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Biodegradation of Azo and Heterocyclic Dyes by *Phanerochaete chrysosporium*

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Biodegradation of Orange II, Tropaeolin O, Congo Red, and Azure B in cultures of the white rot fungus, *Phanerochaete chrysosporium*, was demonstrated by decolorization of the culture medium, the extent of which was determined by monitoring the decrease in absorbance at or near the wavelength maximum for each dye. Metabolite formation was also monitored. Decolorization of these dyes was most extensive in ligninolytic cultures, but substantial decolorization also occurred in nonligninolytic cultures. Incubation with crude lignin peroxidase resulted in decolorization of Azure B, Orange II, and Tropaeolin O but not Congo Red, indicating that lignin peroxidase is not required in the initial step of Congo Red degradation.

Dyes are released into the environment in industrial effluents from two major sources, the textile and the dyestuff industries (12, 13). A necessary criterion for the use of these dyes is that they must be highly stable in light and during washing. They must also be resistant to microbial attack. Therefore, they are not readily degradable and are typically not removed from water by conventional wastewater treatment systems (1, 16). While most dyes are not particularly toxic (2, 12), they are considered to be a pollution problem.

Azo dyes are the largest class of dyes with the greatest variety of colors (1, 9, 12). They also exhibit great structural variety, so, as a group, they are not uniformly susceptible to microbial attack (12). Azo dyes are not typically degraded under aerobic conditions (1, 14, 15, 20); however, under anaerobic conditions, the azo linkage can be reduced to form aromatic amines which are colorless but which can be toxic and carcinogenic (12, 20).

The lignin-degrading system of the white rot fungus, *Phanerochaete chrysosporium*, is able to degrade a wide range of structurally diverse organic pollutants (3). Although azo dyes are generally considered to be nonbiodegradable under aerobic conditions, we hypothesized that the nonspecific nature of the lignin-degrading system could reasonably be expected to be effective in degrading these dyes.

In this report, we demonstrate the aerobic biodegradation of three azo dyes, Tropaeolin O, Congo Red, and Orange II, and one heterocyclic dye, Azure B (see Fig. 1), by the white rot fungus, *P. chrysosporium*.

MATERIALS AND METHODS

Abbreviations. The trivial names of all dyes were used for convenience. The following names of these dyes are those recognized by the Chemical Abstracts Service: Azure B, 3-(dimethylamino)-7-(methylamino)-phenothiazin-5-ium chloride; Tropaeoline O, 4-[(2,4-dihydroxyphenyl)azo]benzenesulfonic acid monosodium salt; Orange II, 4-[(2-hydroxy-1-naphthalenyl)azo]benzenesulfonic acid monosodium salt; Congo Red, 3,3′-[[1,1′ biphenyl]-4,4′-diylbis-(azo)]bis[4-amino-1-naphthalenesulfonic acid] disodium salt. The structures of these dyes are shown in Fig. 1.

Chemicals. All dyes were purchased from Aldrich Chemical Co. (Milwaukee, Wis.).

Microorganism. *P. chrysosporium* BKM-F-1767 was obtained from the U.S. Department of Agriculture Forest Products Laboratory (Madison, Wis.) and was maintained on malt agar slants at room temperature. Subcultures were routinely made every 30 to 60 days.

Culture conditions. Nutrient nitrogen-limited cultures of *P. chrysosporium* were incubated at 39°C in liquid culture medium similar to that previously described (6, 10) in 250-ml Wheaton bottles fitted with Teflon-lined caps. This medium consists of 56 mM glucose, 1.2 mM ammonium tartrate, 1.5 mM veratryl alcohol, mineral salts, and thiamine (1 mg/liter) in 20 mM dimethylsuccinate (sodium) buffer (pH 4.5). Nitrogen-sufficient cultures were grown in the same medium, except the ammonium tartrate concentration was increased to 12 mM. Cultures were established by inoculating the media with spores as described previously (10). The cultures were grown in air during the first 3 days of incubation and were then gently flushed with O_2 on day 3 and every 3 days thereafter.

Biodegradation. Nutrient nitrogen-limited and nitrogen-sufficient cultures of *P. chrysosporium* were allowed to grow for 6 days as described above. On day 6, one of the dyes was added to each culture. All dyes were prepared in water at 1.0 mg/ml and were added to give final concentrations of 16, 63, 57, and 76 μM for Azure B, Tropaeolin O, Orange II, and Congo Red, respectively. Dye disappearance was determined spectrophotometrically by monitoring the absorbance at or near the wavelength maximum for each dye at specified intervals for 5 days. Results are reported as the mean amount of decolorization for four replicate cultures.

