P. chrysosporium*. Throughout, we have attempted to correlate dye decolorization and the ligninolytic activity by *P. chrysosporium* by comparing the effect of various physiological parameters, mutations, and inhibitors on both processes. Dye decolorization, like ligninolytic activity, appears to be a secondary metabolic process. It was repressed by nutrient nitrogen and only occurred after the nitrogen in the cultures had been consumed. Dye decolorization paralleled lignin degradation temporally; the rate of dye decolorization reached an optimum in ~8-day-old cultures. Further, the amount of dye decolorization in a secondary metabolic mutant (10) and a phenotypic revertant strain paralleled the capacity of these strains to degrade lignin. All of these results indicate that dye decolorization is a secondary metabolic process and suggest that the LDS or a part of it is responsible for decolorization.

As with the LDS (2), oxygen appears both to induce fungal dye decolorization and to activate the catalytic system when it is already present. An example of this is in the case of Poly B or Poly R. The transfer of cultures from a 100% O_2 atmosphere to an air atmosphere after the addition of the dye reduced the rate of decolorization by ~50%. In addition, the transfer of cultures from an air atmosphere to a 100% O_2 atmosphere increased the rate of decolorization by three- to fourfold. Similar results are obtained when ^{14}C-lignins (2, 13) and ^{14}C-lignin model compounds (16, 17) are used as substrates. This also suggests that the LDS is responsible for dye decolorization. The effect of various lignin degradation inhibitors on dye decolorization was examined to test this hypothesis. Azide and thiourea, potent ·OH scavengers (1) and inhibitors of lignin degradation, were very effective at inhibiting dye decolorization, although mannitol, a weaker inhibitor of lignin degradation, appeared to have no effect on dye decolorization. The strong inhibitory effect of azide on respiration may also contribute to its inhibition of dye degradation. 4′-*O*-Methylisoeugenol and lignin were also effective as inhibitors of dye decolorization and may have been acting as competitive inhibitors. KCN (1 mM) was also an effective inhibitor, but its effect only lasted ~6 h. It is not clear whether this transitory effect was caused by a loss through purging with oxygen, cyanide-insensitive respiration, or fun-

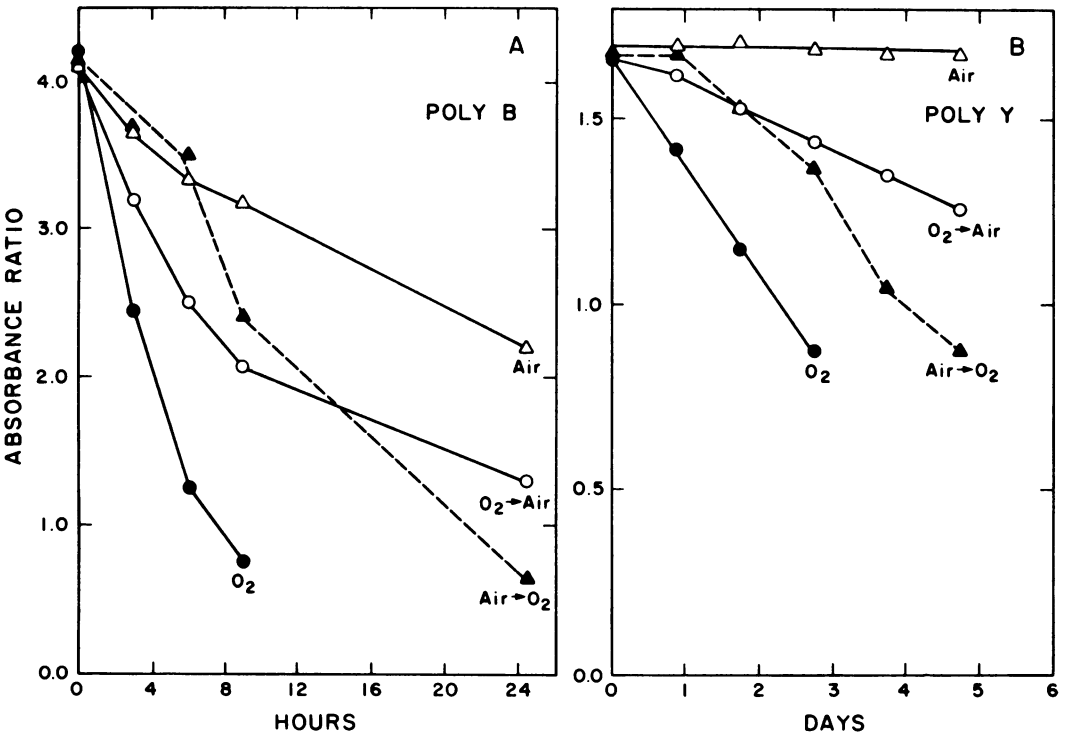

FIG. 5. Effect of oxygen concentration on dye decolorization. Six-day-old O_2-grown or air-grown cultures were utilized as indicated. Decolorization of Poly B (A) and Poly Y (B) was examined. The following regimes were utilized: ●, O_2-grown cultures, O_2 after the addition of dye; ○, O_2-grown cultures, transferred to air after the addition of dye; △, air-grown cultures, air after the addition of dye; ▲, air-grown cultures, transferred to O_2 after the addition of dye.

gal transformation of the cyanide, although the latter appears most likely.

