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Degradation of Azo Dyes by the Lignin-Degrading Fungus *Phanerochaete chrysosporium*

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Under nitrogen-limiting, secondary metabolic conditions, the white rot basidiomycete *Phanerochaete chrysosporium* extensively mineralized the specifically ^{14}C-ring-labeled azo dyes 4-phenylazophenol, 4-phenylazo-2-methoxyphenol, Disperse Yellow 3 [2-(4′-acetamidophenylazo)-4-methylphenol], 4-phenylazoaniline, *N,N*-dimethyl-4-phenylazoaniline, Disperse Orange 3 [4-(4′-nitrophenylazo)-aniline], and Solvent Yellow 14 (1-phenylazo-2-naphthol). Twelve days after addition to cultures, the dyes had been mineralized 23.1 to 48.1%. Aromatic rings with substituents such as hydroxyl, amino, acetamido, or nitro functions were mineralized to a greater extent than unsubstituted rings. Most of the dyes were degraded extensively only under nitrogen-limiting, ligninolytic conditions. However, 4-phenylazo-[U-^{14}C]phenol and 4-phenylazo-[U-^{14}C]2-methoxyphenol were mineralized to a lesser extent under nitrogen-sufficient, nonligninolytic conditions as well. These results suggest that *P. chrysosporium* has potential applications for the cleanup of textile mill effluents and for the bioremediation of dye-contaminated soil.

Synthetic dyes are used extensively for textile dyeing, paper printing, and color photography and as additives in petroleum products (23, 36, 38). Approximately 10,000 different dyes and pigments are used industrially, and over 7 × 10^5 tons of these dyes are produced annually worldwide (38). It is estimated that 10 to 15% of the dye is lost in the effluent during the dyeing process (33). Based on the chemical structure of the chromophoric group, synthetic dyes are classified as azo dyes, anthraquinone dyes, triarylmethane dyes, etc. (38), and azo dyes constitute the largest of these groups used in industry (38).

Several amino-substituted azo dyes including 4-phenylazoaniline and *N*-methyl- and *N,N*-dimethyl-4-phenylazoanilines are mutagenic as well as carcinogenic (24). The carcinogenicity of an azo dye may be due to the dye itself or to aryl amine derivatives generated during the reductive biotransformation of the azo linkage. In mammals, azo dyes are reduced to the aryl amines by cytochrome P-450 (10) and by a flavin-dependent cytosolic reductase (17). Anaerobic bacteria isolated from human intestinal microflora also reduce azo dyes by using a novel extracellular flavin-dependent azoreductase (30). Although aryl amines themselves apparently are not mutagenic, in mammals, aryl amines can be oxidized to the corresponding *N*-hydroxy derivatives, which are subsequently transformed to reactive electrophiles capable of forming covalent linkages with DNA (32).

Synthetic dyes, which often contain substitutions such as azo, nitro, and sulfo groups, are recalcitrant to bacterial degradation (21, 25, 27, 31). Consequently, the isolation of soil bacteria which utilize these dyes as a sole source of carbon has proved difficult (37). Zimmerman et al. (37) isolated two bacterial strains capable of totally degrading the azo dyes carboxyorange I and II, but not the corresponding sulfo analogs orange I and II. Haug et al. (16) have recently demonstrated the mineralization of the sulfonated azo dye Mordant Yellow 3 by a bacterial consortium. In all cases, bacterial degradation is initiated by the reduction of the azo linkage to generate aromatic amines, which are degraded further (15, 37).

The lignin-degrading white rot fungus *Phanerochaete chrysosporium* mineralizes a wide variety of priority aromatic pollutants (4, 6, 15) including chlorophenols (26, 35), nitrotoluenes (9, 34), polycyclic aromatic hydrocarbons (3, 6), and dioxin (6), in addition to lignin. Biodegradative pathways for the metabolism of 2,4-dichlorophenol and 2,4-dinitrotoluene by *P. chrysosporium* have recently been established in our laboratory (34, 35).