After 5 days of incubation with *P. chrysosporium*, some of the dyes remained adsorbed to the fungal mycelia. In an attempt to solubilize any bound dye, the mycelial mat was homogenized in 10 ml of methanol in a Potter-Elvehjem tissue homogenizer. The homogenate was centrifuged at 2,000 × *g* for 5 min, and the mycelial pellet was suspended in an additional 5 ml of methanol and recentrifuged. The two resulting supernatants were combined. The absorbance of the supernatant was then determined, and the amount of dye associated with the mycelial mat was calculated.

Metabolite formation was examined by thin-layer chromatography (TLC) on precoated Silica Gel 60 G F-254 plates (20 by 20 cm; thickness, 250 μm) (E. Merck AG, Darmstadt, Federal Republic of Germany) in a solvent system of propanol:water:acetic acid (90:9:1, vol/vol/vol). The dyes, at the concentrations given above, were incubated with both nu-

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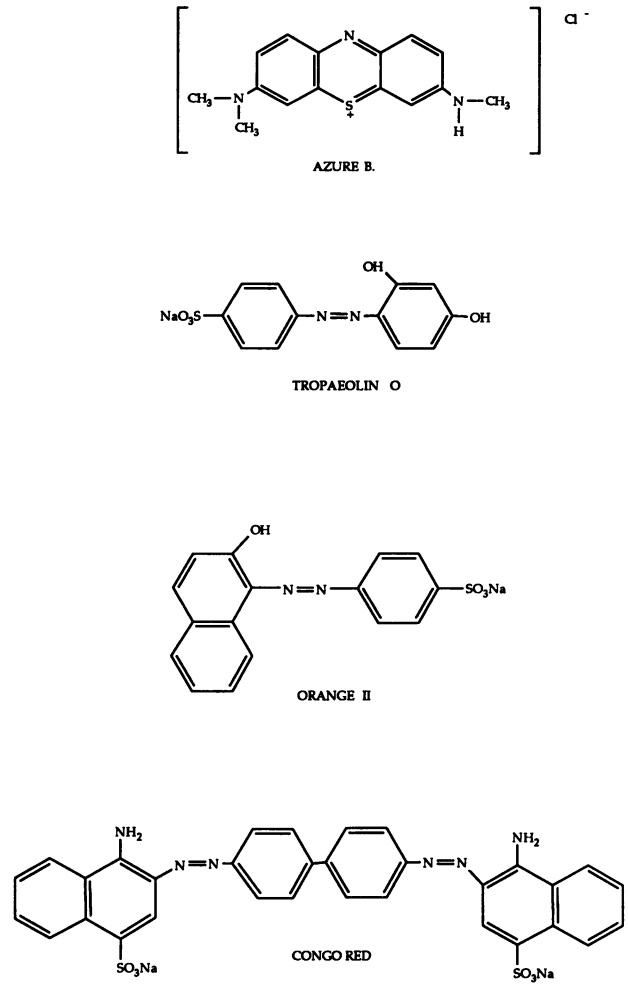

FIG. 1. Structures of Azure B, Tropaeolin O, Orange II, and Congo Red.

trient nitrogen-limited and nitrogen-sufficient 6-day-old fungal cultures for 4 h, except for nitrogen-limited cultures of Azure B and Tropaeolin O, which were incubated for 15 and 30 min, respectively. The extracellular fluid containing the partially decolorized dye was removed, and an equal volume of methanol was added. The dye was concentrated to less than 0.25 ml by rotary evaporation prior to TLC.

