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Phenolic Azo Dye Oxidation by Laccase from *Pyricularia oryzae*

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Laccase oxidation of phenolic azo dyes was examined with a commercially available laccase from *Pyricularia oryzae* as the model. Methyl-, methoxy-, chloro-, and nitro-substituted derivatives of 4-(4′-sulfophenylazo)-phenol were examined as substrates for this laccase. Only the substituents on the phenolic ring were changed. Among the dyes examined, only 2-methyl-, 2-methoxy-, 2,3-dimethyl-, 2,6-dimethyl-, 2,3-dimethoxy-, and 2,6-dimethoxy-substituted 4-(4′-sulfophenylazo)-phenol served as substrates. Preliminary kinetic studies suggest that 2,6-dimethoxy-substituted 4-(4′-sulfophenylazo)-phenol is the best substrate. Laccase oxidized the 2,6-dimethyl derivative of 4-(4′-sulfophenylazo)-phenol to 4-sulfophenylhydroperoxide (SPH) and 2,6-dimethyl-1,4-benzoquinone. The 2-methyl- and 2-methoxy-substituted dyes were oxidized to SPH and either 2-methyl- or 2-methoxy-benzoquinone. Six products were formed from laccase oxidation of the 2,6-dimethoxy-substituted dye. Three of them were identified as SPH, 4-hydroxybenzenesulfonic acid, and 2,6-dimethoxybenzoquinone. A mechanism for the formation of benzoquinone and SPH from laccase oxidation of phenolic azo dyes is proposed. This study suggests that laccase oxidation can result in the detoxification of azo dyes.

Azo dyes constitute the largest group of colorants used in industry (10, 22); however, the environmental fate of these pollutants is not well understood. Azo dyes do not occur in nature and are produced only through chemical synthesis (10, 22). These dyes are resistant to aerobic bacterial degradation (12, 18); nevertheless, anaerobic bacteria readily reduce the azo linkage to yield potentially carcinogenic aromatic amines (3, 5, 17). The wood-degrading white rot basidiomycete *Phanerochaete chrysosporium* is the only organism that can completely degrade a number of azo dyes (16, 19). Azo dye degradation by *P. chrysosporium* occurs in the secondary metabolic stage and coincides with the lignin-degrading phase (16, 19). Two extracellular peroxidases, lignin and manganese peroxidases, produced by *P. chrysosporium* appear to initiate azo dye degradation (4, 6, 7, 14, 15, 20). Recently, we described the mechanism of peroxidase-catalyzed oxidation of the phenolic azo dyes disperse yellow 3 and orange II (4, 20).

Laccases, a class of copper-dependent phenol oxidases, are produced by plants and fungi (1, 21). Laccases oxidize aromatic pollutants, such as anilines and phenols, in the presence of oxygen (1, 2, 8). In this reaction, the substrates are oxidized by one electron to generate the corresponding phenoxy radicals, which either polymerize to yield a phenolic polymer or are further oxidized by laccase to produce a quinone (1). Electrons received from the substrate are subsequently transferred to oxygen, which is reduced to water. In this study, laccase oxidation of phenolic azo dyes was examined with laccase from *Pyricularia oryzae* as the model. The substrate specificity of *P. oryzae* laccase for azo dye oxidation and its mechanism for azo dye oxidation are reported below.

MATERIALS AND METHODS

Chemicals. Aniline, 4-aminobenzenesulfonic acid, and 2-methyl-, 3-methyl-, 2,3-dimethyl-, 2,6-dimethyl-, 3,5-dimethyl-, 2-methoxy-, 3-methoxy-, 2,3-dimethoxy-, 2,6-dimethoxy-, 3,5-dimethoxy-, 2-nitro-, and 2-chloro-phenols were obtained from Aldrich Chemical Co., Milwaukee, Wis. Laccase from *P. oryzae* (EC 1.10.3.2), tyrosinase from mushrooms (EC 1.14.18.1), and HPLC sorbent (particle size, 40 to 63 μm) were obtained from Sigma Chemical Co., St. Louis, Mo.