Glenn and Gold (12) first established that ligninolytic cultures of *P. chrysosporium* decolorize several polymeric dyes. More recently, decolorization of the azo dyes orange II, Tropeolin O, Congo red, Acid Red 114, Acid Red 88, Biebrich Scarlet, Direct blue 15, Chrysophenine, Tetrazine, and Yellow 9 (7, 28, 29) and the triphenylmethane dyes Basic Green 4, crystal violet, brilliant green, cresol red, bromophenol blue, and pararosa-anilines (5) has been demonstrated. Decolorization, however, demonstrates only transformation of the chromophoric group of a dye; it does not demonstrate complete degradation of the dye. In this study, we utilized radiolabeled substrates to establish that *P. chrysosporium* is capable of mineralizing a wide variety of azo dyes including 4-phenylazophenol, 4-phenylazo-2-methoxyphenol, Disperse Yellow 3, 4-phenylazoaniline, *N,N*-dimethyl-4-phenylazoaniline, Disperse Orange 3, and Solvent Yellow 14.

MATERIALS AND METHODS

Chemicals. (Diagrams of the chemicals are illustrated in Table 1.) 4-Phenylazophenol (I) and 4-phenylazoaniline (IV) were purchased from Fluka, Ronkonkoma, N.Y. Disperse Yellow 3 [2-(4′-acetamidophenylazo-4-methylphenol)] (III), Solvent Yellow 14 (1-phenylazo-2-naphthol) (VII), and Disperse Orange 3 [4-(4′-nitrophenylazo)aniline] (VI) were obtained from Aldrich Chemical Co., Milwaukee, Wis. *N,N*-Dimethyl-4-phenylazoaniline (V) and 4-phenylazo-2-methoxyphenol (II) were synthesized in the laboratory as described below. [U-^{14}C]phenol (9.5 mCi/mmol), [U-^{14}C]aniline (60 mCi/mmol), [U-^{14}C]*p*-nitroaniline (48.1 mCi/mmol), and [8-^{14}C]2-

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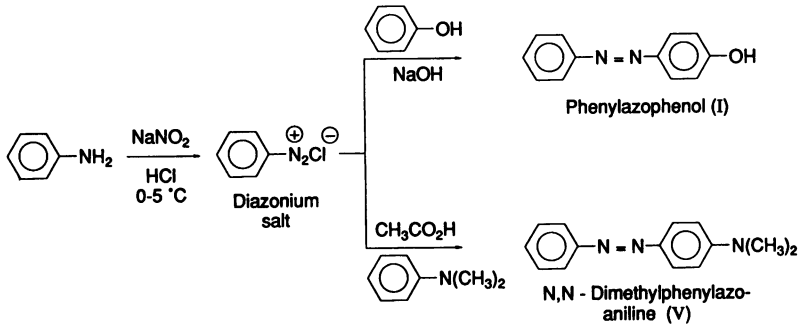


FIG. 1. Syntheses of phenolic and aminoazo dyes.

naphthol (9.8 mCi/mmol) were purchased from Sigma Chemical Co., St. Louis, Mo. [U-^{14}C]2-methoxyphenol and side-chain U-^{14}C-labeled dehydropolymerizate, a synthetic lignin, were prepared in this laboratory as previously described (13, 20).

Syntheses of radiolabeled azo dyes. Each azo dye tested for mineralization by *P. chrysosporium* contained two aromatic rings. To demonstrate that both of the aromatic rings were degraded, we synthesized radiolabeled dyes with one or the other aromatic ring specifically labeled. Thus, except for Disperse Yellow 3 (III) and 4-phenylazoaniline (IV), two specifically radiolabeled derivatives were prepared for each dye.

Phenolic azo dyes. Phenolic azo dye substrates were prepared by coupling the diazonium salt of the aromatic amine with the phenol under basic conditions (Fig. 1) (23, 38). Typically, an aromatic amine (12 to 20 μmol) was reacted with sodium nitrite (1.1 mole equivalent) in 0.25 to 0.4 ml of 2 N HCl at 0 to 5°C. The phenol (12 to 20 μmol), in 0.25 ml of 5 N NaOH, was then added to the resultant diazonium salt. Radioactivity of phenol or amine when used in these syntheses was in the range of 7.5 to 13.5 μCi. Thirty minutes after phenol addition, the reaction mixture was adjusted to pH 2 with concentrated HCl, and the radiolabeled azo dye product was extracted with ethyl acetate.

