Enzymatic Decolorization of Textile Dyeing Effluents

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ABSTRACT

Commercial azo, triarylmethane, antraquinonic, and indigoid textile dyes are efficiently decolorized with enzyme preparations from *Pleurotus ostreatus, Schizophyllum commune, Neurospora crassa, Polyporus sp., Sclerotium rolfsii, Trametes villosa,* and *Myceliophtora thermophila.* The nature of substituents on the dyes' benzene rings influences enzyme activity, and hydroxyl and amino groups enhance decolorization. The presence of lignin peroxidase and/or manganese peroxidase in addition to laccase (*P. ostreatus, S. commune, S. rolfsii, N. crassa*) increases decolorization by up to 25%. The effect of textile dyeing auxiliaries depends on the individual enzymes. *Polyporus sp.* and *T. villosa* are inhibited up to 20% by copper and iron chelating agents and anionic detergents, while the *S. commune* enzymes lose up to 70% of their activity.

Large amounts of dyestuffs are used for textile dyeing as well as for other industrial applications. As a characteristic of the textile processing industry, a wide range of structurally diverse dyes can be used in a single factory, and therefore effluents from the industry are extremely variable in composition. This underlines the need for a largely unspecific process for treating textile waste water. It is known that 90% of reactive dyes entering activated sludge sewage treatment plants will pass through unchanged and be discharged in to rivers [29]. Interest in the pollution potential of textile dyes has been primarily prompted by concern over their possible toxicity and carcinogenity. Not all dyes currently used can be degraded or removed with physical and chemical processes, and sometimes the degradation products are more toxic [30]. Several combined anaerobic and aerobic microbial processes using adapted mixed populations are believed to enhance the degradation of textile dyes [4, 20], but under anaerobic conditions, azo reductases usually cleave azo dyes into the corresponding amines, many of which are mutgenic or carcinogenic [7, 9]. Furthermore, azo reductases have been shown to be very specific enzymes, thus cleaving only the azo bonds of selected dyes [35, 36]. In contrast, the phenoloxidases—lignin peroxidase (ligninase, LiP), manganese peroxidase (MnP), and laccase—act more unspecifically on aromatic rings and thus have the potential to degrade a wide range of aromatic structures [31].

Lignin peroxidase (ligninase, LiP), manganese peroxidase (MnP), and laccase are involved in the biodegradation of lignins, which are complex polyaromatic polymers constituting the main noncarbohydrate component in wood and are among the most abundant groups of biopolymers in the biosphere. A great number of white-rot fungi have been reported to produce the ligin-degrading enzymes LiP, MnP, and laccase, or at least one of these enzymes [14, 15, 33]. Manganese peroxidase (EC 1.11.1.13; Mn (II): hydrogen peroxide oxidoreductase) and ligninase (EC 1.11.1.14; diarylpropane: oxygen, hydrogen peroxide oxidoreductase) belong to the class of peroxidases that oxidize their substrates by two consecutive one-electron oxidation steps with intermediate cation radical formation. While MnP only attack phenolic substrates using Mn^{2+}/Mn^{3+} as an intermediate redox couple, LiP with a higher redox potential prefers nonphenolic methoxy-substituted lignin subunits as substrates [33].

Laccases (benzenediol : oxygen oxidoreductase, EC 1.10.3.2) have very broad substrate specificity with respect to the electron donor. They catalyze the removal of a hydrogen atom from the hydroxyl group of *ortho-* and *para*-substituted mono- and poly-phenolic substrates and from aromatic amines by one-electron abstraction, to form free radicals capable of undergoing further depolymerization, repolymerization, demethylation, or quinone formation [18].

Using laccases and manganese peroxidases for pulp bleaching and effluent treatment has gained considerable interest in the last few years, and several new processes have been patented [18]. Laccases can be used on both chlorolignins and phenolic compounds of pulp mill effluents [3]. The enzymes render phenolic compounds less toxic by means of polymerization reactions and cross-coupling of pollutant phenols with naturally occurring phenols [21]. Several processes using laccases as well as laccases immobilized on activated carbon have been developed for phenolic effluent treatment [5, 12]. In this study we assess the potential of phenol oxidases from the fungi *Pleurotus ostreatus, Schizophyllum commune, Neurospora crassa, Polyporus sp., S. rolfsii, Trametes villosa,* and *Myceliophtora thermophila* to decolorize textile dyes, taking into account the influence of dyeing auxiliaries.