Syntheses of azo dyes. Dyes were synthesized by coupling the diazonium salt of 4-aminobenzenesulfonic acid with an appropriate phenol under alkaline conditions as previously described (10, 14). The synthesis of 4-(4′-sulfophenylazo)-phenol is described as an example. A solution of 4-aminobenzenesulfonic acid (125 mmol) and sodium nitrite (125 mmol) in 1 N NaOH (12.5 ml) was cooled in an ice bath. After the addition of 3 N HCl (8.5 ml) to the sulfonic acid solution, the diazonium salt of 4-aminobenzenesulfonic acid was formed and precipitated from the solution. The diazonium salt suspension was then reacted with a cold solution of phenol (125 mmol) in 1 N NaOH (12.5 ml) for 10 min. The product was boiled on a steam bath until all of the precipitate dissolved. The dye solution was then acidified with concentrated HCl, and the product was allowed to crystallize under ambient conditions. The crystalline product was filtered with a Büchner funnel, rinsed with water, and air dried. The azo dye was further purified by crystallization from boiling water. Dye purity was analyzed by high-performance liquid chromatography (HPLC). If HPLC analysis indicated that an azo dye was impure, then that dye was further purified by silica gel column chromatography, with 10% methanol in ethyl acetate as the eluent. Azo dye structures were confirmed by fast atom bombardment-mass spectrometric analysis as previously described (4).

HPLC and mass spectral analyses. HPLC analyses were performed with a reverse-phase C_{18} column (Separations Group, Hesperia, Calif.). Compounds were eluted with a gradient consisting of phosphate buffer (0.1 M; pH 7) and a water-methanol (1:1) mixture. Initially, the phosphate buffer concentration was maintained at 100% for 5 min. Then the water-methanol mixture was increased from 0 to 100% over 10 min and maintained at 100% for an additional 10 min. The solvent flow rate was 1 ml/min. Compound elution was monitored at 254 nm.

Gas chromatography-mass spectrometry (GC-MS) and electrospray ionization (ESI)-MS analyses were performed as previously described (4).

Azo dye oxidation by laccase. A commercial sample of *P. oryzae* laccase (1 mg) was incubated with an azo dye (50 μM) in an oxygen-saturated phosphate buffer (20 mM; 1 ml) with shaking (150 rpm) under ambient conditions for 1 h. Dye oxidation was determined by monitoring the decrease in absorbance at the λ_{max} for the dye with a UV-visible spectrophotometer (model UV-265; Shimadzu Corp., Kyoto, Japan).

Tyrosine and 2,6-dimethoxyphenol oxidation. Laccase (5 or 50 μg) or tyrosinase (10 or 80 μg) was incubated with tyrosine (100 μM) or 2,6-dimethoxyphenol (0.5 mM) in phosphate (pH 6.5; 50 mM) at room temperature. Tyrosine oxidation was monitored by the increase in A_{280}. 2,6-Dimethoxyphenol oxidation was determined by the increase in A_{469} as previously described (13). One unit of phenol oxidation activity was defined as the amount of enzyme that caused an increase in A_{280} or A_{468} of 1 per min.

Isolation and identification of azo dye oxidation products. In these experiments, a laccase sample which had been dialyzed against 20 mM phosphate (pH 6.5) was used. Dialyzed laccase (5 μg/ml) was incubated with an azo dye (50 μM) in 20 mM phosphate (pH 6.5) with shaking (150 rpm) under ambient conditions for 1 h. The products were analyzed by HPLC. To identify the quinone products, the reaction mixture was extracted with ethyl acetate and the extract was evap-

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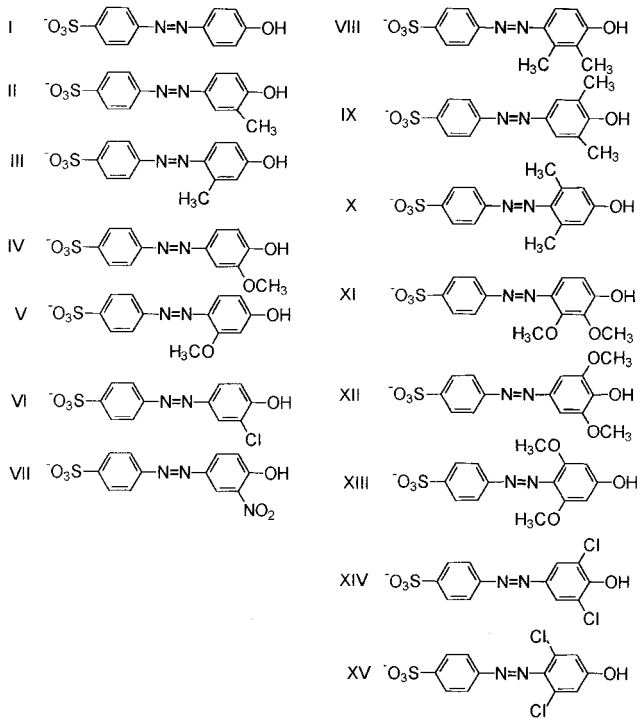