Preparation of lignin peroxidases. Lignin peroxidase used in this study was purified from the extracellular fluid of 150-ml agitated cultures of *P. chrysosporium* BKM-F-1767. The culture medium consisted of 56 mM glucose, 1.2 mM ammonium tartrate, mineral salts, thiamine (1 mg/liter), 1.5 mM veratryl alcohol, and Tween 80 (0.05%) in 10 mM sodium acetate buffer (pH 5.0). The fungal cultures were inoculated with a spore suspension (10% total volume at an optical density at 650 nm of 0.75) and were grown in 500-ml Erlenmeyer flasks at 39°C and at 200 rpm (Series 25 Incubator Shaker; New Brunswick Scientific Co., Inc., Edison, N.J.) in air for 6 days. The extracellular fluid was separated from the mycelia by filtration through glass wool and was concentrated about 10-fold by using a Minitan concentrator (Millipore Corp., Bedford, Mass.) with a molecular exclusion limit of 10,000. The concentrate was frozen overnight, thawed, and centrifuged (10,000 × *g* for 15 min) to remove mucilaginous material. The fluid was further concentrated by using an Amicon concentrator (Amicon Corp., Lexington, Mass.) with a molecular exclusion limit of 10,000 and was then dialyzed overnight against 10 mM sodium acetate (pH 6.0). This material is subsequently referred to as crude lignin peroxidase. Individual isozymes were isolated by fast protein liquid chromatography on a Mono Q HR 10/10 column (Pharmacia, Uppsala, Sweden) as previously described (4, 19). Peak fractions were collected, and purity was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and nondenaturing isoelectric focusing.

Enzyme assays. Lignin peroxidase activity was determined by the method of Tien and Kirk (18). One unit of enzyme activity oxidized 1 μmol of veratryl alcohol in 1 min at room temperature. Dye oxidation was monitored by scanning from 700 to 200 nm and calculating the change in absorbance at the visible wavelength maximum of each dye. The reaction mixture consisted of 32 μM dye, 20 μg of crude lignin peroxidase (concentrated extracellular fluid), and 200 μM H_2O_2 in 50 mM sodium tartrate (pH 4.5) in a total volume of 1 ml. The reaction was initiated by the addition of H_2O_2 and scanned at 1, 4, 7, 10, and 20 min after initiation. Metabolite formation after incubation with crude lignin peroxidase was examined by TLC as described above. Azure B and the azo dyes were incubated for 4 and 20 min, respectively, in the above reaction mixture prior to the addition of methanol and subsequent rotary evaporation and TLC.

RESULTS

Dye decolorization by cultures of *P. chrysosporium*. Extensive biodegradation of all four dyes by cultures of *P. chrysosporium* was demonstrated, as evidenced by the decrease in absorbance of the culture medium (Table 1). In all cases, most of the color loss occurred within the first 6 h. Within 24 h, over 90% of the initial color from all four dyes had disappeared from the culture medium of the nitrogen-limited cultures (i.e., the ligninolytic cultures). With continued incubation for a total of 48 h, the absorbance decreased to undetectable levels for Azure B. Orange II incubated with

TABLE 1. Dye decolorization by cultures of *P. chrysosporium*

Dye	Absorption maximum (nm)	Initial concn (μM)	% Decolorization after:									
			1 h		6 h		24 h		48 h		5 days	
			NL[a]	NS[b]	NL[a]	NS[b]	NL[a]	NS[b]	NL[a]	NS[b]	NL[a]	NS[b]
Azure B	647	16	90 ± 3	32 ± 4	97 ± 1	53 ± 14	100 ± 1	59 ± 18	100 ± 0	60 ± 18	100 ± 0	63 ± 16
Tropaeolin O	395	63	78 ± 7	58 ± 5	91 ± 1	76 ± 6	95 ± 1	82 ± 5	96 ± 1	85 ± 4	96 ± 1	87 ± 2
Orange II	489	57	57 ± 11	46 ± 8	67 ± 9	69 ± 17	91 ± 5	81 ± 18	98 ± 1	87 ± 14	100 ± 0	93 ± 6
Congo Red	506	76	58 ± 7	55 ± 5	87 ± 3	83 ± 3	93 ± 1	91 ± 1	95 ± 1	93 ± 1	97 ± 1	93 ± 1

[a] Cultures grown in nitrogen-limited media.
[b] Cultures grown in nitrogen-sufficient media.

nitrogen-limited cultures was completely decolorized after 5 days of incubation, and Tropaeolin O was completely decolorized after 12 days of incubation (data not shown). The rate of decolorization was slower for the nitrogen-sufficient cultures, and complete loss of color from these culture fluids did not occur for any dye even after 12 days of incubation (data not shown). No decrease in absorbance was observed in sterile control cultures.

