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Bioresource Technology 97 (2006) 407–413
BIORESOURCE TECHNOLOGY

Decolorisation, biodegradation and detoxification of benzidine based azo dye

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Received 27 May 2004; received in revised form 6 December 2004; accepted 16 March 2005
Available online 4 June 2005

Abstract

The present study deals with the decolorisation, biodegradation and detoxification of Direct Black-38, a benzidine based azo dye, by a mixed microbial culture isolated from an aerobic bioreactor treating textile wastewater. The studies revealed a biotransformation of Direct Black-38 into benzidine and 4-aminobiphenyl followed by complete decolorisation and biodegradation of these toxic intermediates. From cytotoxicity studies, it was concluded that detoxification of the dye took place after degradation of the toxic intermediates by the culture.

Keywords: Direct Black-38; Benzidine; Cytotoxicity; Biodegradation; Decolorisation

1. Introduction

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 (Powell et al., 1979; Carliell et al., 1995; Chang et al., 2001). Synthesis of most azo dyes involves diazotization of primary aromatic amines followed by coupling with one or more nucleophiles (Zollinger, 1991). Benzidine (BZ)-based azo dyes have been found to be tumerogenic (Haley, 1975) and carcinogenic (Brown, 1977; National Institute for Occupational Safety and Health, 1980) due to their biotransformation to BZ (Heiss et al., 1992).

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 (Carliell et al., 1995). Electrochemical destruction (Jia et al., 1999), photocatalysis (Davis et al., 1994) and sorption (Vandevivere et al., 1998), though effective as tertiary treatments, are not economically viable. Primary, physical and chemical treatments of azo dye bearing wastewater (chemical coagulation and/or flocculation and/or precipitation), combined with conventional aerobic biological treatment, are effective but may generate significant amounts of chemical sludge categorized in India as hazardous waste (Ministry of Environment and Forests, 2003), whose disposal in a secure landfill increases process cost. Augmentation of biological treatment process without involving primary or tertiary treatment appears to be a techno-economically viable option. Biological degradation of disperse blue 79, an azo dye, has been reported recently (Melgoza et al., 2004). In the present

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doi:10.1016/j.biortech.2005.03.031

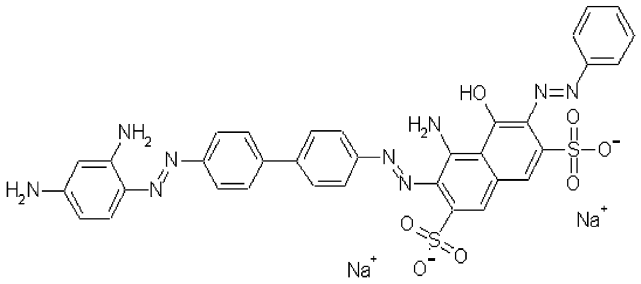

Fig. 1. Structure of Direct Black-38 dye.

study, Direct Black-38, a water soluble, benzidine 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.

2. Methods

2.1. Dye solution preparation

Direct Black-38 dye was dissolved in double distilled water to prepare a stock solution (1000 mg/l). Solutions of the desired concentrations were obtained by successive dilution.

2.2. Screening and selection of microorganisms

Sludge was sourced from an aerobically maintained bioreactor treating textile wastewater. Around 10 g of sludge were added to 100 ml of minimal basal medium (Na_2HPO_4—6 g/l, KH_2PO_4—3 g/l, NH_4Cl—1 g/l, NaCl—0.5 g/l, 1 M $MgSO_4$—1 ml/l) and kept on an orbital shaker for 12 h at 150 rpm and 37 °C. Supernatant was collected after allowing the sludge to settle for 2 h. The microorganisms capable of degrading dye were screened by inoculating the Luria Broth Agar plates (casein enzymic hydrolysate—10 g/l, yeast extract—5 g/l, NaCl—5 g/l, agar—15 g/l; HiMedia Laboratories, India) containing 10 mg/l of Direct Black-38 with the supernatant. The colonies were selected on the basis of their ability to form clear zones on these plates. Such colonies were subsequently transferred to Luria Broth medium containing different concentrations of the dye.

