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Transformation of Textile Dyes by White-Rot Fungus *Trametes versicolor*

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Abstract

We have investigated transformation of eight industrial dyes by a white-rot fungus, *Trametes versicolor*. The fungus was found to decolorize Reactive Golden Yellow R, Procion Red, Reactive Violet 5, Reactive Blue 28, and Ponceau Red 4R at an initial dye concentration of 80 ppm within 72 h of incubation, whereas it took 5 d to completely decolorize Reactive Black 5 (40 ppm). However, it did not significantly decolorize Reactive Red 152 and Novatic Blue BC S/D. During decolorization in liquid medium, laccase and manganese-independent peroxidase (MiP) activities were detected in culture filtrate of *T. versicolor*. Dye-decolorizing activity of the culture was found to be associated with H_2O_2-dependent activity of the culture filtrate. Furthermore, dye-decolorizing activity of the culture filtrate was not influenced by Mn^{2+} or veratryl alcohol, thus suggesting a role of extracellular MiP in decolorization of synthetic dyes by *T. versicolor*.

Index Entries: Decolorization; *Trametes versicolor*; dyes; manganese-independent peroxidase; laccase.

Introduction

A wide variety of synthetic dyes are extensively used for textile dyeing, paper printing, and other industries. About 10,000 dyes with an annual production of more than 7×10^5 metric t worldwide are commercially available *(1)*. These dyes constitute a group of chemically diverse compounds and are classified on the basis of their chromophore group (e.g., azo, diazo, triphenylmethane, phthalocyanine, anthraquinone) or according to their application technologies (e.g., reactive, direct, disperse, and vat) *(2)*. The characteristic structures of azo dyes (the azo linkage and aromatic sulfonic groups) make them resistant to biologic degradation *(3)*. About 10–15% of the amount of synthetic dyes produced annually is discharged in aqueous

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effluents, and because of their recalcitrant nature, they are barely removed from effluents by conventional biologic wastewater treatment *(1)*.

The white-rot fungi, particularly *Phanerochaete chrysosporium*, have been extensively studied for their ability to degrade a broad range of xenobiotic organopollutants *(4,5)*. This potential of white-rot fungi has been ascribed to extracellular oxidation by nonspecific lignin-degrading enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP), laccases, and other oxidases *(6,7)*. The fungus releases LiPs and MnPs in the extracellular environment, which is responsible for nonspecific degradation of a wide range of textile dyes *(5,8)*. However, the ligninolytic enzymes in *P. chrysosporium* are under strict regulation and synthesized under secondary growth phase, which limits its use in real wastewater treatment *(9)*.

Ligninolytic enzymes of *Trametes versicolor* are expressed even under primary growth phase, thus giving it an advantage over *P. chrysosporium* for application in bioremediation of organopollutants *(9,10)*. *T. versicolor* is another white-rot fungus, which has been investigated for its decolorization ability of kraft and pulp mill effluents *(11–13)*. Decolorization of various azo and anthraquinone dyes by *T. versicolor* has been demonstrated *(9,14–16)*. *T. versicolor* produces laccase and MnP as major ligninolytic enzymes; however, the role of these enzymes in decolorization of azo dyes is not yet clear. Laccase and/or MnP activities in culture filtrate of *T. versicolor* were not able to decolorize azo dyes, thus indicating a role of other enzymes or cell-bound component in azo dye degradation *(15)*.

This article reports degradation of several azo dyes by *T. versicolor* and suggests the role of extracellular manganese-independent peroxidase (MiP) in decolorization of these dyes.

Materials and Methods

Culture

T. versicolor was a gift from Institut of Forestbotanisches, Gottingen, Germany. It was grown on 2% malt extract agar plates at 26°C and preserved at 4°C. It was subcultured once in 2 mo.

Chemicals

The dyes (Table 1) used were of industrial grade and obtained from Mantung Dyestuffs and Intermediates, GIDC, Vatva, Ahmedabad, Gujarat, India. All other chemicals used were of analytical grade.