While loss of over 95% of the dye from the culture fluid from the nitrogen-limited cultures occurred within 48 h, some of the dye appeared to be bound to the mycelium. This phenomenon was even more pronounced in the nitrogen-sufficient cultures. Spectrophotometric examination of methanol extracts of the fungal mats after 5 days of incubation with a dye showed that the mycelia of nitrogen-limited cultures incubated with Azure B, Tropaeolin O, and Orange II contained no dye. However, 6% of the Congo Red initially added to the nitrogen-limited cultures could be extracted from the mycelia with methanol. Furthermore, nitrogen-limited cultures containing Congo Red were not completely decolorized even after 12 days of incubation. The nonligninolytic nitrogen-sufficient cultures had greater amounts of the dyes adsorbed to the fungal mycelia after 5 days of incubation: 14% for Azure B, 18% for Tropaeolin O, 11% for Orange II, and 49% for Congo Red. Congo Red could not be completely extracted from the fungal mycelia of the nitrogen-sufficient cultures with methanol and repeated extractions, and homogenization with other solvents, including methylene chloride, ethyl acetate, acetone, ethanol, and water, could not solubilize any additional dye. At the end of these extensive extractions, the mycelia remained dark orange-red in color.

On day 6, when the dyes were added to the cultures, the pH was found to be 4.2 ± 0.1 for nitrogen-limited cultures and 3.7 ± 0.1 for nitrogen-sufficient cultures. On day 12, when the incubations were terminated, the pHs of the nitrogen-limited and nitrogen-sufficient cultures were found to be 4.2 ± 0.1 and 3.9 ± 0.1, respectively.

To ensure that decolorization was not simply a function of pH change, the effect of pH on the visible absorption was assayed between pH 3.5 and 5.0 in 20 mM sodium dimethylsuccinate buffer. The visible absorption spectra of Tropaeolin O and Orange II were unaffected by pH over this pH range. The A_{647} of Azure B increased by 15% as the pH was decreased from 5.0 to 3.5, and the A_{506} of Congo Red decreased by 50% as the pH was decreased from 5 to 3.5. It should be mentioned that over this pH range, the absorbance maximum of Congo Red shifted from 492 nm (pH 5.0) to 566 nm (pH 3.5). For Congo Red, disappearance in culture was monitored at 506 nm as a matter of convenience, since the small changes in culture pH that were measured in these experiments had a minimal effect on absorbance at this wavelength. No pH change was observed during the time the dyes were incubated with nitrogen-limited cultures, and a small increase of only 0.2 pH unit was observed when dyes were incubated with nitrogen-sufficient cultures. Thus, these controls confirm that decolorization cannot be due to a pH change.

In a parallel experiment, cultures grown from the same spore suspension and in the same medium were assayed daily for lignin peroxidase activity (Fig. 2). Lignin peroxidase activity, measured in terms of veratryl alcohol oxidation, was first observed on day 4 in the nitrogen-limited cultures and peaked on day 6 at 137 U/liter. These cultures remained ligninolytic at least through day 12. The nitrogen-sufficient cultures showed no lignin peroxidase activity until day 9, when only one of the four replicate cultures secreted lignin peroxidase into the extracellular fluid, probably in response to carbon starvation.

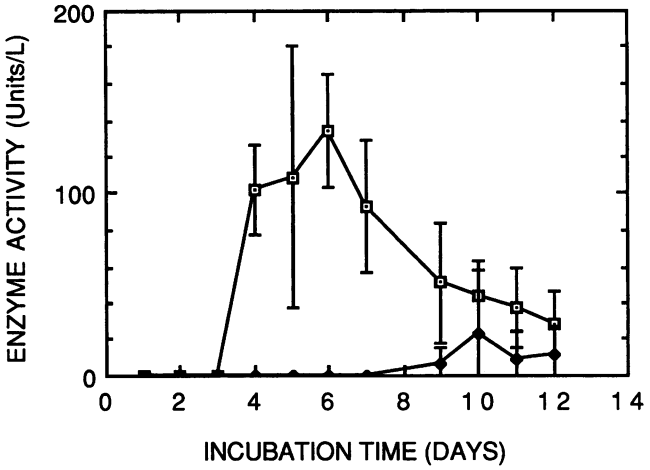

FIG. 2. Lignin peroxidase activity of nitrogen-limited (⊡) and nitrogen-sufficient (◆) cultures of *P. chrysosporium*. Assay conditions are as described in Materials and Methods.

