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Azoreductase and dye detoxification activities of *Bacillus velezensis* strain AB

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Abstract Azo dyes are known to be a very important and widely used class of toxic and carcinogenic compounds. Although lot of research has been carried out for their removal from industrial effluents, very little attention is given to changes in their toxicity and mutagenicity during the treatment processes. Present investigation describes isolation of a *Bacillus velezensis* culture capable of degrading azo dye Direct Red 28 (DR28). Azoreductase enzyme was isolated from it, and its molecular weight was found to be 60 kDa. The enzyme required NADH as cofactor and was oxygen-insensitive. Toxicity and mutagenicity of the dye during biodegradation was monitored by using a battery of carefully selected in vitro tests. The culture was found to degrade DR28 to benzidine and 4-aminobiphenyl, both of which are potent mutagens. However, on longer incubation, both the compounds were degraded further, resulting in reduction in toxicity and mutagenicity of the dye. Thus, the culture seems to be a suitable candidate for further study for both decolourization and detoxification of azo dyes, resulting in their safe disposal.

Keywords Direct red 28 · Azoreductase · Zymogram · Ames test · Biodegradation

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Introduction

Azo dyes constitute the largest and most versatile class of synthetic dyes used for textile dyeing and other industrial applications. As fixation of these dyes to textile fibers is never complete, a significant amount of dyes is lost in wastewater. Release of such coloured compounds into the environment is undesirable not only because of their aesthetic appearance, which may drastically affect photosynthesis in aquatic ecosystem, but also because many of the dyes and/or their breakdown products are toxic and mutagenic to life (Chung and Cerniglia 1992). Unfortunately, azo dyes present in the wastewater are normally unaffected by conventional treatment processes. Their persistence is mainly due to sulfo and azo groups, which do not occur naturally, making the dyes xenobiotic and recalcitrant to oxidative biodegradation (Kulla et al. 1983). The persistence of azo dyes has resulted in several reports, which show that decolourization of azo dyes requires an initial cleavage of azo bonds, after which the resulting aromatic amines can be biodegraded readily under aerobic conditions (van der Zee and Villaverde 2005). The cleavage of azo bonds is catalyzed by azoreductase enzyme with the aid of an electron donor. Several bacteria capable of decolourizing azo dyes have been identified, and azoreductase enzyme has been isolated and characterized from some of them (Chen 2006). However, in addressing the problem of decolourization of azo dyes, the main issue, i.e., their toxicity and mutagenicity, has received much less attention. There are a few reports about detoxification of azo dyes during biodegradation, but they have not tested changes in the mutagenicity of dyes (Işik and Sponza 2006). It should be noted that toxicity and mutagenicity are two different attributes that need to be tested separately.

In the present investigation, a *Bacillus velezensis* strain was isolated based on its ability to decolourize azo dye Direct Red 28 (DR28). The azoreductase enzyme responsible for decolourization was visualized using a zymogram. The toxicity and mutagenicity of dye, during the course of biodegradation, were also monitored using a battery of carefully selected in vitro tests. The metabolic intermediates resulting from dye degradation were identified using high performance liquid chromatography-mass spectrometry (HPLC-MS). In our knowledge, this is the first report that supplements and correlates dye decolourization data with toxicity and mutagenicity results.

Materials and methods

Isolation of the culture

DR28 was selected as the model azo dye, as it is benzidine-based, and its toxic and mutagenic properties are well established (Morgan et al. 1994), apart from its local availability. A culture capable of decolourizing DR28 was isolated from effluent treatment plant (ETP) of a textile industry. Its decolourization ability was tested in a minimal medium (composition in grams per liter: Na_2HPO_4—1.264, KH_2PO_4—0.326, NH_4Cl—1, $MgSO_4$—0.098, $CaCl_2$—0.044, glucose—1) containing 25 mg l^{-1} of DR28. This medium was used throughout the study. The culture was identified by 16S rRNA sequencing. Genomic DNA was isolated from it as described previously (Ausubel et al. 1987) and purified using Ultrapure prep kit (Bangalore Genei, India). 16S rRNA gene was amplified from the extracted DNA using universal primers (Edwards et al. 1989), and the amplified product was cloned in T-vector (Bangalore Genei) using competent *Escherichia coli XL1-blue* cells (Stratagene, USA) as host. Sequencing was carried out with M13 primer on an automated DNA sequencer (ABI 3100, Applied Biosystems, USA). Based on the 16S rRNA gene sequence, the culture was identified as *B. velezensis* (GenBank accession no. EU048223).

