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ORIGINAL PAPER

Coordinate action of exiguobacterial oxidoreductive enzymes in biodegradation of reactive yellow 84A dye

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Abstract A novel bacterial species identified as *Exiguobacterium* sp. RD3 degraded the diazo dye reactive yellow 84A (50 mg l^{-1}) within 48 h at static condition, at 30°C and pH 7. Lower salinity conditions were found to be favorable for growth and decolorization. Enzymatic activities of an H_2O_2 independent oxidase along with laccase and an azoreductase suggest their prominent role during the decolorization of reactive yellow 84A. Presence of an H_2O_2 independent oxidase in *Exiguobacterium* sp. RD3 was confirmed and hydrogen peroxide produced was detected by a coupled iodometric assay. Azoreductase activity was prominent in presence of cofactors NADH and NADP in mineral salt medium. Considerable depletion of COD of the dye solution during degradation of dye was indicative of conversion of complex dye into simple oxidizable products. Products of degradation were analyzed by HPLC, FTIR and GCMS. A possible product of the degradation was identified by GCMS. Degradation of dye resulted with significant reduction of phytotoxicity, confirming the environmentally safe nature of the degradation metabolites.

Keywords *Exiguobacterium* · Reactive yellow 84A · Laccase · Salinity · Phytotoxicity

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Introduction

Dyes released from the textile processing and dyestuff manufacturing industries results increase in organic load of the natural reservoirs. Pollution caused by dye effluent is mainly due to durability of dyes in waste water, color fastness, stability and resistance of dyes to degradation. The water consumption and wastewater generation from a textile industry depends upon the processing operations employed during textile manufacturing.

Effluent discharge area of textile industry is observed to be rich in biodegradation microbial flora. These can thrive there because of their metabolic adaptability. Biodegradation of textile dyes by living forms occurs naturally in the natural habitats. Azo dyes belong to the largest class with a wide variety of

colors and structure (Bafana et al. 2007). Diverse groups of anaerobic bacteria contribute to the anaerobic decolorization of azo dyes via reduction of azo bonds to produce colorless metabolites (Kim et al. 2007). Under anaerobic conditions, azo dyes act as terminal electron acceptors during microbial respiration (Ramalho et al. 2004).

Many of the dyes when released into natural reservoirs are toxic for the natural fauna and flora. Besides this wastewater from fabric and yarn dyeing impose serious environmental problems because of their color and their high chemical oxygen demand (Dutta et al. 2002). Several reports suggest the biodegradation capability of different living forms individually as well as in consortium. The fungi studied for dye degradation include *Fomes lividus* (Selvam et al. 2003), *Tramates* (Abadulla et al. 2000), *Phanerochaete chrysosporium* (Minussi et al. 2001; Yu et al. 2006), *Lentinus edodes* (Hatvani and Mecs 2001). Fungal enzyme systems need low pH for optimal activity and longer retention times for complete decolorization (Swamy and Ramsay 1999). Bacteria studied for biodegradation include *Pseudomonas* NBAR (Bhatt et al. 2005), *Rhodopseudomonas palustris*, etc. (Liu et al. 2006). Use of microbial consortium for biodegradation of dyes has also been reported (HeFang et al. 2004; Moosvi et al. 2005; Senan and Abraham 2004). Addition of quinoid redox mediators to anaerobically incubated cultures is reported to significantly increase reduction rates for the azo dye (Rau et al. 2002; Van der Zee et al. 2001).

In present study we have studied biodegradation of an azo dye reactive yellow 84A by *Exiguobacterium* sp. RD3 and determined the enzymes possibly involved in biodegradation.

Materials and methods

Dyes and chemicals

The diazo sulfur containing dye reactive yellow 84A dye was obtained from local textile industry Solapur, India. Tartaric acid was obtained from BDH Chemical India. ABTS (2,2′-azino-bis (3-ethyl benzothiazoline-6-sulfonic acid)) and NADH were obtained from Sigma Chemical Company (USA); while guaiacol, veratryl alcohol, potassium iodide, and starch from SRL Chemical, India.

Isolation and identification

The strain used in present study *Exiguobacterium* sp. RD3 was isolated from soil at wastewater contaminated area in the vicinity of textile industry at Solapur. A dry environment in this region is observed most of the year. Growth of pure culture of *Exiguobacterium* sp. RD3 was maintained on the nutrient agar medium having composition (g l^{-1}): peptone 5, NaCl 5, beef extract 3 and yeast extract 1. Optimum temperature for growth was 30°C. Identification of the isolate as an *Exiguobacterium* was done by 16S rDNA analysis at geneOmbio Technologies, Pune, and deposited in the Gene Bank under accession number EF 541141.

