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HPLC and spectrophotometric analysis of biodegradation of azo dyes by *Pleurotus ostreatus*

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Abstract

A white rot fungus *Pleurotus ostreatus* was employed for degradation of two commercially used disperse azo dyes, Disperse Orange 3 (C.I. No. 11005) and Disperse Yellow 3 (C.I. No. 11855). Decolorization and products from fungal degradation of these azo dyes in liquid medium were determined by UV–visible spectrophotometric method and high performance liquid chromatography (HPLC). Decolorization study showed that both azo dyes were removed by more than 50% in 5 days and HPLC analysis determined several degradation products. These results suggest that *P. ostreatus* has potential in color removal from textile wastewater containing disperse dyes.

Keywords: Biodegradation; Disperse azo dyes; *Pleurotus ostreatus*

1. Introduction

A great number of dyes and other chemicals are used in textile wet processing. It is estimated that about 10–15% of dyes are released into processing water during this procedure [1,2]. Being highly colored, dyes are readily apparent in wastewater, which is the reason their breakdown is a priority before discharge into the environment. Azo dyes make up about a half of all known dyestuffs in the world, making them the largest group of synthetic colorants and the most common synthetic dyes released into the environment [1]. In mammals, azo dyes are reduced to aryl amines by cytochrome P450 and a reductase [3].

At present, there is no satisfactory method to economically and reliably decolorize and detoxify textile wastewater. Recent research points toward the potential of fungal wastewater treatment for textile industries. The satisfactory ability of white rot fungi to depolymerize lignin is well known. Their ability to degrade synthetic chemicals, such as azo dyes, is also very important because these dyes are usually recalcitrant to microbial degradation and cause problems in biotreatment of industrial effluents [4]. The most studied white rot fungus, *Phanerochaete chrysosporium* has been reported to decolorize dyes with enzymes involved in lignin degradation, such as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase. Other white rot fungi, such as *Trametes versicolor*, *Pleurotus ostreatus*, *Bjerkandera fumosa*, *Thelephora* sp., have been reported to be able to decolorize various classes of dyes [1,5,6].

Disperse dyes are widely used in the textile industry because they are the only dyes which can be used for dyeing polyester fibers. It is desirable to understand the potential of fungal degradation on this kind of dyes. However, there are some difficulties in determining the degradation of disperse dyes in liquid media because of their low solubility in water and the presence of surfactants.

In the research reported herein, we studied the fungal degradation of two widely used disperse dyes by *P. ostreatus* using reversed phase high performance liquid chromatography (HPLC) and UV–visible spectrophotometry.

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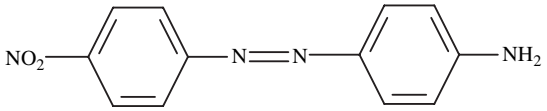
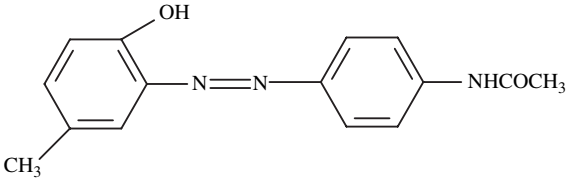


Disperse Orange 3 (C.I. 11005)



Disperse Yellow 3 (C.I. 11855)

Fig. 1. Structure of studied azo dyes.

2. Materials and methods

2.1. Chemicals

C.I. Disperse Orange 3 (C.I. No. 11005, 20% dye content, Fig. 1a) and C.I. Disperse Yellow 3 (C.I. No. 11855, 30% dye content, Fig. 1b) were purchased from Aldrich Chemical Co. (Milwaukee, WI). These commercial dyes contain dispersing agents and were used as such. Acetonitrile (Aldrich) and methanol (EMD Chemicals, Gibbstown, NJ) used in HPLC analysis and sample preparation were of HPLC grade. Phosphoric acid (85%), potassium dihydrogen phosphate and sodium hydroxide pellets were of analytical grade (J.T. Baker, Phillipsburg NJ). All other chemicals used throughout this study were reagent-grade chemicals. Purified water was obtained from an ion exchange and membrane filtration system from U.S. Filter (Warrendale, PA).

2.2. Culture conditions and biodegradation

P. ostreatus (strain Florida) was obtained from the laboratory of Dr. Karl-Eric Eriksson at the University of Georgia. The culture was maintained on malt agar plates (malt extract 20 g/L, agar 15 g/L) at 30 °C, with subcultures routinely made every month. Nitrogen-limited cultures of *P. ostreatus* grown in Kirk's medium [7] were incubated at 30 °C in 250 ml Erlenmeyer flasks at pH 5.0. Cultures were established in the incubator, shaken at 200 rpm, and allowed to grow for 3 days.