FIG. 1. Structures of the phenolic azo dyes tested as substrates for *P. oryzae* laccase.

orated to dryness. The quinone was dissolved in a minimum amount of water and reduced with sodium dithionite. The reduction products were extracted with ethyl acetate, acetylated with pyridine and acetic anhydride (1:2), and analyzed by GC-MS.

To characterize the water-soluble sulfonated products, the reactions were performed in water in the absence of any buffer. Sulfonated degradation products were separated from other hydrophobic products with an HPLC sorbent for reverse-phase chromatography (particle size, 40 to 63 μm; Sigma Chemical Co.) as previously described (4). The HPLC sorbent-treated reaction mixture was lyophilized, and the residue was analyzed by the ESI-MS technique previously described (4).

Kinetics of azo dye oxidation. Laccase (5 to 30 μg), dialyzed against 10 mM phosphate (pH 6.5), was incubated with an azo dye (50 μM) in 10 mM phosphate buffer (pH 6.5; 1 ml) saturated with oxygen. Dye oxidation was determined by monitoring the loss of absorbance at λ_{max} with a spectrophotometer.

RESULTS

The ability of laccase from *P. oryzae* to oxidize variously substituted 4-(4′-sulfophenylazo)-phenols was examined. Only the substituents on the phenolic ring were changed (Fig. 1). The substituents tested included methyl, methoxy, chloro, and nitro groups. Among the 4′-sulfophenylazo-4-phenol derivatives, only the 2-methyl- (compound II), 2-methoxy- (compound IV), 2,6-dimethyl- (compound IX), 2,6-dimethoxy- (compound XII), 2,3-dimethyl- (compound VIII), and 2,3-dimethoxy-substituted (compound XI) dyes were oxidized by laccase from *P. oryzae*. The pH optimum for dye oxidation was broad, ranging from pH 4.5 to 7.5. However, all of the substrates were oxidized at the maximum rate at pH 6.5. Steady-state kinetic studies suggest that among the dye substrates for laccase, 4-(4′-sulfophenylazo)-phenol with a 2,6-dimethoxy substitution (compound XII) is the preferred substrate, followed in order by dyes with 2,6-dimethyl (compound IX) and 2-methoxy substitutions (compound IV) (Table 1). In contrast, 4-(4′-sulfophenylazo)-phenols with 2-methyl, 2,3-dimethyl, and 2,3-dimethoxy substitutions function as poor substrates for this laccase.

TABLE 1. Kinetics of phenolic azo dye oxidation by laccase from *P. oryzae*[a]

Azo dye compound	λ_{max} (nm) (ε, mM^{-1} cm^{-1})	Rate of dye oxidation (nmol/min/mg)
II	358 (1.45)	20
IV	368 (1.08)	200
VIII	362 (1.05)	50
IX	362 (1.07)	447
XI	362 (1.28)	27
XII	377 (0.72)	703

[a] Laccase (5 to 30 μg) was incubated with an azo dye (50 μM) in phosphate buffer (pH 6.5; 10 mM; 1 ml) saturated with oxygen. Dye oxidation was determined at λ_{max}, and the amount of dye oxidized was calculated from the extinction coefficient (ε).

The products formed from laccase oxidation of 4-(4′-sulfophenylazo)-phenols with 2-methyl, 2-methoxy, 2,6-dimethyl, and 2,6-dimethoxy substitutions were also examined (Fig. 2). HPLC analysis of the 2,6-dimethyl-substituted dye oxidation products indicated the presence of two compounds with retention times (R_t) of 3.4 and 22.3 min. The product with an R_t of 3.4 min did not correspond to benzenesulfonic acid, 4-hydroxybenzenesulfonic acid, 3,4-dihydroxybenzenesulfonic acid, or 1,2-benzoquinone-4-sulfonic acid. It was unstable above 60°C. The ESI-MS of this compound indicated mass peaks at 157 and 173. It reacted with iodide to generate triodide, which has an intense yellow color and a strong A_{355}. Among organic compounds, only hydroperoxides and peracids are known to react with iodide to produce triodide (9). The HPLC R_t, ESI-MS, thermal stability, and chemical reactivity of this compound corresponded to those of 4-sulfophenylhydroperoxide (SPH) formed from lignin peroxidase-catalyzed oxidation of 2,6-di-

