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GC-MS AND SPECTROPHOTOMETRIC ANALYSIS OF BIODEGRADATION OF NEW DISAZO DYE BY *Trametes versicolor*

© 2011 H. Ardag Akdogan*, A. Demircali*, C. Aydemir*, N. Pazarlioglu**, F. Karci*

*The University of Pamukkale, Faculty of Science & Arts, Department of Chemistry, P.O. 286, 20017, Denizli, Turkey
e-mail: hardag@pamukkale.edu.tr

**Ege University, Faculty of Science, Department of Biochemistry, 35100 Izmir, Turkey

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In this study; sub-tropical white rot fungi, *Trametes versicolor* was investigated for its ability to degrade 4-(3'-methyl-4'-(4''-nitrophenyl)azo-1'H-pyrazol-5'-ylazo)-3-methyl-1H-pyrazol-5-on in the mediums containing glucose and different concentrations of degrade dye in batch systems. This dye was synthetized at Pamukkale Universtiy of Organic Chemistry research laboratory. Samples were collected on 10 days, and was detected by Shimadzu UV-1600A spectrophotometry. Decolorization study showed that this disazo dye was removed by more than 70% in 10 days. Laccase enzyme activity was detected in samples and then last sample was analyzed by GC-MS. Metabolites weren't showed in GC-MS result. It was concluded that *T. versicolor* could achieve the biodegradation of this new disazo dye.

Azo dyes account for the majority of all textile dye stuffs produced and have been the most commonly used synthetic dyes in the textile, food, paper making, color paper printing, leather and cosmetic industries [1–3]. Synthesis of most azo dyes involves diazotization of primary aromatic amines followed by coupling with one or more nucleophiles [4]. Benzidine (**BZ**)-based azo dyes have been found to be tumerogenic [5] and carcinogenic [6, 7] due to their biotransformation to BZ [8]. Azo dyes are recalcitrant xenobiotics and therefore, conventional aerobic wastewater treatment processes usually cannot efficiently decolorise and degrade azo dye bearing effluents to the regulatory levels [2].

The growing interest in the pyrazole chemistry lies in designing new synthetic approach, theoretical calculations and applications of newer spectroscopic techniques. The usage of many pyrazole derivatives has undoubtedly created considerable attention in developing many different synthetic procedures in pharmaceuticals, agrochemicals, dyestuff. The recent developments in the synthetic routes and the chemistry of pyrazoles have been throughly reviewed [9–15]. The use of heterocyclic intermediates in the synthesis of azo disperse dyes is well established and the resultant dyes exhibit good tinctorial strength and brighter dyeing than those derived from aniline-based diazo components. For instance, amino-substituted thiazole, benzothiazole [16–19] and benzoisothiazole [20] compounds afford highly electronegative diazo components and consequently, provide a pronounced bathochromic effect compared to the corresponding benzoid compounds.

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. 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 [21].

In the present study, new disazo dye, acetonitrile soluble, disazo based azo dye (Fig. 1), was selected for carrying out microbial decolorisation and biodegradation studies. Also, a comparative study of the toxicity potential of the dye before and after degradation treatment was carried out.

MATERIALS AND METHODS

Dye solution preparation. New disazo dye (Fig. 1) was dissolved in acetonitrile to prepare a stock solution (100 mg/l). Solutions of the desired concentrations were obtained by successive dilution.

Organism and culture conditions. The white rot fungus *T. versicolor* (ATCC 11 235) was maintained on 2% (w/v) malt agar slants at 4°C and the fungus was activated at 26°C, for 3 days. The mycelium were harvested with sterile 0.9% NaCl solution and then inoculated into 100 ml of 2% malt extract broth (pH 4.5) in 250 ml Er-

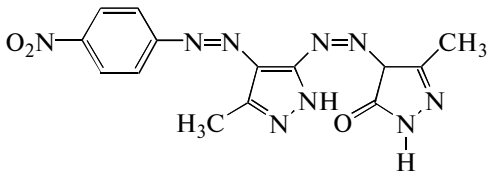

Fig. 1. Molecular structure of new disazo dye.

lenmeyer flasks at 26°C and 175 rpm for 4 days. Pellets were inoculated into the medium consisting of (g/l): glucose – 10.0, $NH_4H_2PO_4$ – 1.0, $MgSO_4 \cdot 7H_2O$ – 0.05, $CaCl_2$ – 0.01, yeast extract – 0.025. Cultivation was carried out in an orbital shaker incubator, at 26°C 175 rpm [22]. At the beginning of the fourth day of incubation, 4-(3'-methyl-4'-(4''-nitrophenyl)azo-1'H-pyrazol-5'-ylazo)-3-methyl-1H-pyrazol-5-on was added to the flasks aseptically at the desired concentrations. Aliquots were assayed for the laccase activity.