Disperse dyes were added to the *P. ostreatus* culture on the fourth day to give a dye concentration of 200 ppm in the dispersion, and biodegradation was conducted at a shaking rate of 150 rpm. Controls were carried out in the same conditions but without dyes or inoculum. Results were reported based on at least three independent assays.

Table 1
Mobile phase program for gradient method

Time (min)	Flow (ml/min)	%A	%B	Curve
0.0	1.0	5.0	95.0	Linear
20.0	1.0	25.0	75.0	Linear
30.0	1.0	40.0	60.0	Linear
40.0	1.0	40.0	60.0	
45.0	1.0	5.0	95.0	Linear

A, acetonitrile; B, 0.025 M phosphate buffer with pH 3.0.

Table 2
Decrease in absorbance – Disperse Orange 3 after degradation (%)

Day	Absorbance decrease (at 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

2.3. Spectrophotometric analysis

Aliquots of 1–2 ml volume of clear dye solution were taken from each reaction flask at regular time intervals and measured immediately using a UV–vis recording double beam spectrophotometer (Shimadzu). 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 determined spectrophotometrically by monitoring the absorbance at the wavelength maximum for each dye, and by the reduction of the major peak area in the visible region for each dye.

2.4. Liquid chromatography

A Hewlett–Packard 1100 series HPLC system (Hewlett–Packard GmbH, Germany), consisting of a model G1311A quaternary pump, G1322A degasser, and a diode array detector (Model G1315A), operated at 254 nm was used to perform the analysis. A stainless steel Ultracarb™ ODS column with 5 μm packing from Phenomenex (150 × 4.6 mm I.D.) was used in the analysis and an RP-C18 guard pre-column was installed to protect the analytical column. A gradient method with the 0.025 M phosphate buffer (pH = 3.0)–acetonitrile mobile phase was employed in the separation (Table 1). HP ChemStation software (version 3.1) was used for data processing and reporting. The injection volume was 100 μl each time to achieve reproducible injection, which was conducted with

Table 3
Decrease in absorbance – Disperse Yellow 3 after degradation (%)

Day	Absorbance decrease (at 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

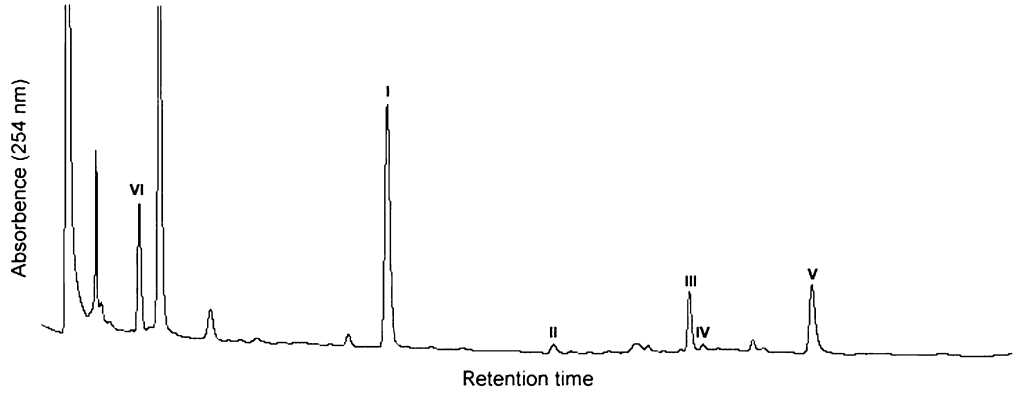


Fig. 2. Chromatograms of fungal degradation samples from Disperse Orange 3. Peak I, veratryl alcohol; peak II, 4-nitroaniline; peak III, nitrobenzene; peak IV, 4-nitroanisole; peak V, Disperse Orange 3; peak VI, unknown metabolite.

automatic injector (Model 1313A). The flow rate was kept at 1 ml/min during the run and the temperature was kept at 25 °C for the separation column.

Three milliliters of supernatant fluid was taken from the white rot fungal culture fluid by pre-autoclaved pipette regularly and the same amount of medium with 200 ppm dyes was replenished after sampling. The culture liquid was then filtered through a 0.45 μm membrane filter (Whatman, Clifton, NJ) before HPLC analysis. Peak identities of the degradation products were confirmed by both retention time and spectra matching of standard compounds.

