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ScienceDirect
Ecotoxicology and Environmental Safety 72 (2009) 960–964
Ecotoxicology and Environmental Safety
www.elsevier.com/locate/ecoenv

Detoxification of benzidine-based azo dye by *E. gallinarum*: Time-course study

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Received 5 June 2007; received in revised form 22 November 2007; accepted 29 November 2007
Available online 21 February 2008

Abstract

Direct black 38 (DB38) dye is a well-established toxic and carcinogenic compound. Present investigation reports isolation of an *Enterococcus gallinarum* strain capable of decolorizing and degrading it. Changes in toxicity and mutagenicity of DB38 and its metabolites were also determined using a battery of carefully selected tests (cytotoxicity, respiration inhibition test and Ames test). Toxicity assays were carried out on *E. gallinarum* itself as this also gave information about suitability of this strain for the dye decolorization operation. The strain was found to reduce both toxicity and mutagenicity of DB38 metabolites. Benzidine and 4-aminobiphenyl (4-ABP) were identified as the DB38 metabolites, responsible for its toxic and mutagenic properties, by HPLC-MS analysis. Further degradation of benzidine and 4-ABP was found to result in the decrease in toxicity and mutagenicity.

Keywords: Toxicity; Mutagenicity; Ames test; Azo dye; Benzidine; 4-Aminobiphenyl

1. Introduction

Azo dyes are characterized by the presence of a chromophore azo group. They constitute the largest and most versatile class of synthetic dyes with the greatest variety of colors. Approximately 10–15% of the dyes are released into the environment during dyeing process, making the effluent highly colored and aesthetically unpleasing (Tan et al., 2000). Moreover, most of the azo dyes and/or their breakdown products have been shown to be toxic and carcinogenic to animals, including humans (Weisburger, 2002). As these dyes are designed to be recalcitrant to different environmental factors like enzymes, light, sweat and detergents, they tend to persist in the environment (Anliker, 1979). It is difficult to remove them by conventional wastewater treatment techniques. In fact, treatments like chlorination result in release of mutagenic compounds from relatively harmless dyes (Oliveira et al., 2006). It has been reported that complete mineralization of dyes is possible by a two-step biological process. First, bacteria reduce the azo bonds of dyes under anaerobic conditions to release potentially toxic and carcinogenic aromatic amines. In the second step, these aromatic amines are mineralized by bacteria under aerobic conditions (van der Zee and Villaverde, 2005). This emphasizes that the treatment process should not only decolorize the dye, but also degrade/remove its toxic/mutagenic components. Hence, toxicity and mutagenicity evaluation is a very crucial parameter to determine the success of a treatment operation.

Present investigation reports isolation of an *Enterococcus gallinarum* strain capable of decolorizing azo dyes. Direct black 38 (DB38) was selected as the model benzidine-based azo dye because its toxicity and mutagenicity are well documented (Robens et al., 1980). Further, a battery of toxicity and mutagenicity tests was carried out on DB38 and products of its degradation by *E. gallinarum*. The degradation intermediates were identified by HPLC-MS. The dye was found to release its toxic and mutagenic components initially, which were slowly degraded by *E. gallinarum*, resulting in reduction in toxicity and mutagenicity. In our knowledge, this is the first report to demonstrate dye decolorization activity in *E. gallinarum*

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0147-6513/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ecoenv.2007.11.013

coupled to reduction in mutagenicity of dye/its degradation products and correlate it with the reduction in toxicity.

2. Materials and methods

2.1. Chemicals

DB38 was obtained from a local supplier. Benzidine, 4-aminobiphenyl (4-ABP) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemicals (USA). Diethyl ether and methanol were obtained from Merck (India). Growth media Luria broth and Luria agar were obtained from Himedia, India. The S9 preparation from aroclor-induced Sprague-Dawley rat was purchased from Molecular Toxicology Inc. (USA).

2.2. Isolation of culture

Effluent treatment plant sludge from a textile industry was plated on agar plates containing DB38 dye. A colony showing zone of decolorization was picked up and maintained on Luria agar. It was identified as *E. gallinarum* by 16S rRNA sequencing (GenBank DQ864487).

2.3. DB38 degradation by E. gallinarum

Overnight culture of *E. gallinarum* was inoculated in Luria broth containing 100 mg/l DB38. It was incubated under static condition and samples were collected at 0, 5, 10 and 20 days. These samples were subjected to HPLC-MS to identify and quantify the degradation intermediates, and to different toxicity and mutagenicity assays.

