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Short Communication

Decolorisation and detoxification of Direct Blue-15 by a bacterial consortium

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Abstract

Studies were carried out on decolorisation and biotransformation of the dye Direct Blue-15 into 3,3′-dimethoxybenzidine (*O*′-dianisidine) and a sulphonated derivative by a five-member bacterial consortium. Chromatographic studies revealed further complete biodegradation of 3,3′-dimethoxybenzidine coupled with release of ammonia, but the recalcitrant sulphonated derivative persisted. The microorganisms identified in the mixed consortium by 16S rDNA sequence analysis were *Alcaligenes faecalis*, *Sphingomonas* sp. *EBD*, *Bacillus subtilis*, *Bacillus thuringiensis* and *Enterobacter cancerogenus*. The cytotoxicity data showed a significant reduction in the toxicity ($P < 0.001$) of the degraded dye as evidenced from the number of viable human polymorphonuclear leukocyte cells present.

Keywords: Direct Blue-15; Benzidine; Decolorisation; Biotransformation; Cytotoxicity

1. Introduction

The major classes of synthetic dyes used for textile dyeing and other industrial applications include azo, anthraquinone and triaryl methane dyes (Padmavathy et al., 2003; Chang et al., 2001). Azo dyes are by far the most widely used (Manning et al., 1985; Chen et al., 2004) and color index lists more than 2000 azo compounds (SCCNFP, 2002). Around 15% of the total world production of dyes and intermediates are released into environment during synthesis, processing and usage (Spadary et al., 1994; Zollinger, 1991).

Benzidine-based azo dyes contain benzidine attached to other substituents by diazo linkages. Benzidine by itself is tumorigenic (Haley, 1975) and a human urinary bladder carcinogen (Brown, 1977; National Institute for Occupational Safety and Health, 1980).

The NIOSH review also indicated benzidine-based dyes to be carcinogenic due to their biotransformation to benzidine.

According to the EU (European regulations, SCCNFP, 2002), Direct Blue-15 is one of the azo dyes, which splits into carcinogenic amine. Although Direct Blue-15 has been banned in India since 1997 (Ministry of Environment and Forest, 1997), it is still available in the world market (EEC, 2000) and is manufactured in India for export. The aim of the present study was to carry out decolorisation and biodegradation studies on benzidine-based Direct Blue-15 using a mixed bacterial culture. Further, the toxicity potential of the dye on human polymorphonuclear lymphocytes was compared before and after microbial treatment.

2. Methods

2.1. Reagents

Commercial grade Direct Blue-15 was obtained from a dye-manufacturing unit. A stock solution of the dye

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(1000 mg L^{-1}) was prepared in de-ionized water and used for all studies. Diethyl ether, dichloromethane, methanol and DMSO used were of HPLC grade (Merck, India). All other chemicals used in the experiment were of analytical grade (Merck, India).

2.2. Experimental setup

2.2.1. Screening and selection of microorganisms

Acclimated activated sludge was sourced from an aerobically maintained bioreactor treating textile wastewater. Around 10 g of sludge was added in 100 mL of minimal basal medium (g L^{-1}, Na_2HPO_4 – 6, KH_2PO_4 – 3, NH_4Cl – 1, NaCl – 0.5, and 1 M $MgSO_4$ – 1 mL L^{-1}) and kept in orbital shaker for 12 h at 150 rpm and 37 °C. Supernatant was collected after allowing the sludge to settle down for 2 h. The microorganisms capable of degrading dye were screened by inoculating the Luria broth agar plates (g L^{-1}, Casein enzymic hydrolysate – 10, yeast extract – 5, NaCl – 5, agar-15; Hi-media laboratories, India) containing 10 mg L^{-1} of Direct Blue-15 with the supernatant. The bacterial growth was picked up on the basis of its ability to form clear zones on these plates. These cultures were subsequently transferred to Luria broth medium containing different concentrations of the dye. For identification, these cultures were further isolated on Luria agar and maintained on slants. Five colonies were picked up according to their different morphological appearance and identified on the basis of 16S rDNA sequence analysis.