3. Results and discussion

3.1. UV–visible spectrophotometric analysis

The ultraviolet and visible absorbance (from 200 to 800 nm) of dye samples were monitored by a UV–vis spectrophotometer to examine the biodegradation rate of azo dyes by *P. ostreatus*. A visible decolorization occurred in the medium of *P. ostreatus* for both dyes. Most color removal in the first day may due to dye absorption by mycelium of fungi, which took on the color of the dye. To accurately reflect the full degree of decolorization, both the wavelength maximum of the dye and the area under the curve in the visible regions (400–800 nm) were employed in the calculation.

Disperse Orange 3 (Fig. 1a) contains two substituted aromatic rings, one with an amino group and the other with a nitro substituent, typical of many monoazo disperse dyes. The visible portion of the spectrum of Disperse Orange 3 shows a major peak at 415 nm. The biggest color removal occurred in the first 24 h. The maximum wavelength of absorbance decreased by 57% and the reduction of the area of the major peak in the visible region was 65%, indicating substantial decolorization (Table 2). A second disperse dye examined was Disperse Yellow 3 (Fig. 1b), a widely used yellow disperse dye in the United States. It also has been indicated as a possible carcinogen [8]. The use of *P. ostreatus* caused 57% decolorization of this dye after 5 days of treatment (Table 3), which was indicated by calculation of decrease in peak height at the maximum absorption wavelength and area in the visible region. No new peaks appeared in the UV region after decolorization.

3.2. HPLC analysis of biodegradation products

Several degradation products were determined by HPLC analysis. Nitrobenzene, 4-nitroanisole, and 4-nitroaniline were identified by comparison of the retention time and UV–visible spectrum of sample peaks from Disperse Orange 3 with the standards (Fig. 2). A metabolite (VI) could not be determined at this time. Veratryl alcohol (I) was found in

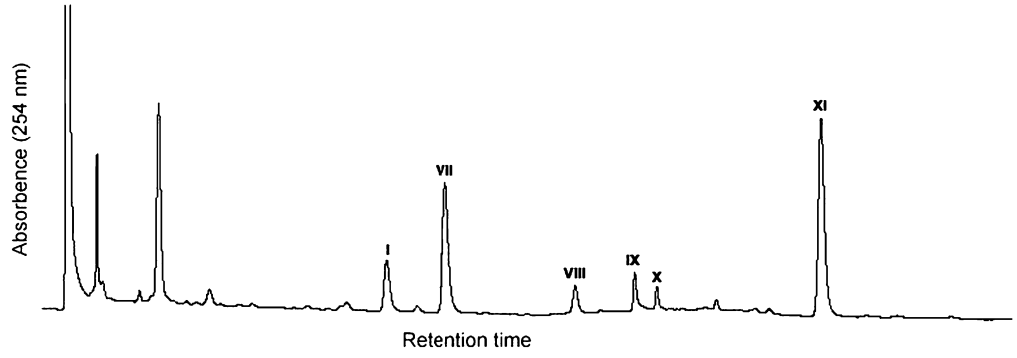


Fig. 3. Chromatograms of fungal degradation samples from Disperse Yellow 3. Peak I, veratryl alcohol; peak VII, acetanilide; peak VIII, unknown metabolite 1; peak IX, unknown metabolite 2; peak X, unknown metabolite 3; peak XI, Disperse Yellow 3.

both the liquid medium of Disperse Orange 3 and Disperse Yellow 3 during fungal degradation. Veratryl alcohol is a secondary metabolite synthesized de novo from glucose by fungi and is a physiological cofactor of lignin peroxidase (LiP) [9,10]. The major function of veratryl alcohol is as a redox mediator for LiP and protecting LiP during the redox cycle from hydrogen peroxide [11,12].

In our assay of Disperse Yellow 3, one major degradation product, acetanilide, was determined as well as three minor products being detected (Fig. 3). The identification of these minor metabolites could not be determined at this time and will be done later by mass spectrometry. Spadaro and Renganathan [8] have showed that culture of *P. chrysosporium* degraded Disperse Yellow 3 to acetanilide via a peroxidase oxidation mechanism.

Our study showed that *P. ostreatus* could degrade both phenolic and non-phenolic disperse azo dyes with both enzymatic and non-enzymatic systems involved in the decolorization of these dyes with liquid medium. Further study of the mechanism of degradation of disperse dyes with similar structures by *P. ostreatus* is underway.

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