2.3. Experimental setup for the decolorisation and biodegradation studies

Experiments were performed in flasks. Different concentrations of Direct Black-38 dye (25 mg/l, 50 mg/l and 100 mg/l) were added to Luria Broth medium inoculated with 0.10 OD (600 nm) of culture. These flasks were incubated at 37 °C under static conditions till decolorisation and degradation were completed. The samples were withdrawn at different time intervals and analyzed for decolorisation and biodegradation. The biodegradation was monitored by ammonia release, HPLC and GC/MS analyses and cytotoxicity studies, while decolorisation was determined at 487 nm using an UV–Visible spectrophotometer.

2.4. Analytical methods

2.4.1. Decolorisation study

The absorbance peak (λ_{max}) of the Direct Black-38 dye was determined by UV scanning and found to be at 487 nm.

For the decolorisation study, 5 ml sample was taken and centrifuged at 12,000*g* for 10 min. The absorbance of the supernatant was spectrophotometrically determined at 487 nm (Perkin Elmer UV–Vis–NIR Lambda-900) at different time intervals (0, 4, 8, 12 and 24 h on the first day and thereafter every 24 h on subsequent days for a total period of 10 days).

2.4.2. Biomass and biosorption study

The pellet from the centrifuged sample was suspended in 5 ml of distilled water. This was then vortexed and filtered using pre-weighed Whatman GF/C glass fibre filter paper circles. The biomass on GF/C was determined gravimetrically. The OD of the filtrate was measured at 487 nm for the biosorption study.

2.4.3. Ammonia estimation

Ammonia was estimated by the Nesslerization method according to APHA, AWWA, WPCF (1991).

2.4.4. Extraction and HPLC analysis

The dye degradation was monitored by HPLC (Waters, Model No. 501) as the decolorisation continued. Ten millilitres samples were taken at intervals (days 0, 4 and 10), centrifuged and filtered through a 0.45 μm membrane filter (Millipore). The filtrate was then extracted three times with diethyl ether and flash evaporated in a rotary vacuum evaporator with a 45–50 °C water bath, after which the residue was dissolved in 2 ml methanol. Extracted samples were analyzed by HPLC using a mobile phase of 50:49.6:0.4% methanol:water:disodium hydrogen phosphate, a C-18 column (void volume 2.9 ml, length 250 mm and internal diameter 4.6 mm) with an eluant flow rate of 0.8 ml/min, chart speed of 1 cm/min and UV detector at 280 nm. Benzidine and 4-aminobiphenyl standards were procured from Sigma-Aldrich Chemicals, USA.

2.4.5. Extraction and GC/MS analysis

Samples on the fourth day and after the conclusion of the experiment were centrifuged and filtered through a 0.45 μm membrane filter. The filtrate was then extracted three times with diethyl ether and the pooled extract was flash evaporated in a rotary vacuum evaporator with a 45–50 °C water bath. Residue was dissolved in methylene chloride for GC/MS analysis.

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

2.4.6. Cytotoxicity study

The toxicity studies were carried out using a trypan blue exclusion assay (Kiang et al., 1998) on human polymorphonuclear leukocyte cells. A 2 ml sample was taken and extracted three times with diethyl ether. The pooled extract was flash evaporated and leftover residue was dissolved in 1 ml of 0.1% dimethyl sulphoxide. This extract was used to challenge the cells in the cytotoxicity study.

3. Results and discussion

3.1. Biosorption and decolorisation of dye

Considerable decolorisation was noticed in the supernatant at different time intervals of incubation (Fig. 2). An additional observation worth reporting is the appearance of biomass with a strong black color after

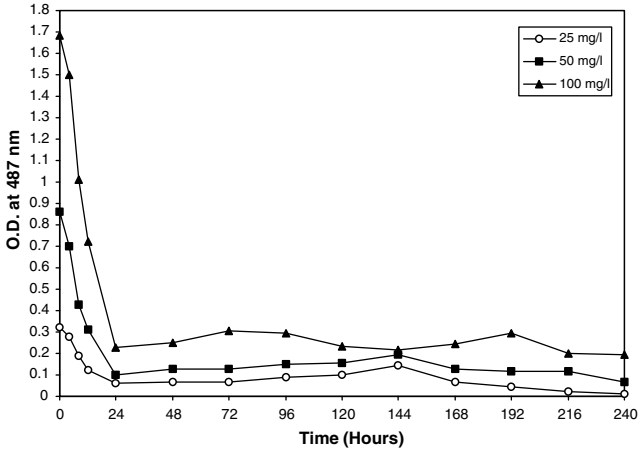

Fig. 2. Decolorisation of Direct Black-38.