Medium

Basal salt medium (BSM), used to study dye decolorization, contained the following ingredients: 5.0 g/L of glucose, 1.0 g/L of KH_2PO_4, 0.5 g/L of $MgSO_4$, 0.5 g/L of KCl, 0.5 g/L of yeast extract; the pH was adjusted to 5.5. The medium was sterilized by autoclaving at 121°C for 15 min.

Table 1
Characteristics of Dyes Used for Decolorization Studies

Dye	Class	Absorbance maximum (nm)
Reactive Blue 28	Mono azo, metal complex	572
Reactive Violet 5	Mono azo, metal complex	558
Reactive Black 5	Diazo	598
Reactive Red 152	Azo	535
Reactive Golden Yellow R	Azo	418
Procion Red H7B	Azo	515
Novatic Blue BC S/D	Anthraquinone	588
Ponceau Red 4R	Azo (food color)	506

Decolorization Studies Using Dye Agar Plates

Initially, the ability of *T. versicolor* to decolorize different types of dyes was tested using dye agar plates *(17)*. Dye agar plates for all different dyes were prepared using BSM containing 100 mg/L of individual dye. Plates were inoculated centrally with a block of uniform growth of *T. versicolor* in quadruplicate for each dye. Uninoculated dye agar plates as abiotic controls and inoculated plates were incubated at 26°C.

Decolorization in Liquid Media

Fifty milliliters of sterilized medium in 250-mL flasks was inoculated with a block (1 × 1 cm) of uniform growth of *T. versicolor*, to which an aliquot of dye was added from an autoclaved stock (2000 ppm) at a final concentration of 80 ppm for all dyes except for Reactive Black 5. Decolorization of Reactive Black 5 was studied at a concentration of 40 ppm in the medium. Flasks were incubated on a rotary shaker at 26°C and 150 rpm. Uninoculated dye containing liquid medium served as a control. On incubation mycelial pellets were separated from growth media by filtration through Whatman no. 1 filter paper. The dye content in the culture filtrate was monitored by reading absorbance at its λ_{max} using a UV-VIS diode array scanning spectrophotometer (Hewlett Packard 8452). This experiment was performed in quadruplicate.

Enzyme Production and Dye Decolorization

As already mentioned, flasks containing 50 mL of medium and Reactive Blue 28 (100 ppm) were inoculated with *T. versicolor* and incubated under shaking conditions at 26°C and 150 rpm. Each day the contents of one flask were filtered and culture filtrate was analyzed for residual dye content and different enzyme activities. A parallel set without dye was inoculated, and the culture filtrate was analyzed for the enzyme activities each day.

Enzyme Assays

MiP activity was estimated by monitoring the oxidation of 1 m*M* 2,6-dimethoxyphenol (DMP) in 100 m*M* sodium tartrate (pH 4.5), using 0.1 m*M* H_2O_2. The reaction mixtures used to determine MnP activity contained 1 m*M* DMP, 0.1 m*M* H_2O_2, 1 m*M* $MnSO_4$, and 100 m*M* sodium tartrate (pH 4.5). MnP activity was corrected for MiP activity by subtracting the activity obtained in the absence of $MnSO_4$. Oxidation of DMP was measured as described by Martinez et al. *(18)*. LiP activity was determined as described by Tien and Kirk *(19)*. Laccase was measured by monitoring oxidation of *O*-dianisidine as described by Palmieri et al. *(20)*. The reaction mixtures used to monitor dye-decolorizing activity of culture filtrate contained dye, 0.1 m*M* H_2O_2, and 100 m*M* sodium tartrate buffer. All enzyme reactions were started by adding 200 µL of culture filtrate, and the enzyme activities were calculated from the linear phases of the reaction. One unit of enzyme activity was defined as the amount of the enzyme that transformed 1 µmol of substrate/min.

Results and Discussion

Decolorization on Solid Media

To determine the spectrum of dyes decolorized by *T. versicolor*, dye decolorization was studied using dye agar plates. Decolorization began with the formation of clear zones around growth by the second day for all dyes except Novatic Blue BC S/D and Reactive Red 152. Complete decolorization was examined as the total disappearance of color without any visible sorption to the biomass. Reactive Blue 28, Reactive Violet 5, and Ponceau Red 4R were decolorized completely in 5 d of incubation whereas it took 7 d for complete decolorization of Procion Red and Reactive Golden Yellow R. Reactive Black 5 was initially converted to red, which was further decolorized to give a colorless zone and took 10 d for complete decolorization. No decolorization was observed in uninoculated plates (Table 2).