Disperse yellow 3 (III) was prepared by coupling *p*-cresol (16.6 μmol) in 5 N NaOH (65 μl) with diazotized [U-^{14}C]4-amino-acetanilide (14.8 μmol). The latter was prepared from [U-^{14}C]4-nitroaniline. Acetylation of [U-^{14}C]4-nitroaniline (14.8 μmol) with acetic anhydride (50 μl) at 80°C yielded the corresponding acetanilide. Reduction of the nitro group of [U-^{14}C]4-nitroacetanilide with stannous chloride (87 μmol) in ethanol-ethyl acetate (1:1, 50 μl) at 75°C for 2 h generated [U-^{14}C]4-aminoacetanilide (2), and this was used in the synthesis of dye III without further purification.

Amino azo dyes. Amino azo dyes were synthesized by coupling the diazonium salt of an aromatic amine (12 to 20 μmol) with another appropriate aromatic amine (12 to 20 μmol) in glacial acetic acid (20 to 40 μl) for 30 min (Fig. 1) (23). The radioactivity of the diazonium salt or amine when used in these syntheses was 10 μCi. After the coupling step, the pH of the reaction mixture was adjusted to 9 with NaOH, and the precipitated amino azo dye was extracted with ethyl acetate. [U-^{14}C]*N*,*N*-dimethylaniline, required in the synthesis of *N*,*N*-dimethyl-4-phenylazo-[U-^{14}C]aniline (Va), was prepared by methylating [U-^{14}C]aniline (0.1 mmol in 0.7 ml of tetrahydrofuran) with formaldehyde (37% solution, 50 μl) and sodium borohydride (27.2 mg) under acidic conditions (11). The *N*,*N*-dimethylaniline obtained was used in the synthesis of labeled *N*,*N*-dimethyl-4-phenylazoaniline (V) without further purification.

Purification of radiolabeled dyes. All the dyes were purified initially by silica gel thin-layer chromatography with hexane-ethyl acetate (7:3). All except for 4-phenylazophenol (I) and 4-phenylazo-2-methoxy phenol (II) were further purified by high-pressure liquid chromatography (HPLC) with a C-18 reverse-phase column (μBondapak; Waters Associates) and a water-methanol gradient (20 to 100% methanol, 10 min, 1 ml/min) as the eluant. *N*,*N*-Dimethyl-4-phenylazoaniline (V) was contaminated with the corresponding monomethylated and unmethylated dyes. To remove these impurities, we acetylated the reaction mixture with acetic anhydride, which acetylates monomethylated and unmethylated products only. *N*,*N*-Dimethyl-4-phenylazoaniline (V) was separated from the acetylated impurities by thin-layer chromatography with hexane-ethyl acetate (7:3). Further purification by HPLC yielded the pure dye. Specific activities of pure radiolabeled azo dyes ranged from 70 to 200 μCi/mmol.

Identification of radiolabeled dyes. Purified radiolabeled dyes were analyzed by HPLC with a C-18 reverse-phase column (μBondapak) and a water-methanol gradient (20 to 100% methanol, 10 min, 1 ml/min) as eluant. All purified dyes displayed only a single peak in the HPLC analysis and cochromatographed with the standard unlabeled dye. Also, each synthesis was performed with unlabeled intermediates at the same scale as the corresponding radioactive synthesis; products were purified and identified by mass spectral analyses. Mass spectral characteristics of all synthesized dyes were identical to those of standard dyes.

Culture conditions. *P. chrysosporium* OGC 101 (1) was grown from a conidial inoculum (optical density at 650 nm of ~10.0, 0.1 ml) at 37°C in stationary culture (20 ml in a 250-ml Erlenmeyer flask) as described previously (8, 19). The medium composition was the same as described previously (19) except that 3% glucose, 1% Tween 80, 30 μM $MnSO_4$, and either 1.2 or 24 mM ammonium tartrate were used. The medium was buffered with 20 mM sodium dimethylsuccinate (pH 4.5). Cultures were incubated under air for 3 days, after which they were purged with 99.9% O_2 every 3 days for 30 min.