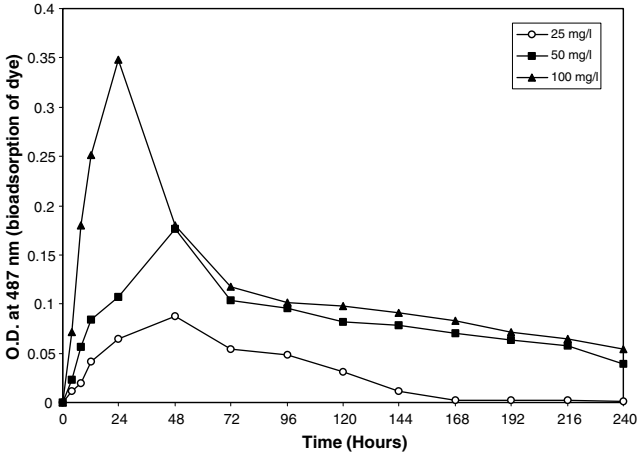

Fig. 3. Adsorption of Direct Black-38 on the cell surface.

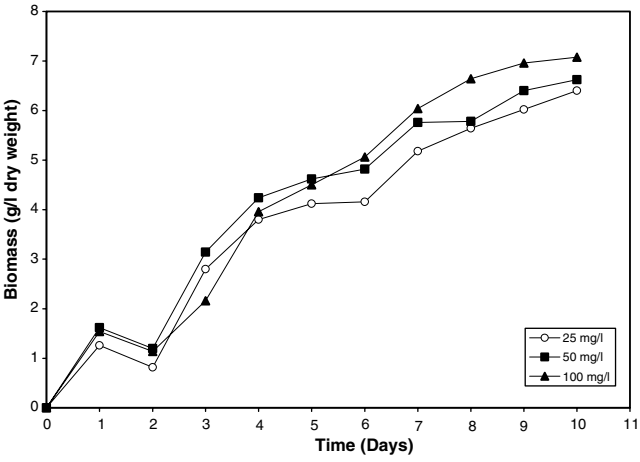

Fig. 4. Dry weight of cells in Direct Black-38 amended medium following incubation.

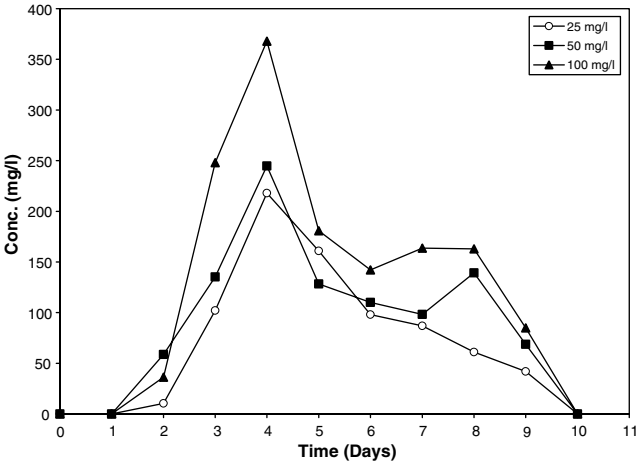

Fig. 5. Ammonia released from biodegradation of Direct Black-38.

24 h of incubation, which started fading after 48 h. The color from the biomass, on extraction and spectophotometric measurement, showed an absorption maximum at 487 nm (Fig. 3), suggesting biosorption, as has also been reported elsewhere (Ambrosio and Campos-Takaki, 2004). The biomass, however, started increasing

