BIOTREATMENT APPROACH TO DECOLORIZING TEXTILE WASTE EFFLUENTS

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Abstract. A very promising area for removing unwanted color from textile wastewater is biotreatment that is targeted at breaking down the dye molecules to basic elements (mineralizing them), and doing so in a way that has much less potential for environmental impact than conventional methods. Previous work in our laboratories demonstrated the success of using white rot fungi for this purpose. The nature of the degradation fragments produced is of great interest because of concern that these may increase the aquatic toxicity of the overall effluent. We report the development of method to identify the degradation products using thin layer chromatography, HPLC, capillary electrophoresis and mass spectrometry.

INTRODUCTION

Azo dyes, which account for approximately one-half of all known dyes, are commonly used as coloring agents in the food, pharmaceutical, and textile industries. As a result, they are the most common synthetic colorants released into the environment (Bumpus 1995). Because they are highly colored, azo dyes are readily visible in effluent water and can be the focus of significant environmental complaints centered around that visibility. These compounds are also of concern because some of the dyes, dye precursors or their biotransformation products, such as aromatic amines, have been shown to be carcinogenic (Razo-Flores et al. 1997). The nonspecific nature of the lignin-degrading systems of fungi are of increasing interest in the study of textile effluent treatment. Although their function in nature is to degrade lignin, they have the capability to degrade other types of compounds. Various investigations have shown that white rot fungi can efficiently degrade various organic pollutants other than lignin. Examples include polycyclic aromatic hydrocarbons, persistent environmental pollutants such as DDT (Bumpus et al. 1985, 1989), alkyl halide insecticides (Kennedy et al. 1990), xenobiotic compounds (Paszczynski et al. 1995), and dinitrotoluene (Valli et al. 1992).

The similarities of the molecular structures of the above compounds to those of dyes was obvious to the early investigators. This initiated interest in using the fungi for decolorization of dye wastewater. This application to the decolorization of azo dyes using the white rot fungus *Phanerochaete chrysosporium* was first described by Cripps et al. (1990). They showed that three azo dyes, Orange II, Tropaeolin O, and Congo Red, could be decolorized by *Phanerochaete chrysosporium.* Other white rot fungi, such as *B. adusta* and *T. versicolor,* also showed the ability to degrade azo dyes efficiently (Heinfling et al. 1997). In our own laboratory, various white rot fungi including *P. chrysosporium, P. cinnabarinus, T. versicolor, C. subvermispora, C. stercoreus, P. ostreatus, P. tremellia, P. oxysponum,* and *P. pini* have been screened for their capability in degradation of commercially used dyes (Cao 2000). This and other work showed that *Pleurotus ostreatus,* had high potential for decolorizing a variety of azo dyes (Cao 2000, Shin et al. 1998). Despite the known ability of the fungi to break down dyes and render them colorless, there have been few attempts to comprehensively examine the dye effluent systems after biotreatment by white rot fungi and determine the molecular nature of the process. Decolorization, by itself, demonstrates only the transformation of the chromophoric group of a dye, but does not reveal much about the mode of degradation of the dye molecules. The objective of this paper is to examine the decolorization of two widely used disperse azo dyes in cultures of *Pleurotus ostreatus* and to analyze the degradation products generated in the biotreatment system. The results gained by treating these two dyes with relatively simple structures will be used to start development of a comprehensive analytical method for investigating the degradation of dyes by white rot fungi.

MATERIALS AND METHODS

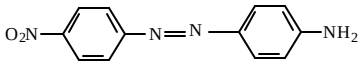
Chemicals

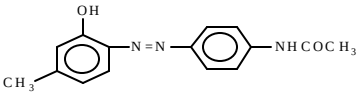
Two disperse dyes of relatively simple structures (see Figure 1) were chosen for this work. Disperse Orange 3 (20 percent purity) and Disperse Yellow 3 (30 percent purity) were obtained from Aldrich Chemical Company (Milwaukee, Wis.), as was Disperse Orange 3 in a 90 percent pure form. The dyes were converted to pure form by recrystallization. In order to determine positively the

degradation products from these dyes, probable compounds were were postulated. These were:

4-aminophenol 4-nitrophenol
1,4-benzoquinone
4-nitroaniline
aniline
1,4-phenylenediamine acetanilide
4'-aminoacetanilide
4-acetamidophenol
4'-nitroacetanilide
6-amino-m-cresol

These degradation "model" compounds were purchased from Aldrich Chemical. The organic solvents employed in the experiments were at least reagent grade and used as purchased.





a.Disperse Orange 3 (C.I. 11005)
b.Disperse Yellow 3 (C.I. 11855)

Figure 1. Structures of the disperse Dyes.

Microorganism. Previous work done in our lab had involved screening a number of white rot fungi for their ability to decolorize a variety of dye molecular structures (Cao, 2000). The most successful candidate was the *Pleurotus ostreatus* fungus (strain Florida) used in the work discussed herein. This fungus was isolated at the University of Georgia (Eriksson, 1987). The culture was maintained on malt agar plates at 30 degrees C, with subcultures routinely made every month.