Mineralization of azo dyes. ^{14}C-labeled azo dye (250 nmol, 2.5×10^4 to 1.2×10^5 cpm) in ethanol (40 μl) was added to cultures on day 3. During O_2 purging, evolved $^{14}CO_2$ was trapped in a basic scintillation fluid as previously described (19) and quantitated with a Beckmann LS-3133 liquid scintillation spectrometer. Four flasks were used for each mineralization experiment. Standard deviation was calculated

by using Quattro Pro commercial software (Borland International Inc.).

RESULTS AND DISCUSSION

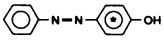
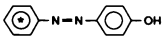
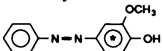
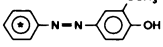
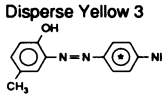
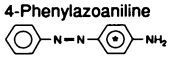
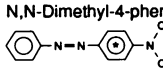
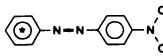
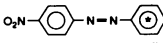
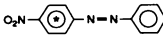
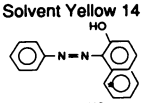
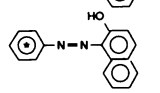
Lignin is an optically inactive, random phenylpropanoid polymer that is relatively recalcitrant to biodegradation (14, 18). White rot basidiomycetous fungi are the only organisms that are capable of degrading lignin extensively to CO_2 and H_2O (14, 18). *P. chrysosporium*, the best-studied lignin-degrading fungus, degrades lignin during secondary metabolic (idiophasic) growth, the onset of which is triggered by the depletion of nutrient nitrogen (19). Ligninolytic cultures of *P. chrysosporium* produce two extracellular peroxidases, lignin peroxidase and manganese peroxidase (14, 18), which, along with an H_2O_2-generating system, are the major extracellular components of its lignin-degrading system (14, 18). Recently, Paszczynski and Crawford (28) observed that lignin peroxidase alone could not decolorize the phenolic azo dyes Biebrich Scarlet and tetrazine; however, in the presence of veratryl alcohol, a lignin peroxidase substrate, the dyes were found to be decolorized efficiently (28).

Recent research has demonstrated that ligninolytic cultures of *P. chrysosporium* are capable of mineralizing many persistent aromatic pollutants (4, 6, 15, 34, 35). Since azo dyes are relatively recalcitrant to bacterial degradation (21, 27, 31), we examined the ability of *P. chrysosporium* to mineralize these compounds. Mineralization of the following specific ^{14}C-labeled azo dyes by *P. chrysosporium* was studied (Table 1): 4-phenylazo[U-^{14}C]phenol (Ia), 4-[U-^{14}C]phenylazophenol (Ib), 4-phenylazo-2-methoxy[U-^{14}C]phenol (IIa), 4-[U-^{14}C]phenylazo-2-methoxyphenol (IIb), [U-^{14}C]acetamidophenyl ring-labeled Disperse Yellow 3 [2-([4′-acetamido[U-^{14}C]phenylazo)-4-methylphenol] (III), 4-phenylazo [U-^{14}C]aniline (IV), *N,N*-dimethyl-4-phenylazo[U-^{14}C]aniline (Va), *N,N*-dimethyl-4-[U-^{14}C]phenylazoaniline (Vb), [U-^{14}C]aminophenyl ring-labeled Disperse Orange 3 [4-(4′-nitrophenylazo)[U-^{14}C]aniline] (VIa), [U-^{14}C]nitrophenyl ring-labeled Disperse Orange 3 [4-(4′-nitro[U-^{14}C]phenylazo)aniline] (VIb), [8-^{14}C]naphthol-labeled Solvent Yellow 14 (1-phenylazo[8-^{14}C]2-naphthol) (VIIa), and [U-^{14}C]phenyl ring-labeled Solvent Yellow 14 (1-[U-^{14}C]phenylazo-2-naphthol) (VIIb). The microbial degradation of these compounds has not been examined previously. All the dyes used in this study, except 4-phenylazophenol (I) and 4-phenylazo-2-methoxyphenol (II), are considered toxic by the Environmental Protection Agency (22). Disperse Yellow 3 is the most widely used yellow dye in the United States (23), and *N,N*-dimethyl-4-phenylazoaniline is known to be a potent carcinogen (24).