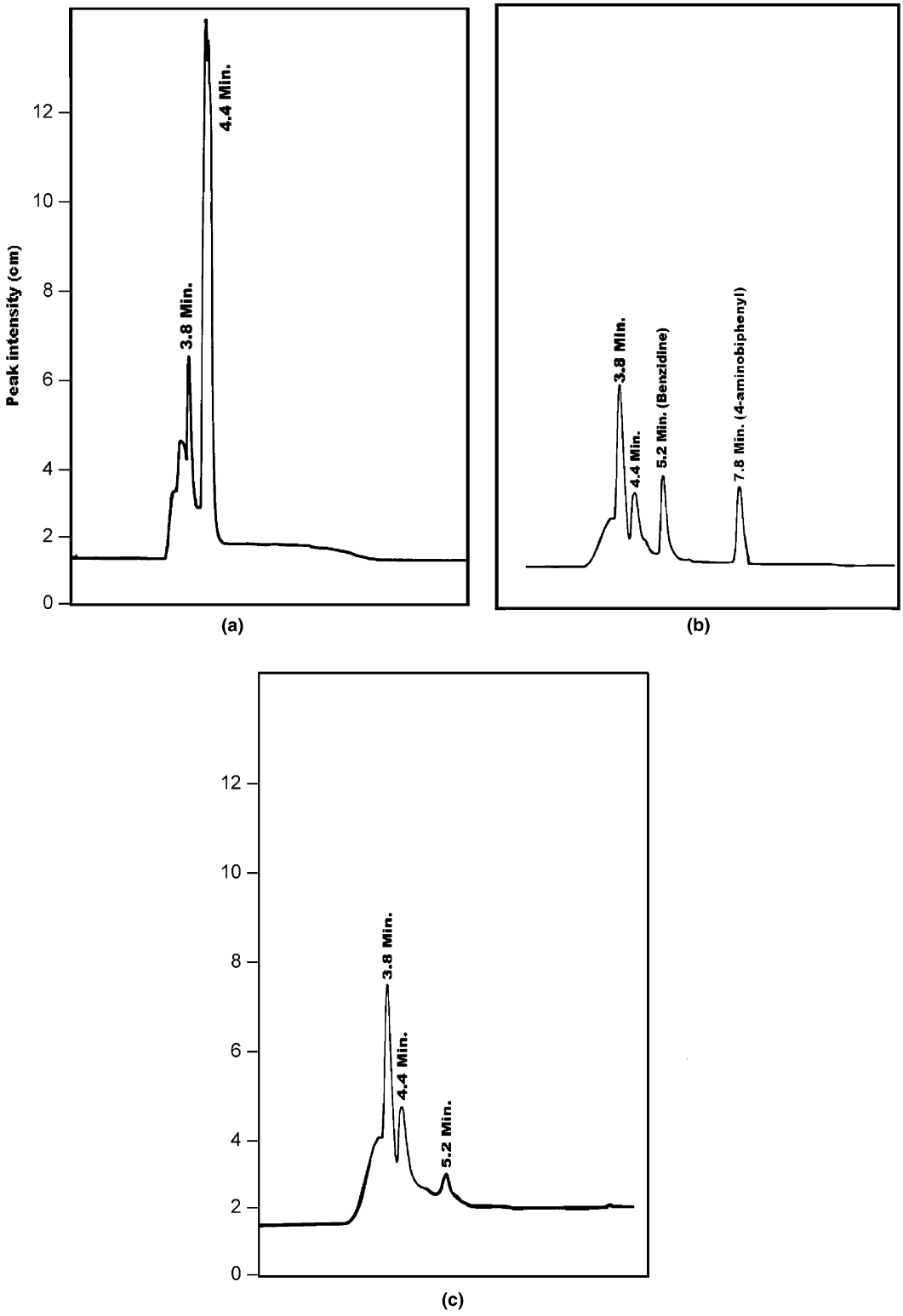

Fig. 6. HPLC chromatograms of degradation study of Direct Black-38 dye (100 mg/l). (a) Direct Black-38 dye (0 day). (b) Transformed Direct Black-38 (fourth day). (c) Biodegraded Direct Black-38 dye (10th day).

after 2 days concomitantly with the biodegradation of Direct Black-38 to benzidine and 4-aminobiphenyl as revealed by HPLC and GC–MS.

3.2. Release of ammonia

As the decolorisation proceeded, the release of ammonia was seen only after 2 days (Fig. 5), commensurate with biomass increase, which was due to monodeamination of benzidine to 4-aminobiphenyl (Puvaneshwari et al., 2002). From the observations on increase in biomass (Fig. 4) and release of ammonia (Fig. 5) beyond 2 days of incubation, it appears that biodegradation of Direct Black-38 dye has a lag period of 2 days and was maximum at the fourth day, which could be correlated with the emergence of 4-aminobiphenyl resulting from deamination of benzidine.