Sampling. Samples (1 ml) were taken every day (total 10 day). Samples were centrifuged to remove suspended biomass and ligninolytic (laccase) activities in supernatants were determined. All experiments were performed in triplicate.

Analytical methodology. *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, Japan). Because of the low water solubility of this dye, an equal volume of acetonitrile 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 this dye, and by the reduction of the major peak area in the visible region for new disazo dye.

Extraction procedure. Fluorene extraction and analysis was performed as described in Yuan et al. [23] and Chang et al. [24]. Briefly, 1 ml of n-hexane was added 1 ml sample. The organic phase was collected. The combined crude extracts of organic phase were dried. The residue was dissolved in acetonitrile (1.5 ml), filtered through a 0.45 μm membrane filter and an aliquot of 5 μl was taken for GC-MS analysis.

GC-MS analysis. Shimadzu GC-2010, gas chromatograph equipped with MS-QP2010 plus mass spectrometer, AOC-20s auto sampler and AOC-20i auto injector were used for analysis. A 30 m × 0.25 mm I.D. × 0.25 μm HP-5MS fuse-silica capillary column was employed. The column temperature program was set as follows: 60°C hold for 1 min, at 10°C/min to 200°C hold for 15 min. The GC injector was held isothermally at 250°C with a splitless period of 3 min. All injection volumes were 1 μl in the splitless mode. The solvent delay time was set at 5 min. Helium was used as the carrier gas, at a flow rate of 10 ml min^{-1} by using electronic pressure control. The GC/MS interface temperature was maintained at 250°C. The MS was operated in electron impact (**EI**) ionization mode with electron energy of 70 eV and scan ranged from 50 to 500 amu (atom to mass unit) to determine appropriate masses for selected ion monitoring.

Enzyme assays. Laccase (**Lac**) production was assessed by measurement of enzymatic oxidation of 2,2'-azinobis-(3 ethylbenzothiazoline-6-sulphonic acid) (ABTS) at 420 nm ($2 = 3.6 \times 10^4$ cm^{-1} M^{-1}) [25]. The reaction mixture contained 300 ml of extracellular fluid, 300 ml of 1 mM ABTS and 0.1 M Na-acetate buffer (pH 4.5). One unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 mmol ABTS in 1 min.

RESULTS AND DISCUSSION

^{1}H-NMR and FT-IR analysis of new diszao dye. The infrared spectra of new synthetized disazo dye (in KBr) showed pyrozol and pyrozolone (N–H) bands at 3169 ve 3103 cm^{-1}, aromatic C–H band at 3068 cm^{-1}, pyrozol and pyrozolone ($-CH_3$) band 2971 cm^{-1}, carbonyl (C=O) bands at 1678 cm^{-1} (Fig. 2).

The ^{1}H-NMR spectra measured in DMSO-*d6* at 25°C showed a singlet peak for pyrozolone at 2.08 ppm ($-CH_3$), a singlet peak for pyrozol at 2.54 ppm ($-CH_3$), a multiplet from 8.30–8.10 ppm for aromatic protons (Aro-H), a broad peak for enol tautomer (–OH) or hydrozo tautomer (–NH) at 11.61 ppm, a singlet peak for pyrozolone proton (–NH) at 13.34 ppm, a singlet peak for pyrozol proton (–NH) at 14.03 ppm (Fig. 3).

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 disazo dye by *T. versicolor*. A visible decolorization occurred in the medium of *T. versicolor* for this dye. 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. 4-(3'-methyl-4'-(4''-nitrophenyl)azo-1'H-