Mineralization of the azo dyes by *P. chrysosporium* was examined under nitrogen-sufficient and nitrogen-limiting culture conditions. Time courses for the mineralization of 4-phenylazophenol (Ia, Ib) and disperse orange 3 (VIa, VIb) are shown in Fig. 2. Only nitrogen-limiting, ligninolytic cultures efficiently mineralized these dyes, although 4-phenylazo-[U-^{14}C]phenol (Ia) was mineralized to a lesser extent under nonligninolytic conditions (Fig. 2). The rate of $^{14}CO_2$ evolution due to 4-phenylazophenol mineralization is maximal in the first 3 days after substrate addition and decreases significantly after 12 days (Fig. 2A). Similar patterns of $^{14}CO_2$ release were obtained with most other radioactive dyes. However, $^{14}CO_2$ evolution due to Disperse Orange 3 mineralization is linear in the first 9 days and then it decreases (Fig. 2B).

All the dyes examined were degraded much more rapidly under nitrogen-limiting conditions than under nitrogen-sufficient conditions (Table 1). However, phenolic ring-labeled 4-phenylazophenol (Ia) and 4-phenylazo-2-methoxyphenol (IIa) were also mineralized extensively under nitrogen-sufficient conditions (Table 1). This observation is extremely interesting and suggests the involvement of enzymes other than lignin-degrading enzymes in the mineralization of Ia and IIa. Except for [8-^{14}C]naphthol-labeled Solvent Yellow 14 (VIIa), all the other radiolabeled azo dyes were mineralized to at least 23% under nitrogen-limiting, lignin-degrading conditions (Table 1). Aromatic rings substituted with a phenolic or an amino or an acetamido function, such as in the dyes Ia, IIa, III, IV, Va, and VIa, are degraded to a greater extent than aromatic rings without such substituents. A comparison of mineralization of 4-phenylazo-[U-^{14}C]phenol (Ia) and 4-phenylazo-[U-^{14}C]2-methoxyphenol (IIa) under nitrogen-limiting conditions suggests that a methoxyl-group substitution into a phenolic ring enhances aromatic ring degradation. Recently, Paszczynski et al. (29) observed that introduction of a guaiacyl (2-methoxyphenol) substructure into azo dyes enhanced dye decolorization by *P. chry-*

TABLE 1. Mineralization of azo dyes by *P. chrysosporium*

^{14}C-Substrate[a]		Mineralization (%) 12 days after substrate addition	
		Low nitrogen culture	High nitrogen culture
Side chain ^{14}C-labeled DHP		21.9 ± 1.2	2.9 ± 0.05
4-Phenylazophenol			
	(Ia)	38.2 ± 1.6	21.9 ± 2.7
	(Ib)	28.4 ± 1.7	6.9 ± 1.2
4-Phenylazo-2-methoxyphenol			
	(IIa)	48.1 ± 3.4	15.1 ± 1.2
	(IIb)	20.7 ± 1.2	0.8 ± 0.3
Disperse Yellow 3			
	(III)	42.7 ± 0.5	5.7 ± 0.38
4-Phenylazoaniline			
	(IV)	25.8 ± 0.2	4.7 ± 0.06
N,N-Dimethyl-4-phenylazoaniline			
	(Va)	46.0 ± 0.4	6.7 ± 0.2
	(Vb)	29.9 ± 0.2	2.2 ± 0.1
Disperse Orange 3			
	(VIa)	40.1 ± 0.5	9.5 ± 0.1
	(VIb)	42.5 ± 0.68	2.5 ± 0.59
Solvent Yellow 14			
	(VIIa)	4.5 ± 0.05	0.0 ± 0.04
	(VIIb)	23.1 ± 0.68	3.4 ± 0.6

[a] *, indicates ^{14}C label. DHP, dehydropolymerizate.