2.2.2. Decolorisation and biodegradation studies

Experiments were performed in flasks. Different concentrations of Direct Blue-15 dye (50,100 and 250 mg L^{-1}) were added in Luria broth medium inoculated with 0.10 OD (600 nm) of culture. These flasks were incubated at 37 °C at static condition till decolorisation and degradation were completed. The samples were drawn at different time intervals, centrifuged at 12,000*g* and analysed for decolorisation and biodegradation as described below.

2.3. Analyses

2.3.1. Decolorisation and ammonia analyses

The UV–Vis spectra of the samples were recorded from 200 to 800 nm using a spectrophotometer (Perkin Elmer UV–Vis-NIR Lambda-900). Decolorisation was measured at different time intervals at the wavelength in the visible range where maximum absorbance was obtained (604 nm). Ammonia was estimated by Nesslerization method as described in Standard Methods (APHA, AWWS, WPCF, 1991).

2.3.2. Biomass study

The pellet from the centrifuged sample (Section 2.2.2) was suspended in 5 mL of distilled water. This was then homogenized using a vortex and filtered using pre-weighed glass fibre circles [GFC (42.5 mm)]. The biomass on GFC was determined gravimetrically.

2.3.3. Extraction and HPLC analysis

The dye degradation metabolites were monitored by HPLC (Waters, Model No.: 501) as the decolorisation continued. Ten milliliter sample was taken at different days interval (day 4 and 10) centrifuged at 12,000*g*, and filtered through 0.45 μm membrane filter (Millipore). The filtrate was then extracted three times with diethyl ether and flash evaporated in rotary vacuum evaporator in temperature controlled water bath (45–50 °C) and residue dissolved in 2 mL methanol. This extracted sample was analysed by HPLC having the mobile phase of 50:49.6:0.4% methanol:water:disodium hydrogen phosphate, C-18 column with a flow rate of 0.8 mL min^{-1}, chart speed of 1 cm min^{-1} and UV detector at 280 nm.

2.3.4. Extraction and GC/MS analysis

Samples on the 4th day, and after conclusion of the experiment, were centrifuged and then filtered through 0.45 μm membrane filter. The filtrate was then extracted thrice with diethyl ether and flash evaporated in rotary vacuum evaporator in temperature controlled water bath (45–50 °C) and residue was dissolved in methylene chloride for GC/MS analysis.

GC/MS conditions: The GC/MS analysis of metabolite(s) was carried out using Varian/Saturn 2200 GC/MS/MS equipped with gas chromatograph CP-3800 with capillary column CP-Sil-8CB. Helium was used as carrier with a flow rate of 1.1 mL min^{-1}. The injector temperature was maintained at 300 °C and the analysis was carried out as per the protocol of EPA-8270. The compounds were identified using NIST library on the basis of mass spectra and retention time.

2.3.5. Cytotoxicity study

The cytotoxicity studies were carried out using trypan blue exclusion assay (Kiang et al., 1998) on human polymorphonuclear leukocyte cells. Two milliliter sample was taken, extracted thrice by diethylether and flash evaporated. The left over residue was dissolved in 1 mL of 0.1% dimethyl sulphoxide. This extract was used to challenge the cells for cytotoxicity study. The average and percentage of cell viability were calculated from the results of at least five replicate experiments. Statistical analysis was done using student "*t*" test by Analyse-it software.

3. Results and discussion

The individual microorganisms identified in the mixed culture by 16S rDNA sequence analysis were *Alcaligenes faecalis*, *Sphingomonas* sp. *EBD*, *Bacillus subtilis*, *Bacillus thuringiensis* and *Enterobacter cancerogenus*. The 16S rDNA sequences of *Alcaligenes faecalis*, *Sphingomonas* sp. *EBD*, *Bacillus subtilis*, *Bacillus thuringiensis* and *Enterobacter cancerogenus* have been deposited in the GenBank