Decolorization of Dyes in Liquid Medium

Decolorization of different dyes was monitored in liquid medium under shaking conditions. Greater decolorization rates were observed in agitated cultures as compared with static cultures of *T. versicolor (16)*. Reactive Blue 28, Reactive Golden Yellow R, and Procion Red were more susceptible to attack by *T. versicolor* and were completely decolorized at uniform rates within 3 d of incubation. Decolorization of Reactive Violet 5 and Ponceau Red 4R occurred at low rates during the first 24 h but increased uniformly to attain maximum rates during the second day of incubation. Maximum decolorization of Reactive Black 5 was observed during the third and fourth days of incubation (Fig. 1).

Visible sorption of these dyes by the biomass was not observed and no dye was recovered on homogenization of biomass resuspended in various

Table 2
Decolorization of Dyes by *T. versicolor* in Solid Medium

Dye	Decolorization (d)[a]
Reactive Blue 28	+ (5)
Reactive Violet 5	+ (5)
Reactive Black 5	+ (10)
Reactive Red 152	–
Reactive Golden Yellow R	+ (7)
Procion Red H7B	+ (7)
Novatic Blue BC S/D	–
Ponceau Red 4R	+ (5)

[a]+, decolorization; –, no decolorization.
Numbers in parentheses represent the time required for complete decolorization.

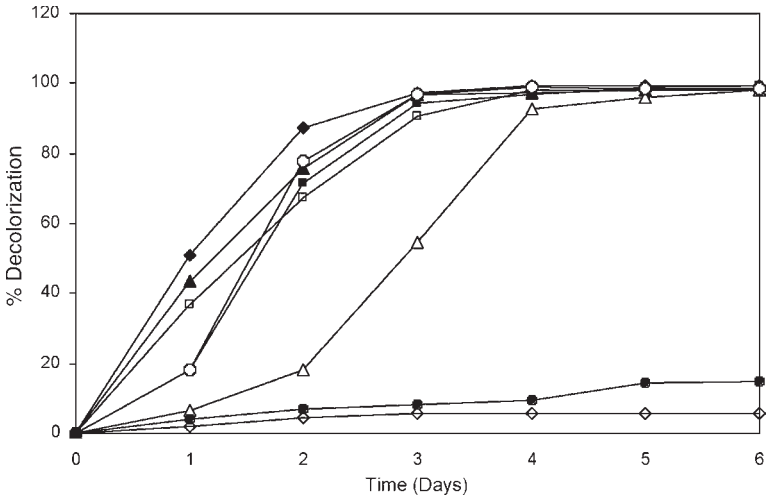

Fig. 1. Decolorization of dyes by *T. versicolor* in liquid medium. ◆, Reactive Blue 28; ■, Reactive Violet 5; ▲, Reactive Golden Yellow R; △, Reactive Black 5; ○, Ponceau Red 4R; □, Procion Red; ◇, Novatic Blue BC S/D; ●, Reactive Red 152.

solvents (data not shown), thus indicating that the decolorization was owing to biotransformation. Note that the concentration of Reactive Black 5 (40 ppm) used for decolorization was half that of the other dyes tested. The lower rates of decolorization for Reactive Black 5 can be correlated to the presence of two azo bonds and a greater number of sulfonic acid groups. An early step in azo dye decolorization is the breaking of the azo bond, the ease of which has been found to depend on the identity, number, and position of functional groups in the aromatic region and the resulting interaction with the azo bond *(21)*. Moreover, susceptibility of dyes under aerobic conditions depends on the aromatic substitution pattern as well as on the microorganism *(22)*. This might be the reason for the inability of