Phylogenic analysis

The partial nucleotide sequence of *Exiguobacterium* sp. RD3 was blasted using the NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the homologous species were used for phylogenic analysis. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length was 261.84316451. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) was shown next to the branches (Felsenstein 1985). The phylogenetic tree was linearized assuming equal evolutionary rates in all lineages (Takezaki et al. 1995). The clock calibration to convert distance to time was 0.01 (time/node height). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and were in the units of the number of base substitutions per site. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 1,417 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007).

Effect of different reactive yellow 84A concentrations

As reported in case of many other reactive azo dyes, effect of different concentrations on decolorization

was studied (Mabrouk and Yusef 2008). Different concentrations of reactive yellow 84A, i.e. 5, 10, 20, 40, 50, 60, and 100 mg were added to the cultured nutrient broths (100 ml each) and incubated at 30°C to observe the decolorization time. Decolorization percentage for each dye concentration was determined using formula: $(A-B)/A \times 100$; where A is initial absorbance and B is observed absorbance on decolorization at 420 nm.

Change in chemical oxygen demand during decolorization

The decolorization medium was observed for the change in chemical oxygen demand (COD) at different time intervals. For determining change in COD, titrimetric procedure was followed in which supernatant refluxed with potassium dichromate, in presence of silver sulfate, mercury sulfate and concentrated H_2SO_4 was titrated with ferrous ammonium sulfate (FAS) using ferroin indicator (Guivarch et al. 2003).

Enzyme preparation and assays

Cells were harvested from nutrient medium after 24 h (control) and after decolorization of dye (test) by centrifugation at 6,000 rpm for 20 min. Under chilling conditions the cell biomass was suspended (100 mg ml^{-1}) in 50 mM potassium phosphate buffer pH 7.4. The chilled suspension was homogenized adequately and then sonicated; giving five strokes each of 30 s at amplitude 50 to get crude enzyme solution. The time gap between two consecutive strokes was 2 min. Crude enzyme solution was centrifuged at 5,000 rpm for 20 min and the resultant solutions were used to study the control as well as induced enzyme activity.

Enzymatic assay of oxidase

The presence of an oxidase was confirmed by performing assays for determination of guaiacol and veratryl alcohol oxidation. Reaction mixture for guaiacol oxidation contained 18 mM guaiacol in 10 mM tris chloride buffer [pH 8] (Deits et al. 1984). For determining veratryl alcohol oxidation the same reaction mixture contained 2 mM veratryl alcohol in place of guaiacol. Formation of tetraguaiacol was measured at 436 nm and that of veratraldehyde at 310 nm. Enzyme activities were calculated using the extinction coefficients of tetraguaiacol product (6.39×10^3 M^{-1} cm^{-1}) at 436 nm (Deits et al. 1984) and of veratraldehyde (9,300 M^{-1} cm^{-1}) at 310 nm (Kapich et al. 2005; Tien et al. 1986).

Production of H_2O_2

A modified Graf and Penniston (1980) method was used for evaluation the abiliy of the H_2O_2 producion by the oxidase from *Exiguobacterium* sp. RD3. In this assay the enzyme activity in reaction mixture of previously mentioned oxidation assay was stopped by boiling the reaction mixture after 3 min followed by addition of 0.2 ml potassium iodide (20 mM) and 0.2 ml starch (2%). The second reaction was initiated by addition of ammonium molybdate having composition 1 mM l^{-1} ammonium molybdate in 0.5 mol l^{-1} H_2SO_4 (247.2 mg of $(NH4)_6MO_7O_{24}{\cdot}4H_2O$ and 5.6 ml of concentrated H_2SO_4, diluted to 200 ml with water); which acts as a catalyst. The blue colored complex formed by starch and free iodine liberated on H_2O_2 action was measured at 530 nm.

The laccase and azoreductase assays were performed as reported earlier (Jadhav et al. 2007). Enzyme activities were calculated using extinction coefficient of oxidized ABTS (3.6×10^4 M^{-1} cm^{-1}) at 420 nm (Bacchi et al. 2004) and of methyl red (23,360 M^{-1} cm^{-1}) at 430 nm (Chen et al. 2005). All the assays were carried out at 30°C using UV–Vis spectrophotometer (Hitachi U-2800) (Table 1).

Effect of salinity on biodecolorization

Effect of different salt concentrations on the growth of the organism and decolorization was studied by using different sodium chloride concentrations 0.5, 1, 2, 3, 4, and 5% in the mineral salt medium. Study of such effect has been also reported earlier (Salah Uddin et al. 2007). Halophilic bacteria are also reported to decolorize azo dye under high salt conditions (Guo et al. 2007). The change in color of the medium and growth of cell biomass were observed at 420 and 620 nm, respectively at different time intervals.