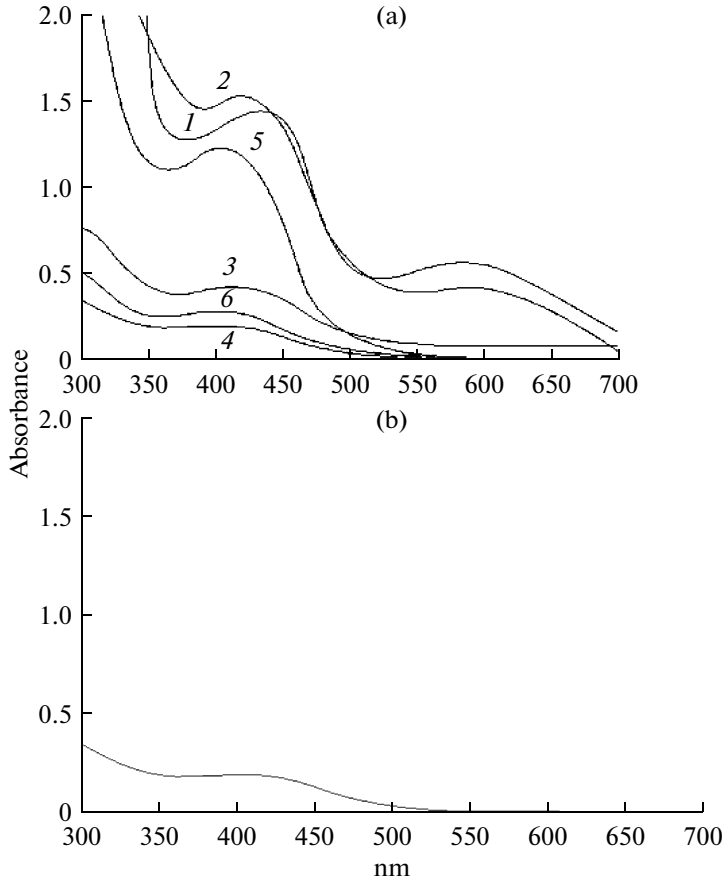

Fig. 2. FT-IR spectrum (a) and the ^{1}H-NMR spectrum (b) of new synthetized disazo dye: *1* – DMSO, *2* – DMF, *3* – acetonitrile, *4* – metanol, *5* – acetic acid, *6* – chloroform.

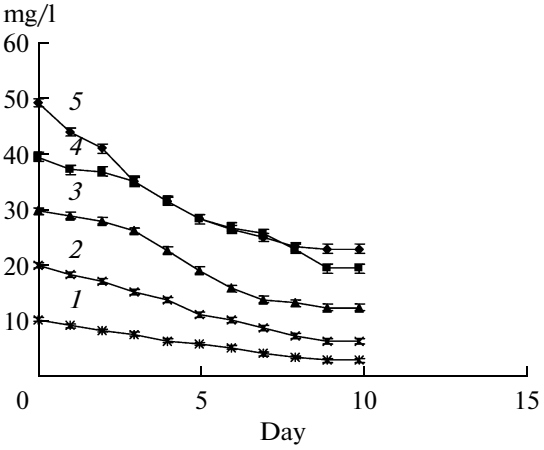

Fig. 3. Biodegradation of disazo dye: *1* – 10, *2* – 20, *3* – 30, *4* – 40, *5* – 50 mg/l of disazo dye.

pyrazol-5'-ylazo)-3-methyl-1H-pyrazol-5-on (Fig. 1) contains one substituted aromatic rings, one with an amino group and the other with a nitro substituent, typical of disazo disperse dye. The visible portion of the dye spectrum shows a major peak at 408 nm in acetonitrile. The biggest color removal occurred in the first 6 day. The maximum wavelength of absorbance decreased by 78% (table). In different concentration of this new disazo dye

removal was exhibited in Fig. 2. Spectrum of new disazo dye and spectrum of medium after degradation were shown Fig. 3.

Ligninolytic enzyme activities in different fluorene concentrations. In this study, laccase activities were measured daily (Fig. 4). Laccase (benzenediol: oxygen oxidoreductase, EC.1.10.3.2) from white-rot fungi are part of the complex enzymatic system for lignin degradation [26, 27] catalyse the oxidation of a wide variety of organic and inorganic substrates, including mono-, di- and polyphenols, aminophenols, methoxyphenols, aromatic amines and ascorbate with the reduction of oxygen [28, 29]. In this study, in order to determine the role of ligninolytic enzymes on new disazo dye degradation, laccase was monitored during the degradation of this compound.

GC-MS analysis. Total ion chromatograms (**TIC**) obtained from GC-MS analysis of the degradation products. TIC shown in Fig. 5 was also present in the control cultures. In order to verify the presence of cell-bound metabolites, the fungal mycelium was extracted with hexane. When the supernatant was extracted with hexane, no metabolites other than those obtained when the extraction was carried out.