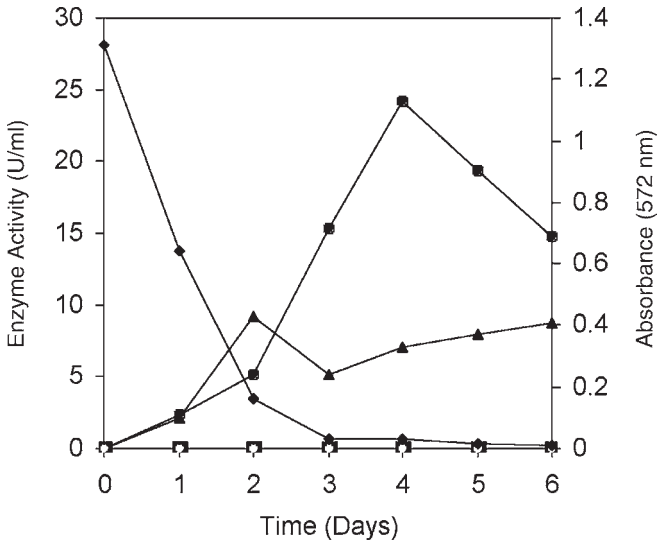

Fig. 2. Enzyme production by *T. versicolor* in liquid medium containing Reactive Blue 28. ◆, Absorbance of residual dye in culture filtrate; ○, LiP; ■, MnP; ▲, MiP; ●, laccase.

T. versicolor to decolorize significantly Novatic Blue BC S/D and Reactive Red 152. Similar studies on the ability of *T. versicolor* to decolorize a wide range of dyes have been reported *(16)*.

Enzyme Production and Dye Decolorization

Further studies were carried out using Reactive Blue 28 since it was most susceptible to decolorization by *T. versicolor* compared with the other dyes used. The time course of various ligninolytic enzyme activities and decolorization of Reactive Blue 28 are shown in Fig. 2. The maximum rate of decolorization of Reactive Blue 28 was observed during the first two days, during which 82% of the dye was decolorized. During decolorization only MiP and laccase activities were detected in culture filtrates whereas MnP and LiP activities were absent. MiP was detected in culture filtrate from the first day, which rapidly increased to a maximum (9.13 U/mL) after 2 d of incubation. Laccase was also detected from the first day onward but was found to increase steadily with maximum activity on the fourth day (24.16 U/mL). Although *T. versicolor* produced both MiP and laccase enzymes when cultivated in BSM without dye, significantly lower activities were observed (Fig. 3). *T. versicolor* produced eightfold higher amounts of MiP in the presence of Reactive Blue 28 compared with that in the absence of dye on the second day. In the case of laccase, only twofold higher activity was observed on the fourth day in *T. versicolor* cultures inoculated with Reactive Blue 28 compared with to those inoculated without dye (Figs. 2 and 3).

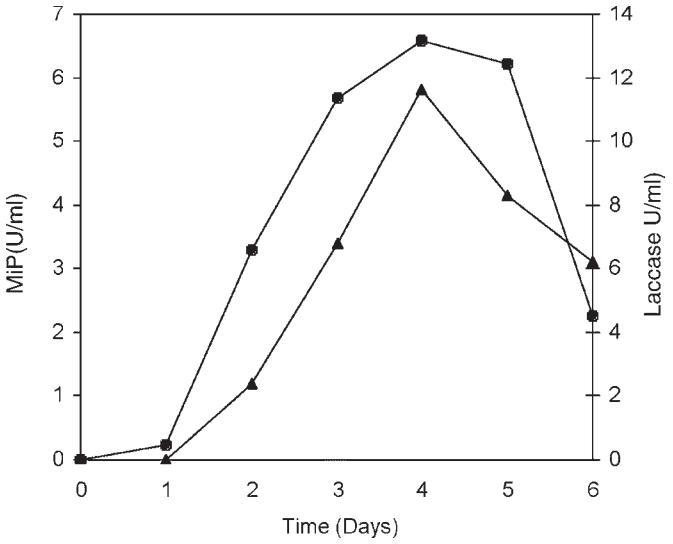

Fig. 3. Enzyme production by *T. versicolor* in liquid medium lacking Reactive Blue 28. ●, Laccase; ▲, MiP.