Effect of cofactors on azoreductase activity

Cofactors NADH, NADP, cytochrome *c*, and riboflavin (50 μg ml^{-1}) were added along with dye in the medium

Table 1 Enzyme activities in *Exiguobacterium* sp. RD3 during reactive yellow 84A degradation

Enzyme activity	Control cells	Cells obtained after decolorization
Exiguobacterial oxidase (*A*)[a]	17.76 ± 0.15	32.41*** ± 0.83
Exiguobacterial oxidase (*B*)[a]	5.89 ± 0.14	25.31*** ± 0.34
Laccase[a]	7.22 ± 0.04	37.03*** ± 0.92
Azoreductase[a]	1.71 ± 0.08	2.90*** ± 0.11

The values are mean of three experiments and SEM (±) is significantly different from the control cells at *** $P < 0.01$ by one-way analysis of variance (ANOVA) test with Tukey Kramer multiple comparison test

A, exiguobacterial oxidase for guaiacol as a substrate; *B*, exiguobacterial oxidase for veratryl alcohol as a substrate

[a] μM of product formed per mg enzyme per minute

containing 24 h old growth of *Exiguobacterium* sp. RD3. Effect of these cofactors on the azoreductase activity was observed by performing azoreductase assay for each cofactor.

Analytical procedures

Supernatant of the decolorized medium was extracted using ethyl acetate in 1:1 proportion and concentrated using rotary evaporator. This concentrated solution was dried and the dry residue obtained was dissolved in HPLC grade methanol and then used for analysis by FTIR, HPLC, and GCMS. FTIR analysis was carried out using Perkin Elmer Spectrophotometer. HPLC analysis was performed in an isocratic Waters 2690 system equipped with dual absorbance detector, using C_{18} column (4.6 × 250 mm) and HPLC grade methanol as a mobile phase.

A QP2010 gas chromatography coupled with mass spectrometer (Shimadzu) was used for GCMS analysis. The analysis was performed in the temperature-programming mode at an ionization voltage 70 eV. Temperature of the Restek column (0.25 mm, 60 m; XTI-5) was kept 80°C for initial 2 min, then raised up to 280°C with rate of 10°C min^{-1}, and held for 7 min. The temperature of injection port and the GC/MS interface was maintained at 280 and 290°C, respectively. The flow rate for helium as a carrier gas was 1.0 ml min^{-1}. NIST spectral library stored in the computer software (version 1.10 beta, Shimadzu) of the GCMS was used for comparison of retention times and mass spectra of degradation metabolites based on their fragmentation pattern.

Phytotoxicity studies

Phytotoxicity studies were carried out by using plants *Sorghum vulgare* and *Triticum aestivum.* The plants were supplemented with equal volumes of water, dye (500 ppm) and degradation metabolites (500 ppm), incubated for 7 days and finally root and shoot development was observed (Table 2).

Table 2 The effect of RY84A and its degradation metabolites on germination and seedlings of *Sorghum vulgare* and *Triticum aestivum*

Parameters	*Sorghum vulgare*			*Triticum aestivum*		
	Plain water	RY84A (500 ppm)	Degradation metabolite (500 ppm)	Plain water	RY84A (500 ppm)	Degradation metabolite (500 ppm)
Germination (%)	100	60	90	100	60	90
Plumule (cm)	4.80 ± 0.07	0.78*** ± 0.75	3.08$^{\$\$}$ ± 0.05	6.87 ± 0.12	2.9*** ± 0.09	4.85 ± 0.81$^{\$\$}$
Radicle (cm)	2.47 ± 0.06	0.28*** ± 0.07	2.10$^{\$\$\$}$ ± 0.35	9.6 ± 0.09	6.01*** ± 0.11	5.92$^{\$\$\$}$ ± 0.84

The values are mean of 10 germinated seeds of three sets SEM (±). Significantly different from the seeds germinated in plain water *** $P < 0.001$ and significantly different from the seeds germinated in reactive yellow 84A at $^{\$\$\$}$ $P < 0.001$ by one-way analysis of variance (ANOVA) test with Tukey Kramer multiple comparison test