Azoreductase activity in crude protein extract

The culture was grown in minimal medium containing 25 mg l^{-1} of DR28 until the dye was completely decolourized. Cells were harvested by centrifugation, washed with 50 mM phosphate buffer (pH 7) and resuspended in the same buffer. Cells were then disrupted by sonication (four cycles of 15 s, 75% amplitude; Vibra Cell, Sonics, USA), and cell debris was removed by centrifugation at 4°C. Supernatant was used as the crude protein extract.

Azoreductase activity in protein extract was assayed as before (Zimmermann et al. 1982). The assay mixture consisted of 50 mM phosphate buffer (pH 7), 2 mM NADH, 20 μM DR28, and 0.1 ml of crude protein extract in a total volume of 1 ml. Dye decolourization was monitored spectrophotometrically at 480 nm. Azoreductase activity in the presence of NADPH and FAD was measured in a similar manner by the addition of 2 and 0.2 mM of NADPH and FAD, respectively, to the assay mixture. Enzyme activity was expressed in units of micromoles of dye reduced per minute. Protein content of the crude protein extract was determined by Lowry's method using bovine serum albumin as standard for determination of specific activity, i.e., enzyme units per milligram protein.

Gel electrophoresis

Native polyacrylamide gel electrophoresis (PAGE) was carried out according to the method of Laemmli (1970) with slight modifications. SDS was excluded form both electrophoresis system and sample buffer. Native gel was cast with 12% resolving gel and 4% stacking gel. 0.1% carboxymethylcellulose was added to the resolving gel to facilitate binding of DR28 dye. Crude protein extract was mixed with sample buffer (without SDS and β-mercaptoethanol) and run on the gel under native conditions. Azoreductase enzyme was located on the gel by activity staining. For this, the gel was washed two to three times in 50 mM phosphate buffer (pH 7) and stained with 100 μM DR28. The gel was then transferred to phosphate buffer containing 2 mM NADH. Appearance of colourless band against red background in 15–20 min indicated the location of active azoreductase enzyme.

The band was carefully cut out, and protein was electroeluted from it. Its dye decolourization activity was confirmed according to the procedure described above. Molecular weight (M.W.) of the enzyme was determined by standard SDS-PAGE using M.W. marker (Bio-Rad, USA) followed by silver staining (Ausubel et al. 1987).

Identification of metabolites

Decolourization of benzidine-based dyes like DR28 is known to result in release of benzidine (Isik and Sponza 2004). To confirm this and check for other potential metabolites, degradation of DR28 was monitored by HPLC-MS. *B. velezensis* was inoculated in minimal medium containing 100 mg l^{-1} of DR28 and incubated under ambient conditions. Samples were collected after 0, 3, 6 and 15 days. For HPLC-MS analysis, samples were centrifuged and clarified by passing through 0.45 μm membrane. They were then extracted thrice with diethyl ether, extracts were pooled and evaporated to dryness, and final residue was dissolved in methanol. Extracted samples were analyzed on Waters HPLC system equipped with

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 mobile phase of methanol is to water (50:50) at a flow rate of 0.8 ml min^{-1}. Pure benzidine and 4-aminobiphenyl (4-ABP) (Sigma Chemicals, USA) 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 electron spray ionization source operated in positive mode. The acquisition parameters were: cone gas flow=105 l h^{-1}, desolvation gas flow=354 l h^{-1}, capillary=3 KV, cone=60 V, source temperature=80°C and desolvation temperature=150°C.