Experimental

Veratryl alcohol, sodium tartrate, sodium malonate, 2,6-dimethoxyphenol (DMP), and manganese(II)sulfate came from Sigma; all other chemicals were from Merck. Complex carbon sources for the cultivation of fungi such as wheat bran flakes, potato starch, malt extract, dextrose, and sucrose were obtained locally. The dyes C.I. Basic Violet 3, Basic Red 9 Base, Acid Violet 17, Acid Blue 74, and Acid Orange 5 came from Sigma; Reactive Blue 221 and Disperse Red 60 came from Sumitomo Chemicals; Reactive Black 5, Direct Blue 71, and Acid Blue 74 came from Ciba; and Reactive Blue 19 came from Höchst (for dye structures see Figure 1). All commercial textile dyeing auxiliaries came from Ciba.

Laccase preparations from *Trametes villosa* and *Myceliophtora thermophila* were kindly provided by Novo-Nordisk. *Pleurotus ostreatus* (ATCC 9427), *Schizophyllum commune* (BT 2115), *S. rolfsii* (ATCC 200224), *Neurospora crassa* (DSM 1258), and *Polyporus sp.* H7 (BT 2313) were cultivated at 30°C in 300 ml Erlenmeyer flasks on a rotary shaker (150 rpm) containing 100 ml media as described previously for *P. ostreatus* [27], *S. commune* [22], *S. rolfsii* [19], *N. crassa* [16], and *Polyporus sp.* [17]. Cultures were harvested after 8 days (*P. ostreatus*), 7 days (*S. commune*), 14 days (*S. rolfsii*), 6 days (*N. crassa*), and 7 days (*Polyporus sp.*), respectively, filtered and centrifuged, and the clear supernatant was used for the enzyme activity assays and the decolorization experiments.

Laccase (benzenediol : oxygen oxidoreductase, EC 1.10.3.2) and manganese peroxidase (MnP, EC 1.11.1.13; Mn (II): hydrogen peroxide oxidoreductase) activities were determined using 2,6-dimetoxyphenol (DMP) as a substrate as described earlier [13]. The reaction mixture contained 50 mmol sodium malonate (pH 4.5), 1 mmol DMP, 1 mmol $MnSO_4$, and 700 μl sample in a total volume of 1 ml. The formation of an orange/brownish dimer was followed spectrophotometrically at 468 nm (laccase activity). MnP activity equaled the increased activity after adding 0.4 mmol hydrogen peroxide to the reaction mixture, and was corrected for laccase activity. Lignin peroxidase (LiP, EC 1.11.1.14; diarylpropane : oxygen, hydrogen peroxide oxidoreductase) activity was determined spectrophotometrically (310 nm) as the H_2O_2-dependent oxidation of veratryl alcohol to veratrylaldehyde in a reaction mixture (1 ml) containing 50 mmol sodium tartarate (pH 2.5), 2 mmol veratryl alcohol, 4 mmol H_2O_2, and 500 μl culture filtrate as described earlier [32].

For the decolorization experiments, typically 0.5 ml dye (250 mg l^{-1} final concentration), 0.5 ml enzyme preparation (diluted to give 0.1 nkat ml^{-1} final laccase activity), and 3.0 ml buffer (0.1 *M* sodium acetate, pH 5.0) were incubated on a rotary shaker at 50°C for 5 hours. Decolorization was followed spectrophotometrically at the maximum absorbance of each dye, and concentrations were calculated from calibration curves recorded at those conditions. The decrease in absorbance was continuously recorded, and the values were taken after 5 hours from trend curves. The effect of commercial dyeing additives (Ciba) and salts on enzymatic decolorization was determined analogously (added to 3 ml of the buffer) in concentrations of 1, 3, and 5 g l^{-1} and 0.1, 0.5, 1, and 2 *M*, respectively. Calibration curves were recorded separately for all additive concentrations. All experiments were done in triplicate using heat inactivated enzyme preparations as controls.