TLC was used to assess metabolite formation after the dyes were incubated in cultures of *P. chrysosporium*. Colored metabolites were observed for Azure B and Tropaeolin O in both nitrogen-limited and nitrogen-sufficient cultures, and a Congo Red metabolite was found only in nitrogen-sufficient cultures (Table 2). No colored metabolites of Orange II were observed.

Dye degradation by lignin peroxidase. Crude lignin peroxidase from nitrogen-limited cultures of *P. chrysosporium* was able to partially decolorize three of the four dyes (Azure B, Tropaeolin O, and Orange II) within the 20-min incubation period (Fig. 3). In addition to the decrease in absorbance, Azure B also underwent a hypsochromic shift from 647 to 630 nm after 1 min. Upon continued incubation, the visible absorption spectrum decayed to a nondescript peak having a broad absorbance maximum from 564 to 647 nm. Tropaeolin O showed a bathochromic shift in absorbance maximum from 408 to 415 nm. The absorbance of Congo Red did not decrease upon incubation with the crude lignin peroxidase, nor was there a shift in the wavelength of maximum absorbance. Similar results were obtained upon incubation of the dyes with isolated isozymes, H1, H2, H8, and H10, of lignin peroxidase (data not shown).

As a control for the possibility that the decrease in absorbance was due to a nonbiological oxidation rather than

TABLE 2. TLC R_f values of visible metabolites of Azure B, Congo Red, Orange II, and Tropaeolin O after incubation with cultures of *P. chrysosporium*[a]

Dye (R_f)	R_f values of visible metabolites[b]	
	Nitrogen-limited cultures	Nitrogen-sufficient cultures
Azure B (0.09)	0.12, 0.28, 0.31, 0.35	0.19, 0.28, 0.56
Tropaeolin O (0.69)	0.0, 0.12	0.48
Orange II (0.64)	ND	ND
Congo Red (0.22)	ND	0.48

[a] The solvent system was propanol:water:glacial acetic acid (90:9:1, vol/vol/vol).
[b] ND, None detected.

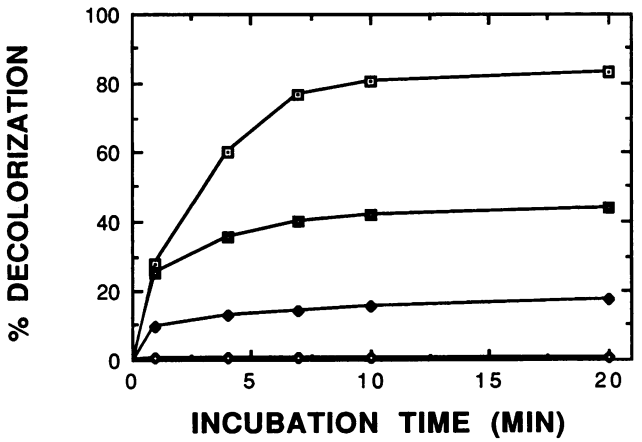

FIG. 3. Dye biodegradation by lignin peroxidase isolated from *P. chrysosporium*, as measured by a decrease in absorbance at the wavelength maximum for the following dyes: Azure B (⊡), Tropaeolin O (◆), Orange II (■), and Congo Red (◇). Incubations were as described in Materials and Methods with 32 μM dye. Reactions were initiated by the addition of H_2O_2.

to biodegradation, the dyes were incubated with 200 μM H_2O_2. The three azo dyes showed no change in absorbance when incubated with H_2O_2. However, Azure B showed a 9% decrease in absorbance after a 20-min incubation. A spectral shift in the absorbance maxima was not observed for any dye upon incubation with H_2O_2.

A number of metabolites were also formed upon incubation of the dyes with crude lignin peroxidase (Table 3). In addition to having colored metabolites, Azure B and Orange II each had a metabolite that could be visualized under UV radiation. No metabolites of Tropaeolin O or Congo Red were detected by TLC.

DISCUSSION

Previously, studies of azo dyes have concluded that, while readily reduced under anaerobic conditions, these dyes could not be degraded aerobically (1, 15, 20). Attempts to develop aerobic bacterial strains capable of degrading azo dyes resulted in organisms that showed strict specificity toward the single azo dye to which the organism had been adapted (11, 20). Because these bacterial strains are so specific, they are not considered to be of any practical value for degrading the mixtures of azo dyes that occur in wastewater. We have shown that substantial biodegradation of three azo dyes occurred in the presence of the white rot fungus, *P. chrysosporium*. The effectiveness of *P. chrysosporium* in decolorizing these dyes varies depending on the structure and complexity of the dye and whether or not the fungus is in idiophase. Of the four dyes examined, the heterocyclic dye, Azure B, was the one most readily degraded by both the fungus and lignin peroxidase, while Congo Red, the largest and most complex of the four examined, was not completely decolorized by the fungus even after 12 days of incubation.