We have examined the use of these dyes as possible substrates for the LDS for several reasons. The dyes allow a simple, quantitative spectrophotometric assay. Due to the rapid spectral changes incurred, this assay can be performed more rapidly than previous assays based on radiolabeled substrates. Second, these attributes may allow the use of the dyes for the isolation of possible enzymes or other catalysts involved in lignin degradation. Finally, the dyes provide an inexpensive and effective method for the selection of fungal mutants with alterations in lignin metabolism in addition to providing a method for the possible screening of additional organisms capable of degrading lignin. The particular dyes described in this paper were chosen because they are polymeric, inexpensive, and commercially available in high purity. Their polymeric nature assures that at least the initial steps in their degradation are extracellular. We have found, recently, that many dyes, including blue dextran, Coomassie brilliant blue, methylene blue, and methyl red are also degraded by ligninolytic cultures of *P. chrysosporium*. This suggests the possible use of this organism for wastewater treatment in dye-related industries (3).

The specific catalysts and reactions involved

TABLE 1. Effect of various inhibitors on the fungal decolorization of Poly B, Poly R, and Poly Y[a]

Inhibitor	% Inhibition		
	Poly B	Poly R	Poly Y
Standard condition	0	0	0
NaN_3 (1 mM)	100	100	100
Thiourea (5 mM)	100	100	100
Urea (5 mM)	0	0	0
Cyanide (1 mM)	95	80	65
Lignin (10 mg per flask)	45	40	ND[b]
4′-*O*-Methylisoeugenol (1 mM)	30	35	ND
Mannitol (0.1 M)	0	0	0

[a] Cultures were incubated under 100% O_2 for 6 days, after which inhibitors and dyes were simultaneously added. Dye decolorization was measured at 3-, 6-, and 9-h intervals for Poly B and Poly R and at 24-h intervals for Poly Y as described in the text.

[b] ND, Not determined.

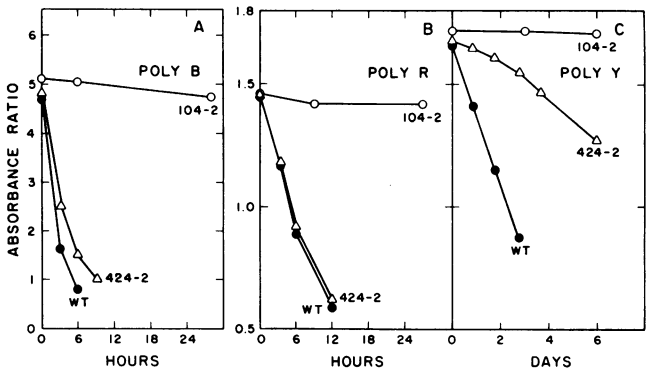

FIG. 6. Dye decolorization by several mutant strains of *P. chrysosporium*. Poly B (A), Poly R (B), or Poly Y (C) was added to 6-day-old O_2-grown duplicate cultures of the wild-type (●), 104-2 mutant (○), and 424-2 revertant (△) strains. Samples were removed at the times indicated and diluted, and the absorbance ratios were measured as described in the text.

in dye degradation by the fungus are unknown. Observed differences between the fungal degradation of lignin and the dyes may be more related to physical, rather than chemical, properties. Lignin is a three-dimensional, heterogeneous, hydrophobic polymer at physiological pH which appears to be degraded only after strong specific binding to the fungus (M. G. S. Chua, S. Choi, and T. K. Kirk, Holzforschung, in press). These polymeric dyes are linear, charged, and hydrophilic. Binding of the dyes appears to be nonspecific and related to the net charge on the molecule. Despite these differences, the results described here suggest that there is probably a single catalytic system responsible for the degradation of both lignin and the dyes but that specific reactions are likely to be dependent on the chemical and physical properties of the substrates. All of the dyes are rapidly bleached by the hydroxyl radical (·OH) produced via Fenton reagent or via UV light-H_2O_2 (unpublished observation). This is consistent with the possible involvement of ·OH in the fungal degradation of lignin as described earlier (8, 14). Further research on the use of these dyes for the isolation and characterization of the catalytic system responsible for their degradation is planned.

ACKNOWLEDGMENT

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