Result and Discussion

Enzyme preparations from the fungi *Pleurotus ostreatus, Schizophyllum commune, Sclerotium rolfsii, Neurospora crassa, Polyporus sp., Trametes villosa,* and *Myceliophtora thermophila* were able to decolorize triarylmethane, azo, antraquinone, indigo, and metal chelate dyes. In all preparations, laccase was the predominant enzyme, but lignin peroxidase and/or manganese peroxidase were also present in the case of *S. commune, S. rolfsii, N. crassa,* and *Polyporus sp.* (Table I). Of the three temperature settings (35, 50, and 65°C) and three pH settings (pH 5.0, 6.5, 7.5), all enzyme preparations were most active at 50°C and pH 5.0. Initial decolorization efficiencies depended on the source of the enzymes and the substrate (dye) used (Figure 1). Of the triarylmethane dyes, Basic Violet 3 was the preferred substrate for most enzymes. Thus, most likely unsubstituted hydroxyl or amino groups on the aromatic rings, which are

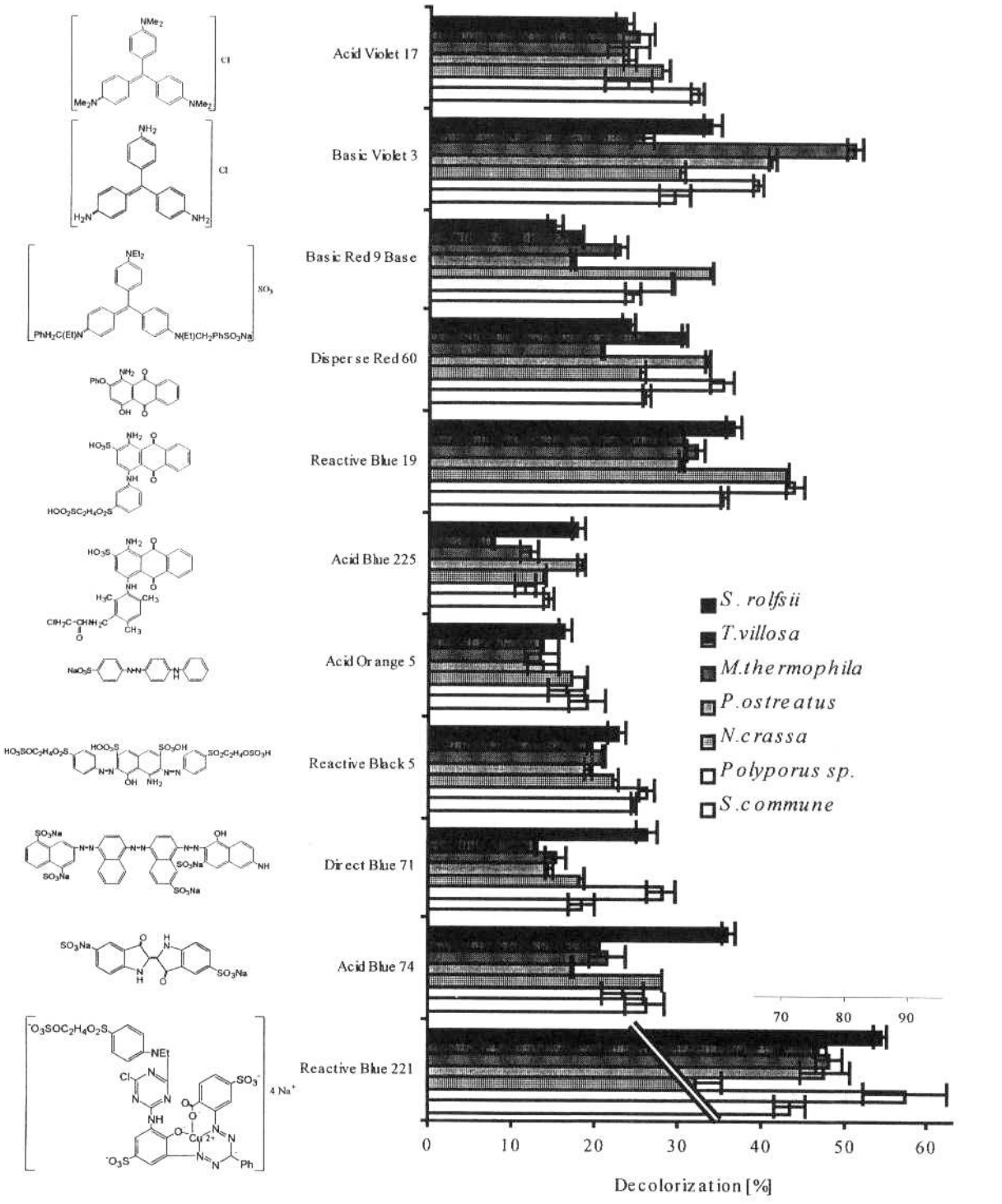

FIGURE 1. Enzymatic decolorization of textile dyes by enzyme preparations from *P. ostreatus, S. commune, S. rolfsii, N. crassa, Polyporus sp., T. villosa,* and *M. thermophila.*

missing on Acid Violet 17 and Basic Red 9 Base, were beneficial for the initial attack by phenol oxidases. These results parallel previous findings on *Phanerochaete chrysosporium* LiP and MnP [28], and they are in agreement with the general catalytic mechanism of laccases, which catalyze the abstraction of a hydrogen atom from

TABLE I. Ratio of laccase activity to manganese peroxidase (MnP) and lignin peroxidase (LiP) activities (nkat ml^{-1}) in various enzyme preparations (laccase activity expressed as 100%).

Enzyme preparation	Laccase	: MnP	: LiP
N. crassa	100	: 32.3	: 27.5
S. rolfsii	100	: 20.2	: 0.5