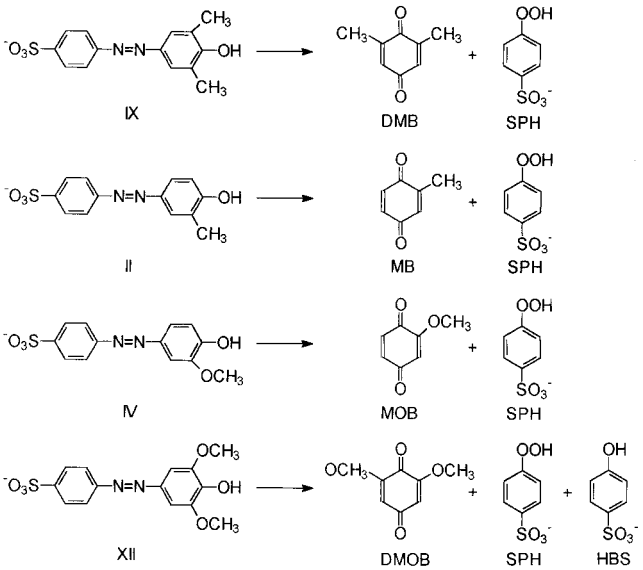

FIG. 2. Products of laccase-catalyzed oxidation of 2,6-dimethyl- (compound IX), 2-methyl- (compound II), 2-methoxy- (compound IV), and 2,6-dimethoxy-substituted (compound XII) 4-(4′-sulfophenylazo)-phenols. DMB, 2,6-dimethyl-1,4-benzoquinone; DMOB, 2,6-dimethoxy-1,4-benzoquinone; MB, 2-methyl-1,4-benzoquinone; MOB, 2-methoxy-1,4-benzoquinone; HBS, 4-hydroxybenzenesulfonic acid.

methyl-substituted azo dye (4). On the basis of these observations, the product with an R_t of 3.4 min was identified as SPH (Fig. 2). The second product (R_t of 22.3 min) was extractable into ethyl acetate. GC-MS analyses of reduced and acetylated derivatives of this compound suggested that it was 2,6-dimethyl-1,4-benzoquinone (*m/z*, 222 [2%]; 180 [10%]; 138 [100%]).

HPLC analyses of 2-methyl- and 2-methoxy-substituted dye (compounds II and IV, respectively) oxidation products indicated the presence of one major product (R_t of 3.4 min) identified as SPH (Fig. 2). GC-MS analyses of reduced and acetylated derivatives of ethyl acetate-extractable compounds indicated the presence of trace levels of 2-methyl-1,4-benzoquinone (*m/z*, 208 [2.3%]; 166 [12.7%]; 124 [100%]) and 2-methoxy-1,4-benzoquinone (*m/z*, 224 [0.8%]; 182 [11%]; 140 [100%]) from the oxidation of 2-methyl- and 2-methoxy-substituted dyes, respectively (Fig. 2). The 2-methoxy-1,4-benzoquinone yield was low because this quinone was very unstable under enzyme reaction conditions. However, since 2-methyl-1,4-benzoquinone was stable, the low yield of this product could not be explained.

HPLC analysis of the 2,6-dimethoxy-substituted dye (compound XII) indicated the presence of six products (R_t of 3, 3.4, 4.3, 18.3, 19, and 20 min). The compounds with R_t of 3.4 and 4.3 min were identified as SPH and 4-hydroxybenzenesulfonic acid by comparison with standard compounds. The compound with an R_t of 19 min was characterized as 2,6-dimethoxy-1,4-benzoquinone by comparison with standard compounds and GC-MS analyses of reduced and acetylated products (*m/z*, 254 [6.5%]; 212 [71%]; 170 [100%]) (Fig. 2). The products with R_t of 3, 18.3, and 20 min could not be identified.