Toxicity assays

Toxicity of the samples was determined using two different assays, i.e., cytotoxicity and respiration inhibition. The assays were carried out in two different test systems: the decolourizer itself, i.e., *B. velezensis* culture and HL-60 cell line. All tests were carried out in triplicate, and statistical analysis of the results was carried out using SPSS suit.

B. velezensis culture

Samples were centrifuged, sterilized by passing through 0.22 μm membrane and directly used in the toxicity assays. For cytotoxicity assay, the test culture was prepared by growing in minimal medium until mid-exponential phase, followed by dilution in sterile saline to a final density of 1×10^4–5×10^4 cells ml^{-1}. Of the cell suspension, 0.8 ml was mixed with 0.2 ml of sample and incubated for 1 h. A control, where sample was replaced by sterile minimal medium, was also maintained for comparison. Viability of cells was then determined by plating on minimal agar medium (minimal medium containing 1.5% agar). All the viable cells were assumed to be plateable, i.e., damaged nonculturable cells, which lose the ability to form colonies but are still alive, were considered to be dead. This is justified as damage to cell is also a measure of toxicity.

To exactly quantify cell death, another test, which assesses cell viability based on respiration measurements, was used. As tetrazolium-based metabolic indicators are well-suited for such studies, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was selected for this test. Actively respiring cells reduce it to formazan, which can be measured spectrophotometrically (Botsford 1998). *B. velezensis* culture for this test was prepared by growing in minimal medium until mid-exponential phase, followed by dilution in sterile saline to OD_{600} of 0.1. 0.8 ml of the cell suspension was mixed with 0.2 ml of sample and incubated for 1 h. MTT and glucose were then added to it at final concentrations of 0.1 mM and 0.01%, respectively, followed by further incubation for 30 min. A control tube with minimal medium replacing the sample was also maintained. Absorbance of all the samples was finally read at 550 nm.

HL-60 cell line

HL-60, a promyletic leukemia cell line, was obtained from NCCS, Pune (India). It grows as a suspension culture (Freshney 1983). In laboratory, it was grown in Roswell Park Memorial Institute (RPMI) medium containing 10% foetal bovine serum at 37°C in a humidified 5% CO_2 incubator. For HL-60 cells, samples were prepared by extraction as in the case of HPLC, except that they were dissolved in dimethylsulfoxide (DMSO) instead of methanol. To estimate cytotoxicity of the samples, 180 μl aliquots of cell suspension (1×10^6 cells ml^{-1}) were dispensed in 96-well polystyrene tissue culture plate, and 20 μl of samples was added to them. Sterile DMSO was added in control. The culture plate was placed in the 5% CO_2 incubator at 37°C for 2 h. Cell viability was then assessed by the trypan blue exclusion test using a haemocytometer slide.

As MTT assay has been shown to be more accurate than the conventional trypan blue exclusion assay, it was also used to assess the toxicity (Zorrilla et al. 2001). After incubating cells with the samples for 2 h as above, 20 μl of MTT solution (5 mg ml^{-1} in phosphate-buffered saline (PBS)) was added to each well, and the plate was re-incubated for 1 h. Cells were then pelleted by low-speed centrifugation, and the supernatant culture medium was carefully aspirated out. Cells were then resuspended in 200 μl of DMSO, and absorbance was measured at 550 nm on a microtiter plate reader.

Ames test

Mutagenicity assay was carried out using Ames *Salmonella typhimurium* strains TA98 and TA100 to detect both frameshift and basepair 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 preincubation 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.

Results

ETP sludge of a textile industry was plated on agar medium containing DR28 dye. A colony showing clear zone was picked up and identified as *B. velezensis* by 16S rRNA sequencing (GenBank accession no. EU048223).