S. commune	100	: 13.9	: 19.2
Polyporus sp.	100	: 19.0	: 0.8
P. ostreatus	100	: 2.68	: 5.01
T. villosa	100	: 0.0	: 0.0
M. thermophila	100	: 0.0	: 0.0

the hydroxyl group of mono- and poly-phenolic substrates and from aromatic amines [31]. Similarly, the azo dye Acid Orange 5 degraded to a lesser extent than Direct Blue 71 and Reactive Black 5.

The nature of substituents on the aromatic ring has been shown to influence enzymatic oxidation. Electron donating methyl and methoxy substituents seemed to enhance enzymatic degradation of azophenols, while electron withdrawing chloro, fluoro, and nitro substituents inhibited oxidation by a laccase from *Pyricularia oryzae* and MnP from *Phanerochaete chrysosporium* [7, 28].

For the degradation of antraquinoid dyes, we did not observe any such general trend. However, Disperse Red 60 is not completely soluble in water, and so the results must not be directly compared with the other dyes. The presence of MnP and/or LiP in addition to laccase in the *S. commune, S. rolfsii, Polyprous sp.*, and *N. crassa* enzyme preparations had a significant, positive effect on the degree of decolorization (up to 25% higher) of all azo dyes, the indigoid dye Acid Blue 74, Reactive Blue 19 (antraquinoid dye), and Basic Red 9 Base (triarylmethane dye). Previously, purified lignin peroxidases have been shown to degrade Reactive Blue 19 and other antraquinonic compounds, while MnP did not seem to be responsible for dye degradation [25]. Other authors have found that its higher oxidation potential allowed LiP to degrade model azo dyes with fluoro substituents on the phenolic ring, which MnP did not cleave [28]. However, a combination of LiP and other oxidative enzymes from *Phanerochaete chrysosporium* seemed to be responsible for degrading the azo compounds [11].