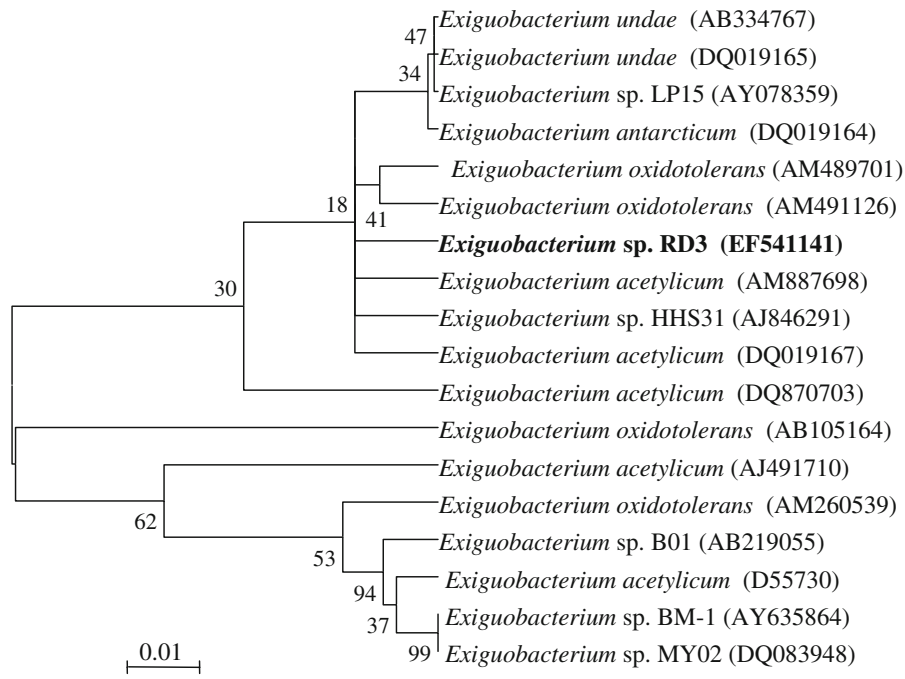

Fig. 1 Phylogenic tree of *Exiguobacterium* sp. RD3

Results and discussion

Phylogenic position of isolate

Phylogenic position of *Exiguobacterium* sp. RD3 in relation to other species of this genus is as illustrated in Fig. 1. The digits adjacent to nodes are the statistical frequency of the indicated species. The numbers shown in parentheses are accession numbers of different species. The homologous strains selected include *Exiguobacterium undae* (AB334767 and DQ0 19165), *Exiguobacterium* sp. LP15 (AY078359), *Exiguobacterium antarcticum* (DQ019164), *Exiguobacterium oxidotolerans* (AM489701 and AM491 126), *Exiguobacterium* sp. HHS31 (AJ846291), *Exiguobacterium acetylicum* (AM887698, DQ019167, D55730, AJ491710 and DQ870703), *E. oxidotolerans* (AB105164 and AM260539), *Exiguobacterium* sp. B01 (AB219055), *Exiguobacterium* sp. BM-1 (AY635864), and *Exiguobacterium* sp. MY02 (DQ083948).

Decolorization studies at different dye concentrations

When the effect of different dye concentrations on the decolorization was studied, 90.05 and 21.05% decolorization was observed at concentrations 50 and 1,000 mg l^{-1}, respectively. This suggests the higher concentration of dye might be inhibiting the enzymatic system required for the degradation (Fig. 2).

Change in COD during decolorization

There is considerable depletion of COD of dye solution, i.e., more than 50% was observed during its

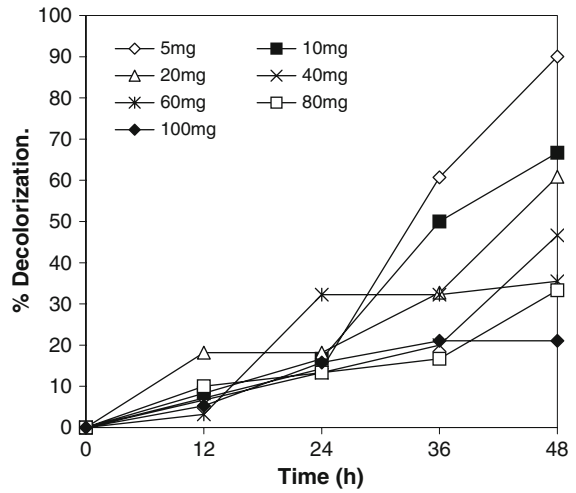

Fig. 2 Effectiveness of decolorization at different concentrations of reactive yellow 84A