Biodegradation results

Concentration of dye, mg/l	Biodegradation, %	Standard deviation
50	54.22	±0.2
40	52.9	±0.5
30	60.22	±0.4
20	70.79	±0.3
10	78.12	±0.6

In our assay of 4-(3'-methyl-4'-(4'-nitrophenyl)azo-1'H-pyrazol-5'-ylazo)-3-methyl-1H-pyrazol-5-on, minor products were detected (Fig. 6). The identification of these minor metabolites could not be determined by

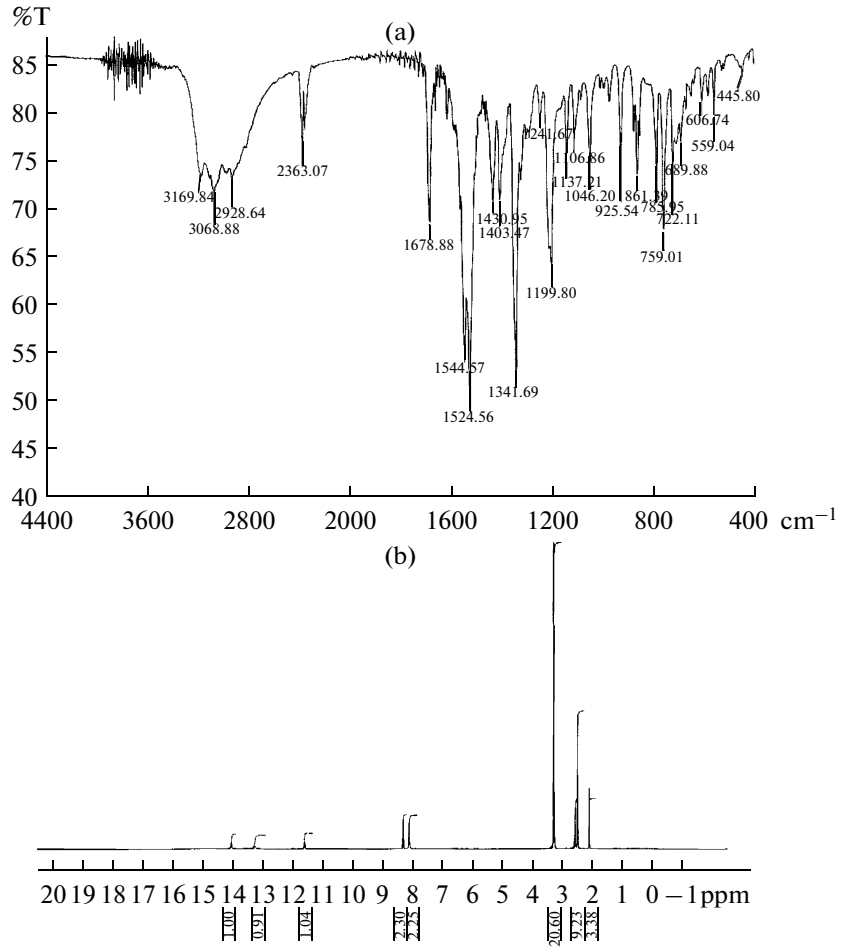

Fig. 4. Spectrum of new disazo dye (a) and of medium after degradation (b).

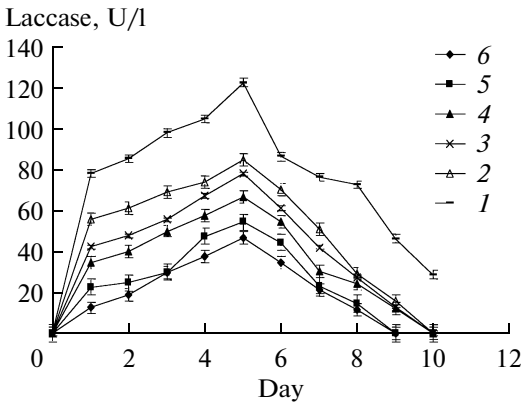

Fig. 5. Laccase activities in different disazo dye concentrations: *1* – 0, *2* – 10, *3* – 20, *4* – 30, *5* – 40, *6* – 50 mg/l.

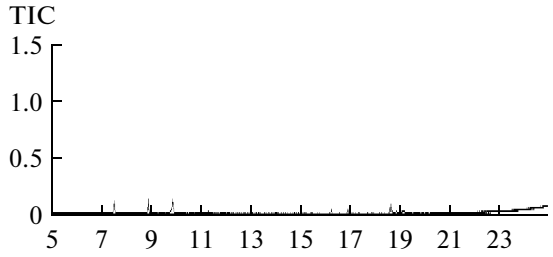

Fig. 6. GC chromatogram of new disazo dye biodegradation products.

mass spectrometry. Our study showed that *T. versicolor* could degrade this new disazo dye, enzymatic and non-enzymatic systems involved in the decolorization with liquid medium.

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