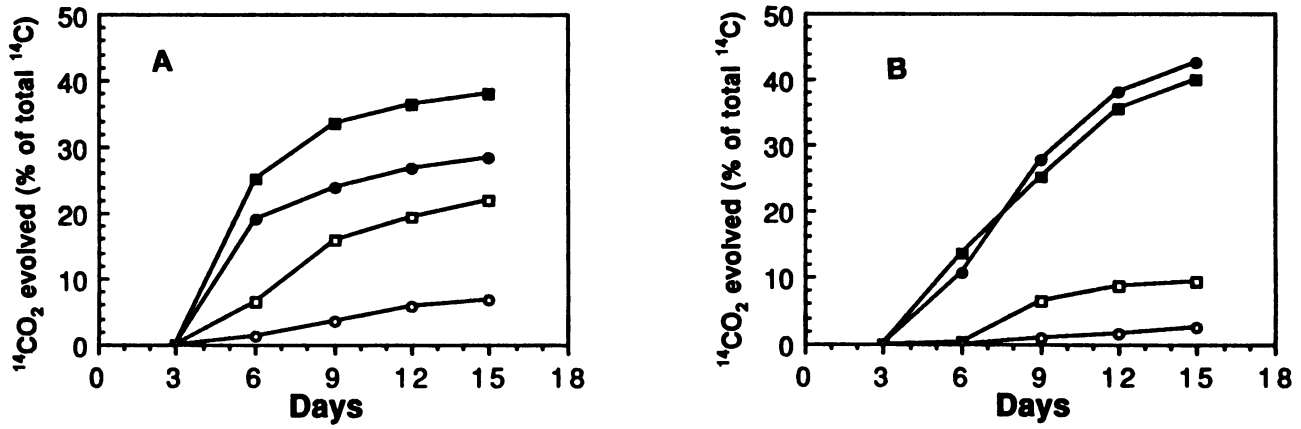


FIG. 2. Mineralization of specifically ^{14}C-ring-labeled azo dyes under low-nitrogen (1.2 mM NH_4 tartrate, closed symbols), and high-nitrogen (24 mM NH_4 tartrate, open symbols) culture conditions. (A) 4-Phenylazo-[U-^{14}C]phenol, Ia (■, □); [4-U-^{14}C]phenylazophenol, Ib (●, ○). (B) 4-(4′-nitrophenylazo)-[U-^{14}C]aniline (Disperse Orange 3), VIa (■, □); 4(4′-nitro-[U-^{14}C]phenylazo)aniline, VIb (●, ○).

sosporium. The present study demonstrates that this substitution also increases the extent of mineralization of these compounds.

Disperse Orange 3 (VI) contains two substituted aromatic rings, one with an amino substituent and the other with a nitro substituent. Surprisingly, both aromatic rings were mineralized to a similar extent (Table 1). A nitro function might have been expected to retard the mineralization process. However, Valli et al. (34) recently demonstrated that the degradation of 2,4-dinitrotoluene by *P. chrysosporium* is initiated by the reduction of a nitro group to the corresponding amine. Similar nitro-group reduction reactions may precede the ring-cleavage reactions in the mineralization of Disperse Orange 3 (VI). The phenyl ring of Solvent Yellow 14 (VIIb) is mineralized to approximately the same extent as the corresponding ring in 4-phenylazophenol (Ib) and 4-phenylazo-2-methoxyphenol (IIb). However, [8-^{14}C]naphthol-labeled Solvent Yellow 14 (VIIa) appears to be mineralized very slowly, probably due to the specific location of the labeled carbon. In a separate experiment, [8-^{14}C]naphthol itself was observed to be mineralized slowly (data not shown), suggesting that the azo dye structure is not suppressing naphthol ring degradation.

In summary, we established (i) that lignin-degrading cultures of *P. chrysosporium* are capable of mineralizing a variety of toxic azo dyes and (ii) that the rate at which *P. chrysosporium* mineralizes aromatic rings of azo dyes is dependent on the nature of ring substituents. Aromatic rings with a hydroxyl, an amino, an acetamido, or a nitro substituent are degraded faster than rings without such substituents. A detailed understanding of dye degradation by *P. chrysosporium* and other white rot fungi is required for developing fungus-based treatment systems for the cleanup of dye industry effluents and for the bioremediation of dye-contaminated soil. Experiments are planned to further examine the pathways and enzymes involved in the degradation of these dyes.

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