2.4. HPLC-MS analysis

Samples were extracted by liquid–liquid extraction as follows. Samples were first clarified by passing through 0.45 μm membrane. Then they were extracted three times with equal volume of diethyl ether. The ether fractions were combined and evaporated to dryness. Finally, the residue was dissolved in 2 ml of methanol for HPLC-MS. The HPLC system (Waters) consisted of Waters 1525 pump and Waters 2487 photodiode array (PDA) detector set at 284 nm. Sample components were separated on Spherisorb 5-μm ODS2 column (4.6 mm × 250 mm), using a mobile phase of methanol:water (50:50) at a flow rate of 0.8 ml/min. Injection volume was 10 μl, and pure benzidine and 4-ABP were used as standards. Compounds detected on HPLC were analyzed by mass spectrometry for additional confirmation. For this, the HPLC system was connected to a mass spectrometer (Micromass Quattro Ultima), equipped with electronspray ionization source operated in positive mode. The acquisition parameters were set as follows: cone gas flow = 105 l/h, desolvation gas flow = 354 l/h, capillary = 3 KV, cone = 60 V, source temperature = 80 °C, desolvation temperature = 150 °C. Each eluted peak appearing on PDA detector was identified using mass spectrometer.

2.5. Toxicity/mutagenicity assays

Samples were sterilized by filtration through 0.22 μm membrane and sterility was confirmed before carrying out the tests. All tests were carried out in triplicate.

2.5.1. Cytotoxicity assay

Cytotoxicity of the samples was assessed towards the decolorizer itself, i.e. *E. gallinarum*. *E. gallinarum* culture was prepared by inoculating a colony from freshly isolated Luria agar plate into Luria broth and growing on shaker to mid-exponential growth phase. The culture was then diluted in sterile saline to appropriate cell concentration (1×10^5–5×10^5 ml^{-1}). For the assay, 0.2 ml of sample was mixed with 0.8 ml of the diluted culture and it was incubated for 1 h. A control with Luria broth replacing the sample was also maintained. Viability of the cells was then determined by plating appropriate dilutions of the samples on Luria agar. It was assumed that all viable cells were platable, i.e. damaged non-culturable cells were treated as dead. This is justified, as ability to damage the cells is also a measure of toxicity.

2.5.2. Respiration inhibition assay

Since the cytotoxicity assay could not differentiate between dead and damaged non-culturable cells, an additional test was set up, which estimated cell viability based on respiration measurements. A modification of the method suggested by Botsford (1998) was used for this. *E. gallinarum* was used as the test organism again. *E. gallinarum* culture was prepared by inoculating a colony from freshly isolated Luria agar plate into Luria broth and growing on shaker to mid-exponential growth phase. The culture was then diluted in sterile saline to OD_{550} of 0.1. For the assay, 0.2 ml of sample was mixed with 0.8 ml of diluted culture. MTT and glucose were then added to final concentration of 0.1 mM and 0.01%, respectively. A control with Luria broth replacing the sample was also maintained. Tubes were incubated for 30 min before recording OD readings at 550 nm.

2.5.3. Ames test

Mutagenicity assay was carried out using Ames *Salmonalla typhimurium* strains TA98 and TA100 to detect both frameshift and base-pair substitution mutations, respectively. The tester strains were obtained from IMTECH (Chandigarh) and tested to confirm their genetic features according to Maron and Ames (1983). The test was performed using standard pre-incubation procedure in the presence of metabolic activation (S9 mix). 0.5 ml of 10% S9 mix and 0.1 ml of sample were used for each plate.

3. Results

3.1. HPLC-MS analysis

During HPLC, standard 4-ABP eluted at 3.11 min while benzidine eluted out at 7.97 min, resulting in a good separation. Peaks appearing in the samples were identified by comparison of their retention time with the standards. Mass spectra of these peaks were also obtained and compared with the standards for additional confirmation. Benzidine and 4-ABP, thus identified in the samples, were quantified based on the peak area. Fig. 1 shows the changes in the levels of 4-ABP and benzidine during biodegradation of DB38. Both were found to be absent from 0 h sample,

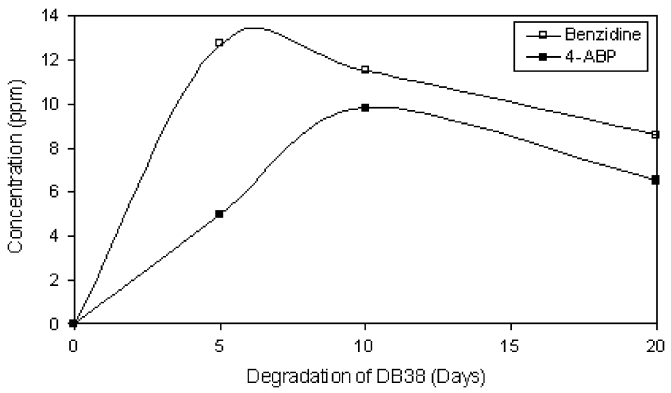

Fig. 1. Changes in the level of benzidine and 4-ABP resulting from DB38 degradation by *E. gallinarum*.