Determination of azoreductase activity

Crude protein extract obtained from *B. velezensis* cells was found to decolourize DR28 dye using NADH as electron donor. Specific activity of the azoreductase enzyme was found to be 0.48 U mg^{-1} protein. Addition of FAD to the assay mixture had no effect on the decolourization activity. When NADH was replaced with NADPH, in the presence or absence of FAD, the crude protein extract could not decolourize the dye at all.

Azoreductase enzyme was visualized from the crude protein extract on a zymogram. The development of zymogram was based on the decolourization of DR28-stained gel by azoreductase in the presence of NADH, which could be seen as a clear band against a red background. It was cut out carefully, and protein was eluted from it. The eluted protein was analyzed by SDS-PAGE resulting in a single band of 60 kDa (Supplementary Figure 1). Azoreductase activity of the eluted protein was confirmed again by incubating it with DR28 and NADH.

Identification of metabolites

Degradation of DR28 by *B. velezensis* was monitored at 100 mg l^{-1} dye concentration using HPLC-MS. Intermediates of degradation were identified and quantified by comparison with chromatograms of standard benzidine and 4-ABP (Supplementary Figure 2). Their identification was further confirmed by comparison of mass spectra obtained using MS. Figure 1 shows changes in the levels of intermediates, thus identified. DR28 (0 h) did not show any peak, indicating that the dye preparation was pure. Benzidine and 4-ABP were found to be released on the 3rd day of biodegradation, followed by reduction in their levels up to 15th day.

Toxicity towards *B. velezensis* culture

Figure 2 shows cytotoxicity of DR28 dye at various stages of biodegradation. Cytotoxicity first increased on the 3rd day, followed by gradual reduction up to 15th day ($p<0.01$). The same trend was observed in the case of respiration inhibition assay ($p<0.01$). Sample, 0 h (i.e., DR28 dye), was found to be slightly toxic in both the assays.

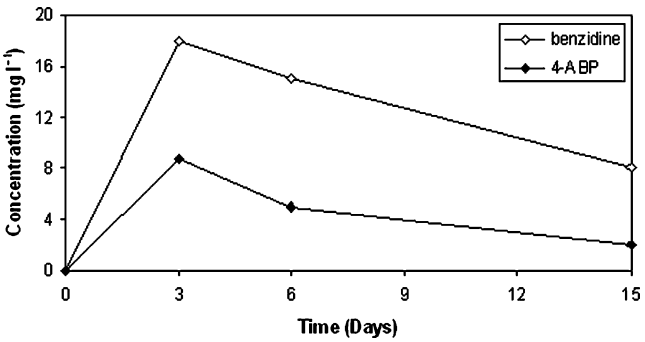

Fig. 1 Formation of intermediates during biodegradation of DR28

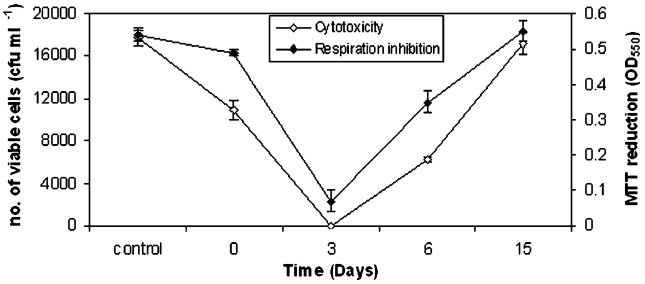

Fig. 2 Toxicity of DR28 dye at various stages of biodegradation, as measured by cytotoxicity and inhibition of respiration (MTT reduction) of *B. velezensis* cells. *Error bars* represent standard deviation from triplicate experiments

Toxicity towards HL-60 cell line

Toxicity towards HL-60 cells followed same trend as in the case of *B. velezensis* culture ($p<0.01$). Thus, toxicity first increased on the third day of biodegradation followed by gradual reduction in both cytotoxicity and respiration inhibition assays (Fig. 3).

Ames test

DR28 dye was found to be non-mutagenic by itself. However, upon biodegradation, mutagenicity sharply increased on the 3rd day, and then, there was steady reduction in mutagenicity up to 15th day ($p<0.01$; Fig. 4). The products of biodegradation were mutagenic to both TA98 and TA100 tester strains used in this study.