3.3. Chromatographic analysis of products formed

The HPLC analysis of dye sample collected at the beginning of static incubation (0 h incubation) shows two major peaks with retention times of 3.8 min and 4.4 min (Fig. 6a). As the decolorisation progressed, the emergence of two additional peaks on the fourth day was observed with retention times of 5.2 and 7.8 min (Fig. 6b). Benzidine was identified as one of the products of reduction of the azo bond by comparing the peak with that of standard benzidine with a retention time of 5.2 min. The other peak at 7.8 min was identified as 4-aminobiphenyl by matching its retention time with a HPLC standard. The emergence of benzidine and 4-aminobiphenyl confirmed azo bond breakage. However, the benzidine peak at 5.2 min was barely present on the 10th day and there was no HPLC detector response at 7.8 min, showing near complete degradation of benzidine and 4-aminobiphenyl on the 10th day of incubation. The initial peaks with retention times of 3.8 and 4.4 min were still present, though the peak height at 4.4 min was drastically reduced (Fig. 6c). The compound with peak at 3.8 min appears to have been recalcitrant.

GC/MS analyses were carried out to investigate the metabolites formed during the biodegradation process. The fourth day extracted sample was injected into the GC/MS. The mass spectrum identified the intermediate 4-aminobiphenyl with retention time of 15.914 min and molecular weight of 169, while benzidine showed a retention time of 19.577 min and a molecular weight of 184 (Fig. 7a and b). 4-aminobiphenyl is a deaminated