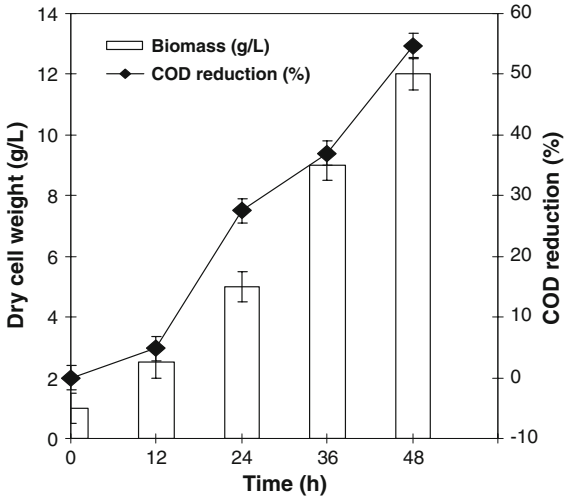

Fig. 3 Change of biomass and in COD during decolorization

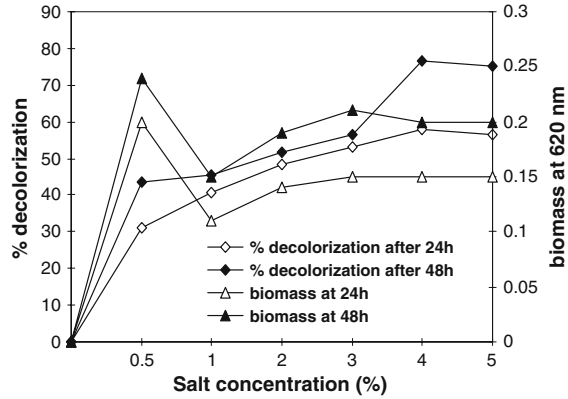

Fig. 5 Effect of salinity, on reactive yellow 84A decolorization

degradation by *Exiguobacterium* sp. RD3 (Fig. 3). Estimations at time intervals of decolorization process showed decrease in COD values. High rate in COD reduction is considered as an indication of mineralization (Hassan and Hawkyard 2002).

Enzyme assays

Since the enzyme used in present study is in crude form, it highlights the combined action of studied oxidative and reductive enzymes during biodegradation of reactive yellow 84A. The analytical studies clear that there is a coordinate action of these mentioned enzymes for breakdown the dye molecules. As reported in case of peroxidases there is requirement of H_2O_2 during catalysis (Lan et al. 2007; Vyas and Molitoris 1995). Fungal Mn peroxidases have been reported to require a Mn dependent oxidation to produce hydrogen peroxide required as co-substrate during catalysis (Francisco et al. 1999). The oxidase in present case does not require H_2O_2 during catalysis. In fact it produces the peroxide molecules which are confirmed by iodometric method (Fig. 4). In coordination to this enzyme there is also significant presence of laccase and azoreductase. During biodegradation, an azoreductase cleaves the azo bonds in the parent as

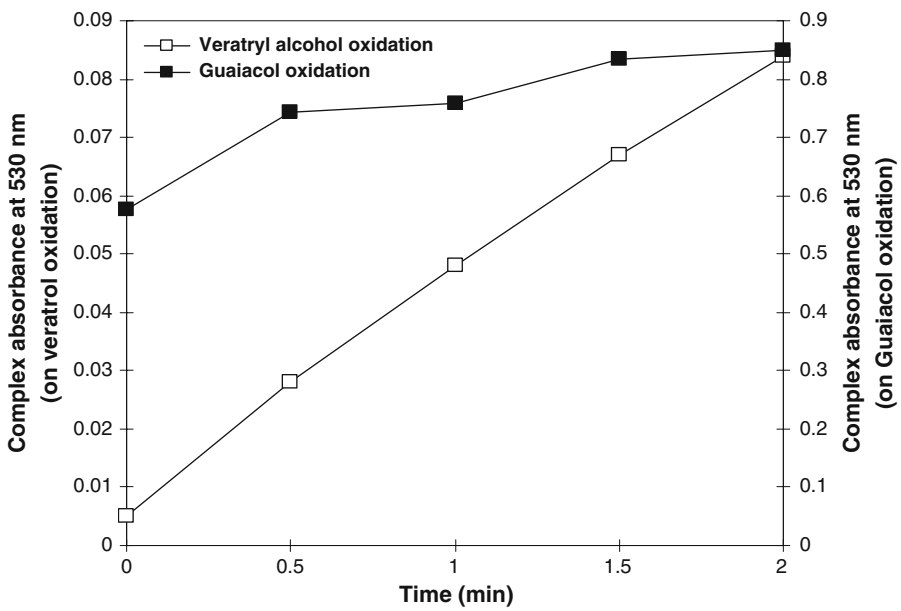

Fig. 4 Hydrogen peroxide formation by exiguobacterial oxidase, analyzed by using coupled iodometric assay

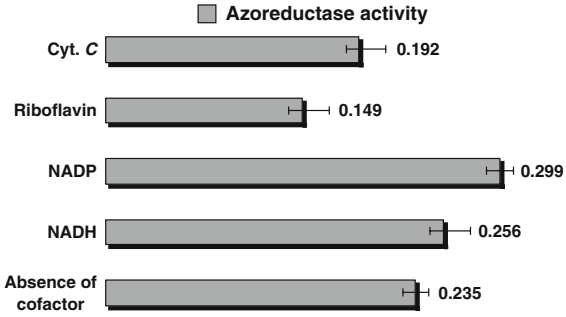

Fig. 6 Effect of different cofactors, on azoreductase activity

well intermediate compounds formed during the degradation. While an oxidase and or laccase acted to make the asymmetric cleavages in the intermediates of degradation of dye molecules.