Discussion

B. velezensis culture isolated in the present study could decolourize 25 mg l^{-1} of DR28 dye within 10 h. Although dye decolourization is known to require anaerobic conditions, it occurred aerobically in this study (van der Zee

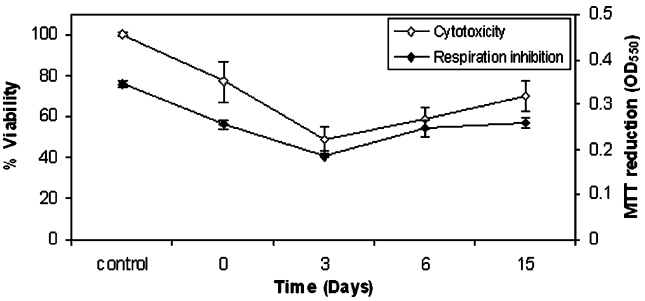

Fig. 3 Toxicity of DR28 dye at various stages of biodegradation, as measured by cytotoxicity and inhibition of respiration (MTT reduction) of HL-60 cell line. *Error bars* represent SD from triplicate experiments

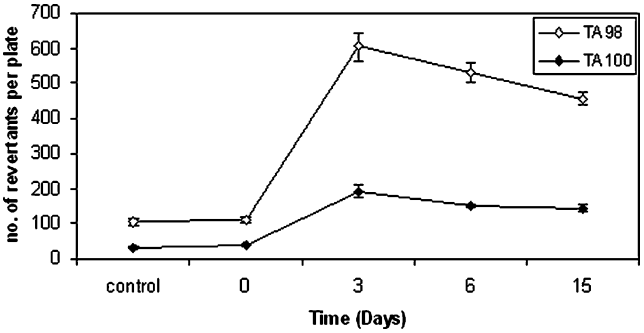

Fig. 4 Mutagenicity of DR28 dye at various stages of biodegradation, as measured towards Ames *S. typhimurium* strains TA98 and TA100. *Error bars* represent SD from triplicate experiments

and Villaverde 2005). To assay the azoreductase enzyme responsible for this, protein extract was obtained from *B. velezensis* cells, and it was also found to decolourize the dye using NADH as electron donor. It did not require removal of oxygen or preincubation with NADH, as described in some of the studies (Maier et al. 2004), indicating that azoreductase from *B. velezensis* is oxygen-insensitive. As many of the azoreductases are known to be flavoproteins, effect of FAD on decolourization was also tested. Addition of FAD did not enhance decolourization process in any way. Also, use of NADPH as electron donor did not result in any decolourization, indicating that the azoreductase enzyme was NADH-specific. Thus, it seems to be different from the azoreductase isolated from *Bacillus* sp. strain SF earlier, which required removal of oxygen and showed enhanced activity on addition of FAD (Maier et al. 2004).

To visualize the azoreductase from crude protein extract, zymogram analysis was carried out. The enzyme was located on DR28-stained native gel by its ability to decolourize dye in the presence of NADH. The decolourized band was carefully cut out, and protein was eluted from it. On analyzing by SDS-PAGE, it showed a single band indicating that azoreductase enzyme had been purified to homogeneity (Supplementary Figure 1). M.W. of the enzyme was found to be 60 kDa, similar to that of azoreductase from *Bacillus* sp. strain SF (Maier et al. 2004). On incubating with DR28 dye and NADH, it could decolourize the dye, re-confirming its azoreductase activity.