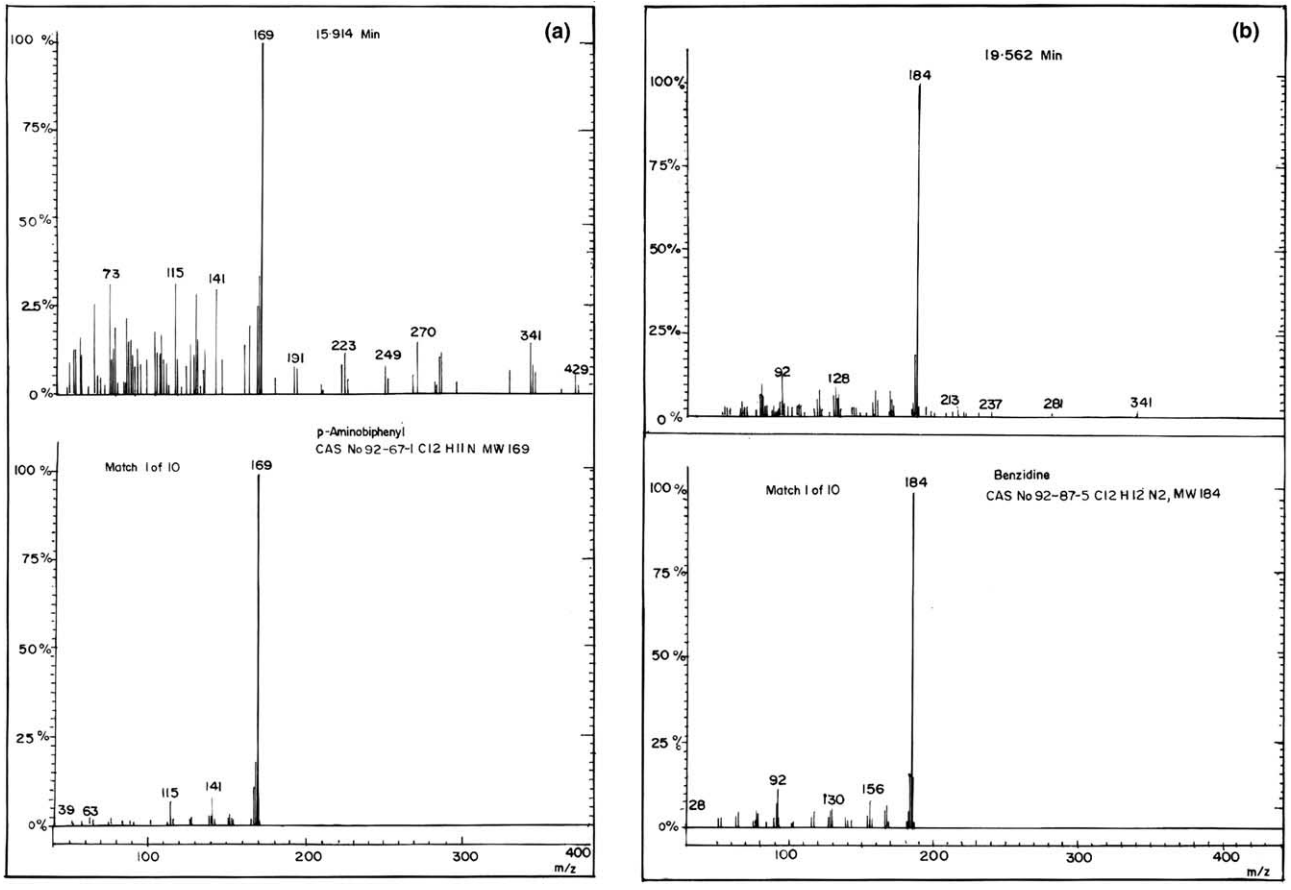

Fig. 7. Identification of metabolites of Direct Black-38 by GC/MS. (a) *p*-Aminobiphenyl, (b) benzidine.

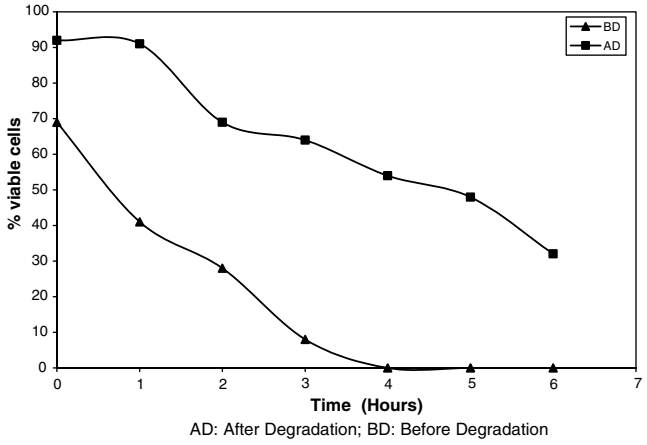

Fig. 8. Cytotoxicity study of Direct Black-38 (50 mg/l).

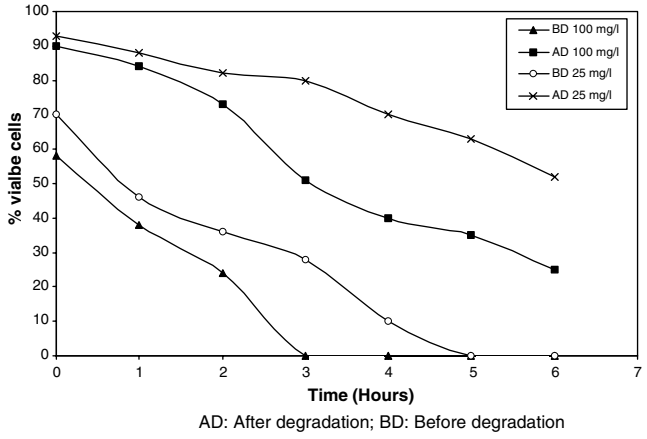

Fig. 9. Cytotoxicity study of Direct Black-38 (25 mg/l and 100 mg/l).

product of benzidine (Puvaneshwari et al., 2002) and biogenic release of amine from Direct Black-38 has been reported recently (Gnamamani et al., 2004). Therefore, it could be concluded that the culture used for decolorisation was not only breaking the azo bonds but also degrading the benzidine to 4-aminobiphenyl. Complete degradation of benzidine and 4-aminobiphenyl in 10 days was confirmed by HPLC.

3.4. Cytotoxicity study

The toxicity of Direct Black-38 dye was evaluated before and after degradation at the cytotoxic concentrations of 25 mg/l, 50 mg/l and 100 mg/l up to 6 h duration. The viability of the control cells was found to be >90% (0.1% DMSO). Cytotoxicity studies revealed that biodegradation of the dye by the isolated culture resulted in detoxification of the dye as could be seen from the absence of significant total cell death at all the time intervals (0–6 h) and at all concentrations (25, 50 and 100 mg/l) tested (Figs. 8 and 9).

4. Conclusions

Cultures isolated from textile dye wastewater achieved a complete decolorisation and biodegradation of benzidine and 4-aminobiphenyl, toxic intermediates of the Direct Black-38 dye. From cytotoxicity studies, it was concluded that detoxification of the dye took place after degradation of the toxic intermediates by the culture.

Acknowledgements

Dr. Sukumar Devotta, Director NEERI is gratefully acknowledged for providing the necessary facilities, his time-to-time suggestions and encouragement. Ms. Dipanwita Dutta is acknowledged for her help in carrying out cytotoxicity studies.

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