Culture Conditions. Nutrient nitrogen-limited cultures of *P. ostreatus* made with Kirk's medium (Kirk et al. 1978) were incubated at 30 degrees C in a liquid culture medium contained in a 250 ml Erlenmeyer flask at pH 5.0.

Decolorization of Commercial Dyes. Cultures were established in the incubator, shaken at 200 rpm, and allowed to grow for three days. On day three, the two commercial azo dyes were dissolved in methanol at 1.0 mg/l concentration. One disperse dye (including dispersing agents) was added to each culture for final dye concentrations of 60 ppm. Controls were run with the same reaction conditions but excluding fungus. These experiments were replicated three times.

Decolorization Determination.

Aliquots of 1-2 milliliters of clear dye solution were taken from the each reaction flask at regular time intervals and measured immediately using a UV-Vis recording double beam spectrophotometer (Shimadzu). Care was taken not to draw out portions of the fungus in the aliquot. All samples from the dyebaths had to be diluted prior to measurement in order to keep the change in absorbance values measured below 2.5 absorbance units per centimeter of pathlength. Because of the low water solubility of these dyes, an equal volume of methanol was mixed with the analytical solution to ensure complete solubilization prior to measurement. Decolorization was assessed in two ways: one way was by monitoring spectrophotometrically the absorbance at the wavelength maximum for each dye; secondly, by the reduction of the major peak area in the visible region for each dye. Both approaches were used because color change may occur during biodegradation with a concommitant wavelength shift. In that case, just using the wavelength maximum of the dye would not be sufficient to accurately reflect the full degree of decolorization. To obtain additional information regarding the changes, the area under the curve in the visible regions (400-800 nm) was integrated.

Dye Purification. In order to analyze degradation products without interference from extraneous materials, the disperse dyes were purified before using in the experiments. This was done by recrystallization in acetonitrile for Disperse Orange 3 and in ethyl alcohol for Disperse Yellow 3. The purity of both dyes was checked by high performance liquid chromatography (HPLC) and differential scanning calorimetry (DSC). Methanol solutions of the purified dyes were added to the fungal cultures to achieve a concentration of 60 ppm of the dye in the culture solution.

Degradation of Purified Disperse Dyes. All dyes were dissolved in methanol at 1.0 mg/mL concentration and were added to the flask containing the fungus system to give a final concentration of 60 ppm. After three days treatment, the degradation samples were prepared for thin layer chromatography analysis.

Thin Layer Chromatography (TLC)

Sample preparation. The mixture of the dye solution and the dispersed fungus constituted a quantity of about 400 milliliters (from three flasks). After 3 days, the solution was filtered and concentrated by rotary vacuum evaporator. The concentrated solution was extracted by methylene chloride of equal volume three times. The organic phases were combined and concentrated to about 10 ml. A salting out procedure was used to facilitate the extraction. The samples were kept at a low temperature

until further analysis. The fungal spores are also extracted by methylene chloride.

Thin Layer Chromatography

Selecto Silica Gel 60, F-254, 5 × 20 cm plates (Selecto Scientific, GA) were used in the analysis. The specimens investigated were applied on the starting line with a volume of 4-5 microliters. The mobile phase for analysis of the Disperse Orange 3 degradation components was an ethyl acetate - hexane (2:1) mixture. An ethyl acetate - hexane (4:1) mixture was used for analysis of the Disperse Yellow 3 degradation solution. The chromatograms were run until the solvent front traveled 80 millimeters. The R_f value of each degradation product was compared to those of the standards for identification of the structure. The samples were run on three different plates and the average R_f value of spots of three runs was used for comparison. The standard compounds were run on the same plate.

RESULTS AND DISCUSSION

The absorptivities are intrinsic properties of a colorant. Measuring the spectral absorption curves of dyes during biodegradation provided a means to determine the reaction rate and gave evidence of the structural change of dyes during biodegradation. Previous experience showed that fading of the original color of the dyes is sometimes accompanied by a color change (wavelength shift) in the solution. Because of this, both the ultra-violet and visible regions were monitored by a UV-Vis spectrophotometer to provide more information about the biodegradation. The decrease of the maximum absorbance of disperse dyes occurred primarily in the first several days of treatment, with no substantial additional decrease in absorbance as the treatment time increased.

This is shown in both Figure 2 and Table 1.

Disperse Orange 3 (Figure 1a) contains two substituted aromatic rings, one with an amino substituent and the other with a nitro substituent, typical of many monoazo disperse dyes. The visible portion of the spectrum of Disperse Orange 3 showed a major peak at 415 nm. After five days of biotreatment, this peak shifted to 388 nm and decreased by 57 percent. The reduction of the area of the major peak in the visible region was 65 percent, which indicated substantial decolorization. A second disperse dye examined was Disperse Yellow 3 (Figure 1b), widely used in the United States. It is also a potential carcinogen (Spadaro et al. 1992). Decolorization with *P. ostreatus* caused a 57 percent decrease in absorption after 5 days (Figure 3 and Table 2), This was indicated by the decrease of absorption at the maximum wavelength and the decrease in the area under the absorption curve in visible region. No new peaks appeared in the UV region after decolorization.