These results indicate that Reactive Blue 28 does have an inducing effect on the production of MiP and laccase activities by *T. versicolor* and, further, that either or both of these activities might be responsible for decolorization of Reactive Blue 28. Studies on decolorization of dyes by *T. versicolor* have reported the production of MnP and laccase during dye decolorization, and also twice as much production of these enzymes was observed by decolorizing cultures against those to which no dye was added *(15)*. Although these results support our findings, MnP was never detected in culture filtrates of *T. versicolor*.

Decolorization by Culture Filtrate

Reactive Blue 28 (100 ppm) was completely decolorized in liquid cultures by *T. versicolor* within 3 d. On complete decolorization on the third day, culture was filtered to separate biomass and cell-free filtrate was used for decolorization of Reactive Blue 28. Culture filtrate did not decolorize Reactive Blue 28 in the absence of H_2O_2; however, as soon as H_2O_2 was added, steady decolorization of Reactive Blue 28 was observed (Fig. 4). H_2O_2 alone caused no decolorization of Reactive Blue 28. Since decolorizing activity was dependent on the presence of H_2O_2, laccase activity does not seem to be directly involved in the decolorization of Reactive Blue 28. Further, the initial decolorization rate of Reactive Blue 28 was not influenced by the presence or absence of Mn^{2+} or veratryl alcohol in the reaction mixture (Table 3). These results indicate a direct role of MiP in decolorization of Reactive Blue 28. Decolorization of Reactive Blue 28 by culture filtrate was monitored spectrophotometrically, by scanning UV-VIS spectra every 30 s for 600 s, on addition of H_2O_2. A decrease in color intensity of the dye

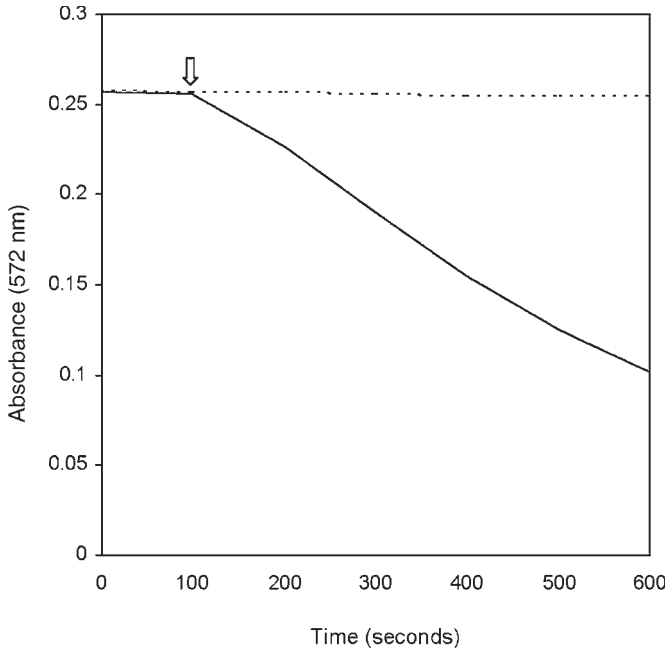

Fig. 4. H_2O_2-dependent dye decolorizing activity of *T. versicolor* culture filtrate. (——) Assay mixture + 0.2 mL of culture filtrate; (– – –) assay mixture + 0.1 m*M* H_2O_2. The arrow indicates the addition of 0.1 m*M* H_2O_2 in the assay mixture. Assay mixtures contained 15 ppm of Reactive Blue 28, 0.1 *M* sodium tartrate (pH 4.5).