Although *B. velezensis* culture could aerobically decolourize DR28, the main issue with azo dyes is their toxicity and mutagenicity rather than persistence. DR28 is a benzidine-based azo dye with well-established toxic and mutagenic potency (Morgan et al. 1994). Hence, testing of reduction in toxicity and mutagenicity of the dye during biodegradation was essential. There are very few such studies reporting detoxification of azo dyes during biodegradation, and none of them has described changes in mutagenicity of the dyes. Also, the test organisms and procedures employed for toxicity assays in these studies are very diverse. For example, inhibition of bioluminescence (Microtox; Hu 2001), toxicity towards *Daphnia* (Isik and Sponza 2004), inhibition of plant seed germination (Pourbabaee et al. 2006), etc., have been used as test systems. However, none of the test organisms is directly relevant to the dye decolourization process, and the results obtained cannot be extrapolated to higher animals. Cytotoxicity and inhibition of respiration by bacteria have been used as simple in vitro models (Isik and Sponza 2004). However, use of undefined mixed bacterial cultures in such studies has made reproducibility of the results difficult.

To address above limitations, a battery of toxicity tests was carefully selected and used in this study. Cytotoxicity towards a known pure culture was used as a simple reproducible model. The biodecolourizer, i.e., *B. velezensis* culture itself was selected as the test organism because the results would then also determine both efficiency and suitability of the culture for dye degradation process. Viability of the cells was determined by plating on agar medium. However, this method underestimates cell viability as it ignores metabolically damaged cells that are alive, but have lost the ability to form colonies on general growth media (Alexander et al. 1999). To overcome this problem, respiration measurement using metabolic indicator MTT was used to assess the cell viability. The linear relationship between amount of MTT reduced and cell viability is well-established (Botsford 1998). Indeed, cell viability estimated from respiration measurements was found to be higher than that obtained from plate count. However, both the assays are essential to determine whether toxicity is through cell death or just metabolic damage to cells.

Although easy to carry out, results obtained from such bacterial systems cannot be directly extrapolated to eukaryotic cells, as both are not only structurally, but also physiologically distinct. Hence, cytotoxicity and respiration inhibition tests were also carried out on human HL-60 cell line. Results obtained were found to correlate well with those obtained from the bacterial system. In all the tests, DR28 dye exhibited slight toxicity at 0 h. However, upon degradation, toxicity sharply increased on the 3rd day followed by a steady decrease up to 15th day ($p<0.01$; Figs. 2, 3). Thus, the *B. velezensis* culture isolated in present study could not only decolourize, but also detoxify the dye.

As toxicity and mutagenicity are different attributes, the toxicity results were supplemented by mutagenicity testing. Mutagenicity was checked by Ames test. The results correlated well with the toxicity results for both the tester strains, i.e., TA98 and TA100. As reported earlier, DR28 was found to be non-mutagenic with only rat liver S9 activation (Novotný et al. 2006). Upon degradation, its mutagenicity reached maximum on the third day and then gradually reduced (Fig. 4). Thus, *B. velezensis* reduced both

toxicity and mutagenicity of DR28 dye ($p<0.01$). In our knowledge, this is the first study showing reduction in both toxicity and mutagenicity of an azo dye by biodegradation.

As toxicity and mutagenicity of DR28 dye followed the same trend during its biodegradation, it was hypothesized that the same degradation intermediates must be responsible for both these attributes. These intermediates were identified as benzidine and 4-ABP by HPLC-MS analysis (Supplementary Figure 2). Toxic and mutagenic potencies of both the compounds are well documented (Morgan et al. 1994). Levels of benzidine and 4-ABP were found to be highest on the 3rd day, followed by their degradation resulting in decrease in their concentration up to 15th day (Fig. 1). Thus, reduction in toxicity and mutagenicity clearly followed changes in the levels of these two intermediates, indicating that they must be responsible for toxicity and mutagenicity of the dye.

As DR28 is a benzidine-based dye, its cleavage by azoreductase must result in release of benzidine (Isik and Sponza 2004). 4-ABP can then be formed from benzidine by a simple deamination reaction (Bafana et al. 2007). Thus, *B. velezensis* could not only decolourize DR28 by azoreductase enzyme, but also, degrade the resulting benzidine moiety to 4-ABP. In our knowledge, this is the first report showing degradation of an azo dye to 4-ABP by a *Bacillus* culture.

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