The *P. oryzae* laccase used in this study was unpurified; thus, it is possible that a peroxidase or tyrosinase contaminant catalyzed dye oxidation. Peroxidase activity did not contribute to dye oxidation because the H_2O_2 required for a peroxidase reaction was not added to the enzyme reaction. The tyrosinase activity of *P. oryzae* laccase was determined by monitoring tyrosine oxidation. In addition, this activity was compared with the 2,6-dimethoxyphenol-oxidizing activity of laccase. *P. oryzae* laccase did not oxidize tyrosine; however, it exhibited a specific activity of 9 U mg^{-1} for 2,6-dimethoxyphenol oxidation. On the other hand, tyrosinase oxidized both tyrosine and 2,6-dimethoxyphenol; the corresponding specific activities were 3 and 0.7 U mg^{-1}, respectively. These findings suggest that *P. oryzae* laccase most probably does not contain tyrosinase and that laccase was responsible for the azo dye oxidation observed.

DISCUSSION

Though hundreds of azo dyes are in industrial use, their environmental fate is not well understood. Laccases are copper-dependent enzymes produced by a number of fungi and plants, and they oxidize phenols and anilines in the presence of oxygen (1, 2, 8, 21). In order to understand the role of laccases in azo dye degradation, the ability of laccase from *P. oryzae* to oxidize 4-(4′-sulfophenylazo)-phenol derivatives was examined. Methyl, methoxy, chloro, and nitro substituents were introduced into the phenolic ring in order to gain insight into the substrate specificity of this laccase. Among the dyes tested, only dyes with electron-donating methyl or methoxy substituents were oxidized. Unsubstituted 4-(4′-sulfophenylazo)-phenol and its 2-chloro and 2-nitro analogs were not oxidized. These observations suggest that the phenolic ring of an azo dye has to be electron rich for oxidation by this laccase. The location of the methyl or methoxy substituent also appears to be important. The 2-methyl and 2-methoxy analogs of 4-(4′-sul-