Effect of salinity on biodecolorization

Satisfactory growth and decolorization was observed upto low salt concentration i.e., up to 4% w/v in mineral salt medium. At salt concentration 5% w/v there is slight decrease in percentage decolorization

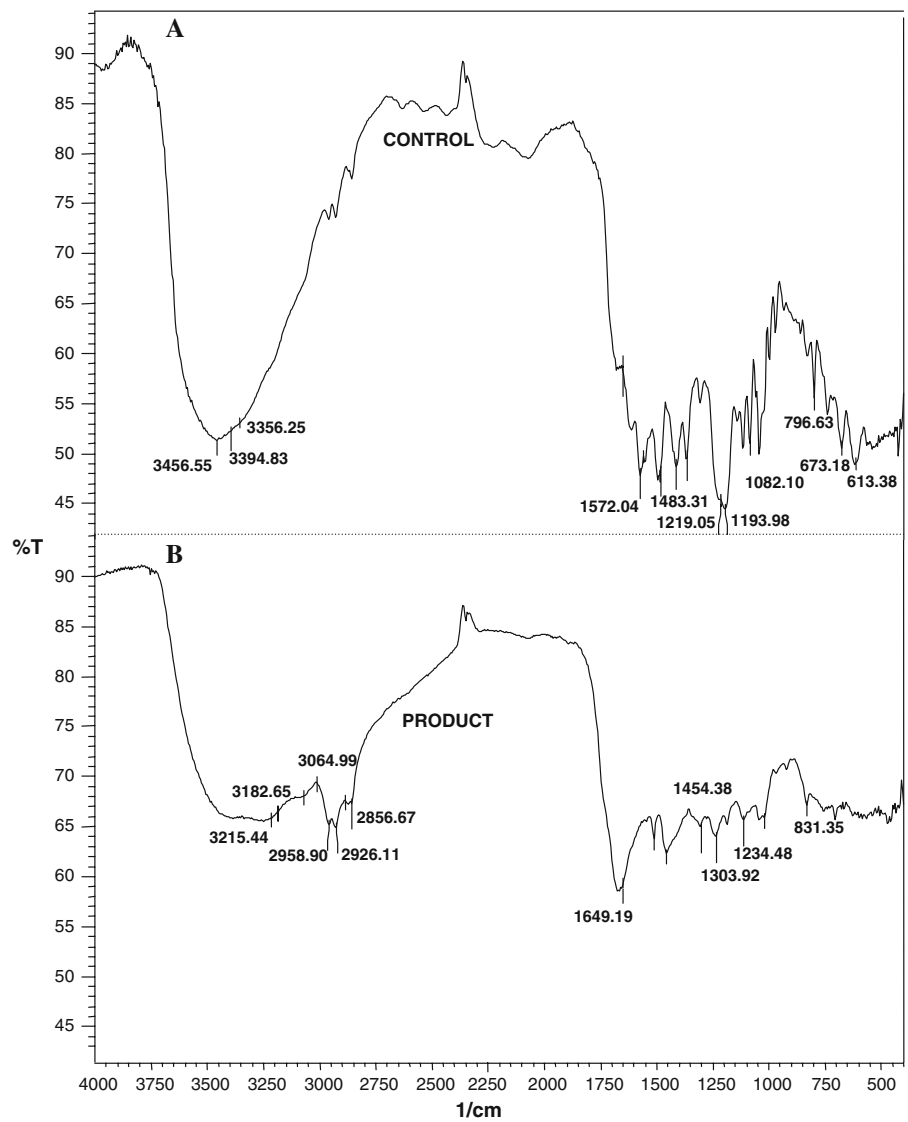

Fig. 7 FTIR spectra of reactive yellow 84A (**a**) and its degradation metabolites (**b**)

(Fig. 5). Inhibition to microorganisms by high salt concentration, may cause plasmolysis and/or loss of activity of cells (Panswad and Anan 1999).

Effect of cofactors on azoreductase activity

Azoreductase activity was found to be more prominent in presence of cofactors NADP and NADH. Whereas presence of riboflavin and cytochrome *c* reduced azoreductase activity up to certain level (Fig. 6). As reported by Rafii et al. (1990) bacteria like *Butyrivibrio* sp. and *Bacteroides* sp. required presence of flavin compounds for azoreductase activity, while for some other species like *Clostridium perfringens*, *Clostridium paraputrificum*, *Eubacterium* sp., no added flavin was required for azoreductase activity.