Table 3
Effect of Mn^{2+} and Veratryl Alcohol
on Dye-Decolorizing Activity of Culture Filtrate[a]

	Initial decolorization rate (mg/[L·h])
AM	ND
AM + 0.1 m*M* H_2O_2	59.4 ± 4.5
AM + 0.1 m*M* H_2O_2 + 1 m*M* Mn^{2+}	55.2 ± 3.6
AM + H_2O_2 + 2 m*M* veratryl alcohol	60.8 ± 6.0

[a]Assay mixtures (AM) contained 15 ppm of Reactive Blue 28 in 0.1 *M* sodium tartrate (pH 4.5) and 0.2 mL of culture filtrate. ND, not detectable.

was observed by the simultaneous formation of a new peak at 324 nm of the spectra (Fig. 5). Further, high-performance thin-layer chromatography analysis shows the appearance of two compounds in culture filtrates of Reactive Blue 28 decolorizing cultures of *T. versicolor* after d 3 until d 5 of incubation, after which they were not detected (data not shown), thus confirming transformation of Reactive Blue 28.

Studies on decolorization of synthetic dyes have demonstrated a direct role of laccase in decolorization of anthraquinone dyes. Azo dyes are not

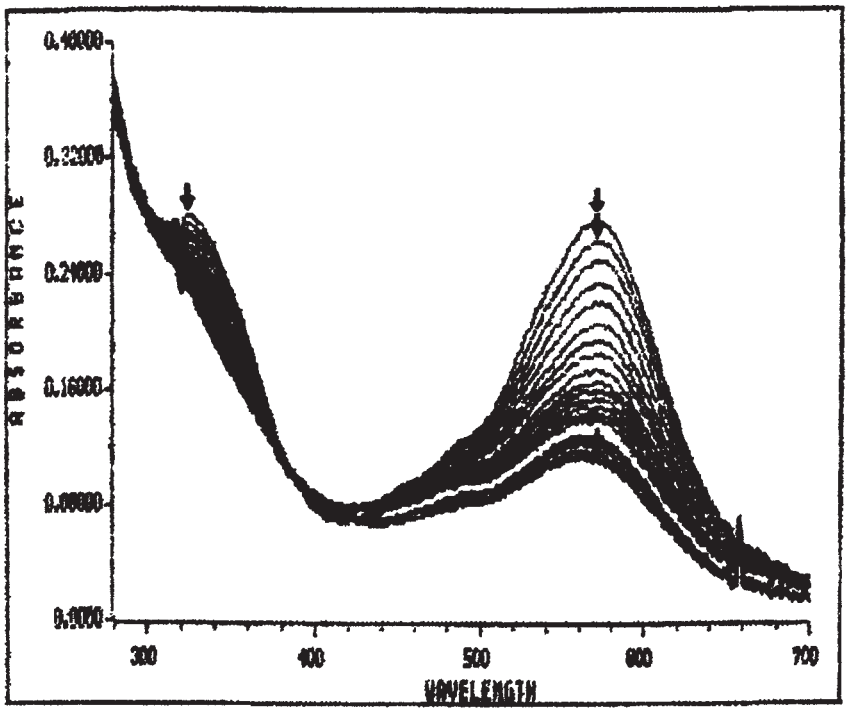

Fig. 5. Absorption spectra of Reactive Blue 28 oxidation by H_2O_2-dependent dye-decolorizing activity of *T. versicolor* culture filtrate. Spectra were recorded at 30-s intervals for 10 min. Assay mixtures contained 15 ppm of Reactive Blue 28, 0.1 *M* sodium tartrate (pH 4.5), and 0.2 mL of culture filtrate. Arrows indicate major changes that occur in the spectra during enzymatic decolorization of Reactive Blue 28.

substrates of laccase, and decolorization of nonsubstrate dyes by laccases requires the presence of mediators such as anthraquinone and ABTS *(9)*. Nevertheless, *T. versicolor* can decolorize azo dyes in the absence of any such mediators in the culture medium, thus indicating a role of other components such as MiP in decolorization of azo dyes. Our results can be useful in understanding the fact that a relationship was not observed between MnP activity and the decolorization rate *(15)*. *Pleurotus ostreatus* produces MiP, which is involved in decolorization of Remazol Brilliant Blue R *(6)*. Heinfling et al. *(23)* have demonstrated transformation of several industrial dyes by MiP isoenzymes purified from *Bjerkandera adusta* and *Pleurotus eryngii*. Our results further support the role of MiP enzyme obtained from *T. versicolor* in dye decolorization.

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