TABLE 3. TLC R_f values of the metabolites of Azure B, Tropaeolin O, Orange II, and Congo Red after incubation with crude lignin peroxidase[a]

Dye (R_f)	R_f values of visible metabolites
Azure B (0.09)	0.0, 0.15, 0.47, 0.56, 0.84[b]
Tropaeolin O (0.69)	ND[c]
Orange II (0.64)	0.82[b]
Congo Red (0.22)	ND

[a] The solvent system was propanol:water:glacial acetic acid (90:0:1, vol/vol/vol).
[b] Visualized with UV radiation.
[c] ND, None detected.

This lignin-degrading fungus has also been shown to degrade a number of polymeric dyes. Glenn and Gold (7) showed that decolorization of these polymeric dyes occurred during idiophase, was suppressed by high nitrogen levels, was dependent on oxygen concentration, and could be inhibited by known inhibitors of lignin degradation, such as KCN, thiourea, and azide, and that decolorization did not occur in the absence of phenol oxidase activity. All of these observations suggest that the lignin-degrading system of the fungus is involved. In addition, examination of a number of fungi showed that dye decolorization occurred only with fungi with known lignin-degrading ability (17). In this report, differences in the extent of degradation were observed between ligninolytic and nonligninolytic cultures. Under ligninolytic conditions, a complete loss of color from both the culture fluid and the mycelia was observed for three of the dyes. In contrast, the nonligninolytic cultures (those grown in nitrogen-sufficient medium and lacking veratryl alcohol oxidase activity) typically had substantial amounts of dye bound to the mycelia (from 11 to 49%) that were not degraded. Nitrogen-sufficient cultures typically exhibit more luxurious growth during the first few days after inoculation. This increased growth results in the depletion of a carbon source, in this case glucose, by day 4 (C. Cripps, unpublished observations). Carbon starvation is also known to induce idiophasic metabolism and lignin peroxidase production in this fungus (5, 8). Lignin peroxidase activity was typically detected in some, but not all, of the nitrogen-sufficient cultures after day 11. In those nitrogen-sufficient cultures that exhibited lignin peroxidase activity, complete decolorization (except for Congo Red) of both the culture fluid and the mycelial mat occurred (C. Cripps, unpublished observations).

Lignin peroxidases are important enzymes in the lignin-degrading system and can be readily isolated from the extracellular fluid of ligninolytic cultures of *P. chrysosporium*. While these enzymes appear to be important in lignin degradation, lignin peroxidases are only one family of enzymes involved in lignin degradation and the degradation of azo dyes. The heterocyclic dye, Azure B, showed a substantial decrease in absorbance within the 20-min incubation period, but the extent of decolorization of the three azo dyes was much less than when the dyes were incubated in the presence of the fungus. In fact, no change in absorbance was observed with Congo Red. A number of differences were also observed between the metabolites formed upon incubation of the dyes with the fungus or with the purified enzyme. Because near complete decolorization of all four dyes will occur upon incubation with ligninolytic fungal cultures, but not in the presence of lignin peroxidase, and because incubation of the dyes with fungal cultures or lignin peroxidase results in different metabolites being formed, it appears that a number of enzymes, or enzyme systems other than lignin peroxidase, expressed at the onset of idiophasic metabolism, must be involved in the breakdown of these dyes and are perhaps responsible for the initial step in the degradation of the azo dyes.

Much of the initial, rapid loss of dye from the culture fluid

appears to be due primarily to adsorption to the mycelia. An initial sorption phase ranging from 8 to 24 h has been described for azo and triphenylmethane dyes in bacterial systems (13, 14). In waste treatment systems, the elimination of colored effluents is not typically based on biodegradation but rather on physical and chemical processes such as adsorption, concentration, chemical transformation, and incineration (12). We have shown that biodegradation and adsorption to the fungal mycelia are both important processes in removing dyes from the incubation media of *P. chrysosporium*. Commercial development and applications by using this fungus for dye wastewater treatment will be able to take advantage of both of these dye removal processes.

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