Characteristic of metabolities

FTIR spectrum of control, i.e., dye reactive yellow 84A (Fig. 7a) shows peak 613.38 cm^{-1} for terminal or monosubstituted deformations of alkynes. A peak at 796.63 cm^{-1} confirms presence of chloride. Peak at 1,082.10 cm^{-1} confirms aromatic nature of the dye. Peak 1,138.04 cm^{-1} indicates presence of sulfone group (S=O). Peak 1,193.38 cm^{-1} indicates presence of ester group (R–COOR′). Peak at 1,219.05 cm^{-1} indicates C–N vibration. Peak at 1,365.65 cm^{-1} shows NO_2 stretching. Peak 1,411.94 cm^{-1} shows O–H deformation. Peak at 1,483.31 cm^{-1} is indicative of alkanes (–CH_2). Peak 1,572.4 cm^{-1} confirms presence of azo bond with N=N stretching. Peak 3,356.25 cm^{-1} indicates primary amines with N–H stretching, while peak 3,456.55 cm^{-1} shows secondary amines with N–H stretching.

FTIR spectrum of degradation metabolite of reactive yellow 84A (Fig. 7b) shows peak 1,020.38 cm^{-1} for primary alcohol with C–OH stretching and peak 1,111.03 cm^{-1} for secondary alcohol. Absence of peak at 796.63 cm^{-1} shows removal of chlorine. Peak 1,184.33 shows presence of esters with S=O stretching. Peak 1,234.48 confirms presence of sulfur group. Peak at 1,508 cm^{-1} shows presence of aromatic nitroso group with N=O stretching. Peak at 1,649.19 confirms presence of secondary amines. Peak 2,856.67 shows presence of aldehyde group with C–H stretching. Peaks 2,926.11 and 3,064.99 cm^{-1} shows alkanes with C–H stretching. Peak at 3,182.65 indicates R–COOH group with O–H stretching and peak at 3,315.44 indicates presence of amide with N–H stretching.

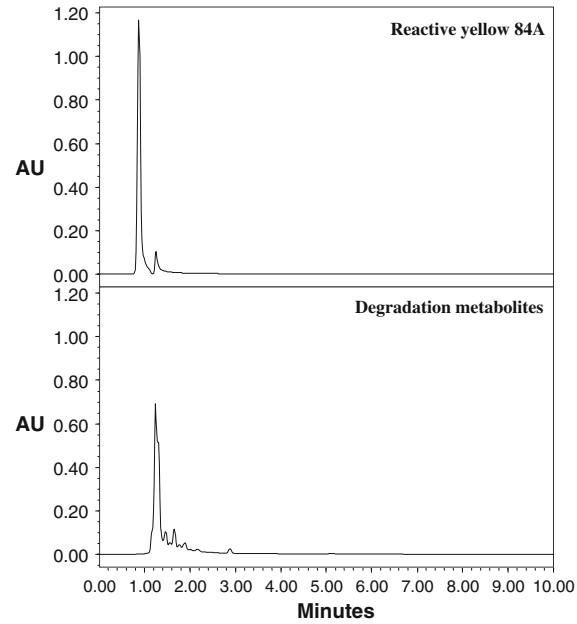

Fig. 8 HPLC elution profile of the reactive yellow 84A and its degradation metabolites

HPLC elution profiles of dye reactive yellow 84A and its product (Fig. 8) show peaks with different retention times and absorbances. In case of product there is decrease in absorbance of peak at retention time 1.5 which is maximum for dye. In addition there is appearance of smaller but prominent peaks with different retention times ranging from 1.6 to 3.0 min. This elution profile confirms breakdown of dye and formation of its metabolites.

GCMS analysis showed appearance of distinct mass peaks. On the basis of fragmentation pattern of these mass peaks, a degradative pathway for the reactive yellow 84A molecule was proposed (Fig. 9). In this pathway the intact dye molecule undergoes an oxidative cleavage and an azo bond breakage to remove an aminobenzene 2,3,5 triol (MW 141; *m*/*z* 141; RT 19.81; %area 5.75) molecule from parent structure to form an intermediate-1. Further this intermediate undergoes cleavage to remove an aniline molecule. The produced intermediate-2 undergoes asymmetric cleavage to form p (1,3 diamino 5-chlorophenyl) aminobenzene. (MW 195; *m*/*z* 195; RT 20.66; %area 10.66) and a second intermediate-3. Finally there is azo bond break in this intermediate to