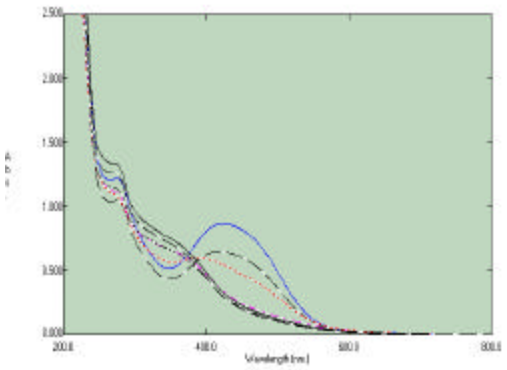


Figure 2. UV-Vis spectra of Disperse Orange 3 before and after biodegradation by *P. ostreatus*. The curves represent 1 day intervals.

Table 1. Decrease in Absoption of Disperse Orange 3 After Degradation (%)

Day	Absoption decrease (@ λ_{max}) (%)	Decrease in peak area in visible region (%)
0	0	0
1	36	35
2	39	40
3	53	57
4	59	65
5	57	65

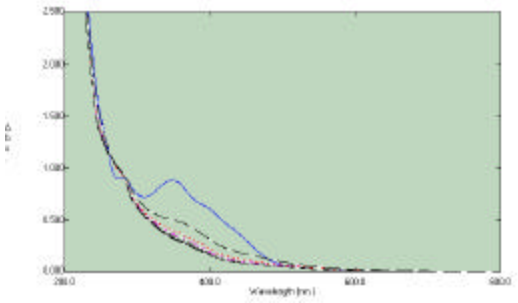


Figure 3. . UV-Vis Spectra of Disperse Yellow 3 before and after biodegradation by *P. ostreatus*. The curves represent 1 day intervals.

Table 2. Decrease in Absoption of Disperse yellow 3 after Degradation (%)

Day	Absoption decrease (@ λ_{max}) (%)	Decrease in peak area in visible region (%)
0	0	0
1	26	22
2	32	29
3	44	32
4	50	40
5	57	57

The monitoring of the UV-Vis spectra supplied information on the nature of the decolorization, particularly with the shifts in the absorption maxima. These could only be the result of chemical change in the conjugated double bond structure, rather than just removal of the unchanged dye molecules. However, complex degradation products produced by the actions of the enzymes from the white rot fungi could only be identified by separation, followed by an identification scheme. To this purpose, thin layer chromatography was employed to identify the degradation products. A number of solvents, including benzene, methanol, acetone, chloroform, tetrahydrofuran, toluene, n-hexane, and ethyl acetate, were screened for effectiveness in the thin layer chromatography approach. The combination of n-hexane and ethyl acetate proved to be the best system for separation.

The ratio of ethyl acetate and n-hexane was changed from 8:1 to 1:2 at intervals of 2:1. Satisfactory separation of the degradation products of Disperse Orange 3 was achieved with ethyl acetate and n-hexane in a ratio of 2:1. A mixture of ethyl acetate and n-hexane in a ratio of 4:1 was very successful in the separation of the degradation products of Disperse Yellow 3.

After treatment with the white rot fungi for the three days, two degradation products were detected from treatment of Disperse Orange 3 and one degradation product from Disperse Yellow 3. The nine compounds which were postulated as degradation products -- 4-aminophenol, 4-nitrophenol, 1,4-benzoquinone, 4-nitroaniline, aniline, 1,4-phenylenediamine, acetanilide, 4'-aminoacetanilide, 4-acetamidophenol, 4'-nitroacetanilide, and 6-amino-m-cresol -- were run under the same conditions as the treated dye solution samples for the qualitative identification of the separated spots on the plates. Among these compounds, 4-nitroaniline showed its possibility to be one of degradation products from Disperse Orange 3. After running both samples and standard on the same plate and changing mobile phase to ethyl acetate-benzene (4:1), it showed that 4-nitroaniline was likely to be one of major products of Disperse Orange 3 after degradation (Data is not shown here). Because the disperse dyes were added into the fungal culture in the methanol solution, the effect of methanol on the degradation of dyes is investigating because the mechanism of biodegradation of azo dyes has not been elucidated yet.

CONCLUSIONS

UV-Vis spectra showed that *P. ostreatus* degraded water unsoluble azo dyes in the decolorization. The absorption of new compounds was indicated from the spectra, which indicated the degradation products generated in the decolorization. At least two major degradation compounds of Disperse Orange 3 and one degradation products of Disperse Yellow 3 had been confirmed by the results from thin layer chromatography.

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