We tested various salts and textile dyeing auxiliaries (Ciba), which are usually applied in combination with textile dyes, for potential inhibitory effects on enzymes. We chose C.I. Acid Orange 5 and *T. villosa, Polyporus sp.*, and *S. commune* for these experiments. Both sodium acetate and sodium chloride at high concentrations decreased the decolorization efficiency by up to 80% (2 *M* NaCl) and even led to partial precipitation of proteins (data not shown). This effect most likely resulted from both inactivation and precipitation of enzymes caused by increased surface tension and hydrophobic interaction. Cibacel DBC, which is used for iron and copper inactivation during textile dyeing, significantly (up to 70%), inhibited all enzymes (Figure 2). Laccases belong to the class of blue oxidases typically containing four copper atoms per polypeptide chain distributed in three different copper binding sites [23] or one copper, one iron, and two zinc atoms per protein molecule [26, 34]. All of these copper ions are apparently involved in the catalytic mechanism. Thus, it is obvious that chelation of copper and iron can inactivate this class of enzymes.

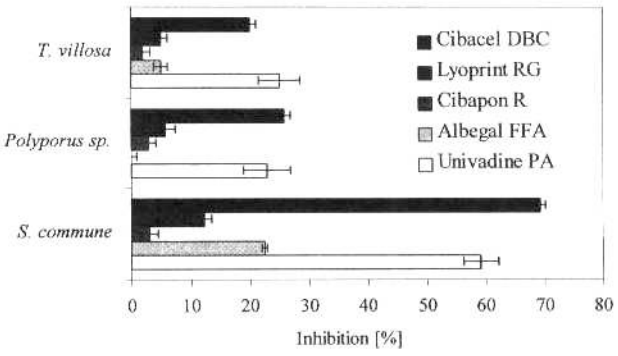

FIGURE 2. Inhibition of C.I. Acid Orange 5 decolorizing enzymes by dyeing auxiliaries.

Anionic detergents (Univadine PA) and cationic agents such as N-tetraalkylammoniumhalogenids (Tinegal MR) seemed to partially denaturate proteins (enzymes) responsible for decolorization. Also migration and penetration auxiliaries (Albegal SET) inhibited the enzymes, while soaping agents (Cibapon R), wetting agents (Albegal FFA, alkylarylpolyglycolethersulfate; Albegal FFC, alkylarylethoxylate) did not show any significant effect. As shown for *S. commune, Polyporus sp.*, and *T. villosa* (Figure 2), the individual enzyme preparations were inactivated to a different extent. While *Polyporus sp.* and *T. villosa* were inhibited up to 20% by additives (3 g l^{-1}), the *S. commune* enzymes lost up to 70% of their activity. Thus, knowledge not only about substrate specificities but also about the effect of auxiliaries is important in selecting suitable enzymes for dye decolorization under industrial conditions.

Previously, various colored industrial effluents have been treated using immobilized bacteria or fungi, including some strains examined in our study [1, 24]. The white rot fungus *S. commune* has been used for degrading pulp mill effluents [2], *N. crassa* has been found to degrade azo compounds [10], and *P. ostreatus* has been

shown to mineralize polycyclic aromatic hydrocarbons [6]. However, the microorganisms were cultivated under physiological conditions and had to be supplemented with nutrients not present in the effluents. Using enzymes instead of whole organisms would allow treatment of effluents with varying compositions under industrial conditions (higher temperatures and pH, etc.). Furthermore, bacterial azo reductases produce aromatic amines, many of which are carcinogenic, while laccases and LiP have been shown to degrade azo bonds to nitrogen [7, 8].

Conclusions

In this study we have shown that enzyme preparations from *Pleurotus ostreatus, Schizophyllum commune, Sclerotium rolfsii, Neurospora crassa, Polyporus sp., Trametes villosa,* and *Myceliophtora thermophila* efficiently decolorized a variety of structurally different dyes. However, initial reaction rates depend on the individual enzymes (laccase, LiP, and MnP) in the preparations. Some dyeing additives dramatically lower decolorization rates. Thus, the effect of dyeing auxiliaries on enzyme activity must be considered when evaluating new enzymes and processes. Future investigations should focus on the immobilization of selected enzymes to form the base for industrial application of enzymatic decolorization.

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