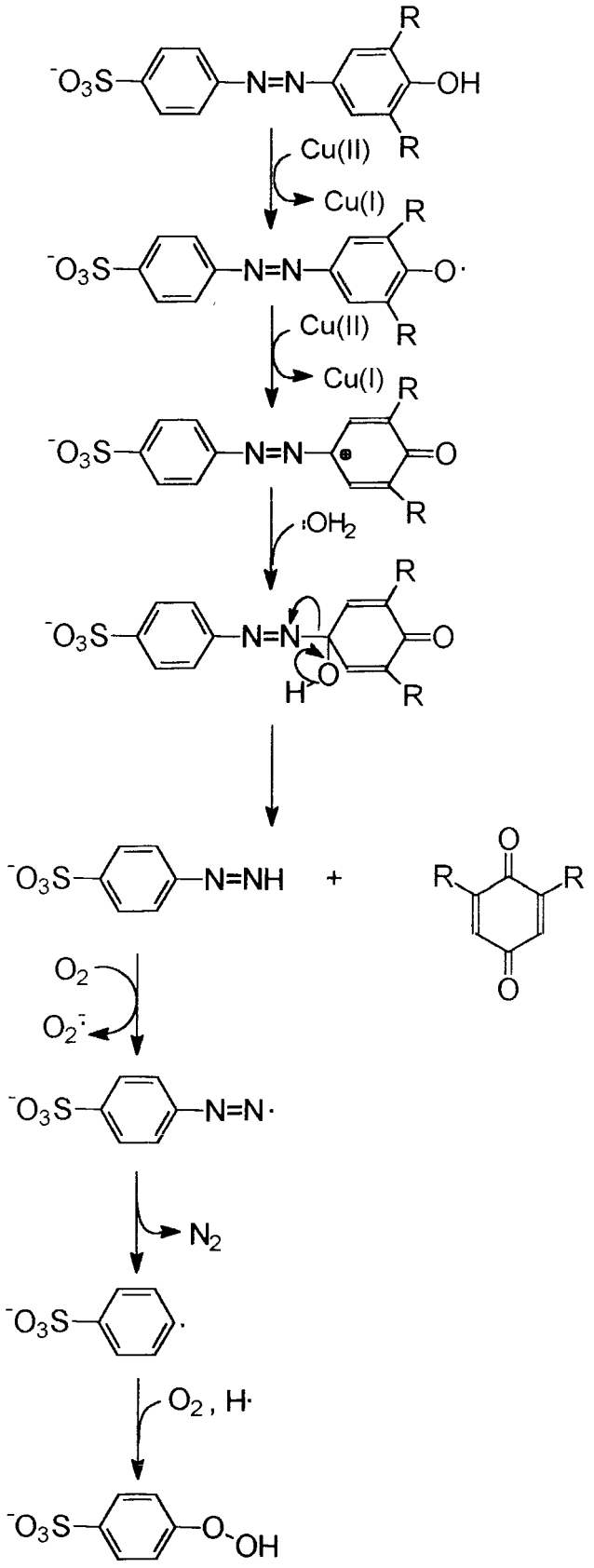

FIG. 3. Proposed mechanism for the degradation of phenolic azo dyes by *P. oryzae* laccase.

fophenylazo)-phenol are laccase substrates, whereas the corresponding 3-methyl analogs are nonsubstrates. Similarly, 2,6-dimethyl, 2,6-dimethoxy, 2,3-dimethyl, and 2,3-dimethoxy analogs serve as substrates, whereas 3,5-substituted derivatives do not function as substrates for this laccase. Among the azo dye substrates identified, the 2,6-dimethoxy-substituted dye is the preferred substrate, followed in order by 2,6-dimethyl- and 2-methoxy-substituted dyes (Table 1). Thus, it appears that laccase from *P. oryzae* has a narrow substrate specificity for azo dye oxidation. In a similar study on the effects of aromatic substitution patterns on peroxidase-catalyzed degradation of phenolic azo dyes, Pasti-Grigsby et al. (14) identified 2-methyl-, 2-methoxy-, 2,6-dimethyl-, and 2,6-dimethoxy-substituted 4-(4′-sulfophenylazo)-phenols as preferred substrates for horseradish peroxidase, manganese peroxidase, and *Streptomyces chromofuscus* peroxidase.

Laccase oxidation of 2,6-dimethyl-, 2-methyl-, 2-methoxy-, and 2,6-dimethoxy-substituted 4-(4′-sulfophenylazo)-phenols produced a quinone from the phenolic portion of the azo dye and SPH from the sulfophenyl ring (Fig. 2). Recently, we identified similar products from lignin peroxidase-catalyzed oxidation of phenolic azo dyes and proposed a mechanism to explain product formation (4). A mechanism similar to that of lignin peroxidase might be applicable to laccase (Fig. 3). According to our proposal, laccase oxidizes the phenolic ring by one electron to generate a phenoxy radical which is oxidized once again by the enzyme to produce a carbonium ion in which the charge is localized on the phenolic ring carbon bearing the azo linkage. Nucleophilic attack by water produces 4-sulfophenyldiazene and a benzoquinone (4, 7) (Fig. 2). 4-Sulfophenyldiazene is presumably unstable in the presence of oxygen, which oxidizes it to the corresponding phenyldiazene radical. The latter readily loses molecular nitrogen to produce a sulfophenyl radical, which is scavenged by O_2 to ultimately yield SPH (Fig. 2). SPH is an unusual peroxide and is known to be formed only from peroxidase oxidation of sulfonated azo dyes; whereas organic peroxides are unstable in the presence of transition metal ions, SPH is stable (4). The mechanism of 4-hydroxybenzenesulfonic acid formation from compound XII oxidation is unknown; it could be derived from SPH.

Bollag and coworkers observed dimerization and polymerization of phenoxy radicals in *Rhizoctonia praticola* laccase-catalyzed reactions (1, 2). *P. oryzae* laccase does not appear to polymerize phenolic azo dyes. Radical coupling occurs at the *ortho* or *para* position of the phenol. In the phenolic azo dye substrates for *P. oryzae* laccase, the *para* position has the phenylazo substitution and either or both of the *ortho* positions are replaced with a methyl or methoxy substituent. Such a substitution pattern could reduce or eliminate radical coupling reactions.

In summary, this study demonstrates that laccase from *P. oryzae* is capable of oxidizing phenolic azo dyes. Many wood-degrading basidiomycetes produce laccase (21). Such laccases might contribute to the oxidative degradation of azo dyes in the environment. The azo linkage is susceptible to reduction, which generates potentially carcinogenic aromatic amines (5, 11). Laccase oxidation might detoxify azo dyes because this reaction releases azo linkages as molecular nitrogen, which prohibits aromatic amine formation. Laccase and peroxidase appear to utilize similar mechanisms for azo dye oxidation.

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