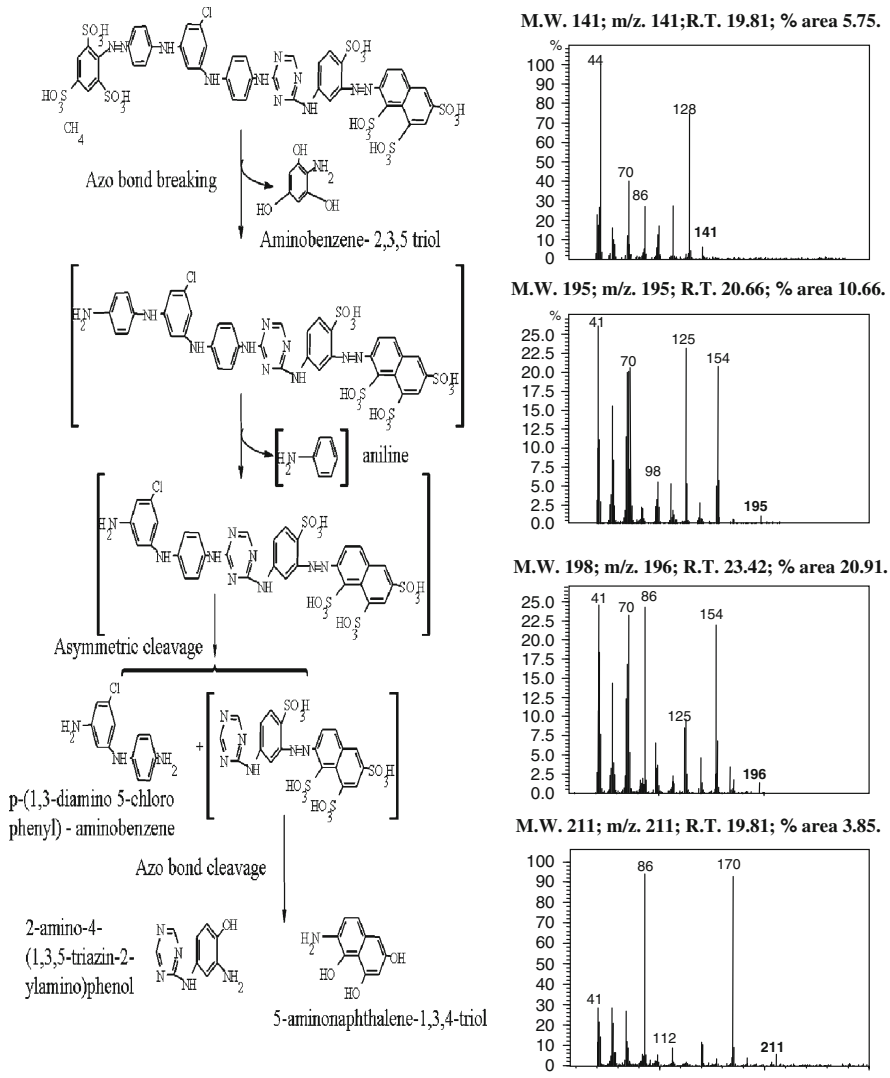

Fig. 9 Proposed pathway for the degradation of reactive yellow 84A and the related mass peaks

form 2-amino-4-(1,3,5-triazin-2-ylamino) phenol (MW 198; *m/z* 196; RT 23.42; %area 20.91) and 7-aminonaphthalene-1,3,8-triol (MW 211; *m/z* 211; RT 19.81; %area 3.85). Even though the intermediates do not show peripheral mass peaks in the spectra, appearance of mass peaks with molecular weight matching to proposed molecules in degradation and the enzymatic status of the *Exiguobacterium* sp. RD3 suggests the cleavage outline. Thus analytical studies confirmed the biodegradation of reactive yellow 84A dye in which the smaller molecular weight intermediates are formed by consecutive action of enzymes present in *Exiguobacterium* sp. RD3.

Phytotoxicity studies

Phytotoxicity studies revealed that there is diminished germination with stunted root and shoot growth in presence of the dye. Whereas in presence of degradation metabolites of the dye, studied plant seeds germinated adequately as well as there was good root and shoot development. This confirmed the nontoxic nature of the degradation metabolites of reactive yellow 84A. Reduction in phytotoxic nature of dye molecules have also been reported earlier by bacterial and fungal sources (Shedbalkar et al. 2008).

Conclusion

An isolate *Exiguobacterium* sp. RD3 efficiently degraded the reactive yellow 84A dye. The metabolites produced were environmentally safe since they did not show phytotoxicity. Presence of hydrogen peroxide independent oxidase, along with laccase and azoreductase highlighted their coordinated action, for the breaking of reactive yellow 84A.

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