Journal of Hazardous Materials 163 (2009) 735–742

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Ecofriendly biodegradation and detoxification of Reactive Red 2 textile dye by newly isolated *Pseudomonas* sp. SUK1

D.C. Kalyani, A.A. Telke, R.S. Dhanve, J.P. Jadhav*

Department of Biochemistry, Shivaji University, Kolhapur 416004, India

ARTICLE INFO

Article history:
Received 1 February 2008
Received in revised form 24 April 2008
Accepted 4 July 2008
Available online 12 July 2008

Keywords:
Pseudomonas sp. SUK1
Textile dyes
Biodegradation
Lignin peroxidase
Detoxification

ABSTRACT

The aim of this work is to evaluate textile dyes degradation by novel bacterial strain isolated from the waste disposal sites of local textile industries. Detailed taxonomic studies identified the organisms as *Pseudomonas* species and designated as strain *Pseudomonas* sp. SUK1. The isolate was able to decolorize sulfonated azo dye (Reactive Red 2) in a wide range (up to 5 g l^{-1}), at temperature 30 °C, and pH range 6.2–7.5 in static condition. This isolate also showed decolorization of the media containing a mixture of dyes. Measurements of COD were done at regular intervals to have an idea of mineralization, showing 52% reduction in the COD within 24 h. Induction in the activity of lignin peroxidase and azoreductase was observed during decolorization of Reactive Red 2 in the batch culture, which represented their role in degradation. The biodegradation was monitored by UV–vis, IR spectroscopy, HPLC. The final product, 2-naphthol was characterized by GC-mass spectroscopy. The phytotoxicity study revealed the degradation of Reactive Red 2 into non-toxic product by *Pseudomonas* sp. SUK1.

1. Introduction

The number of studies on the biodecoloration of dyestuffs has been steadily increasing in recent years [1]. The textile industry is one of the greatest generators of liquid effluent pollutants, due to the high quantities of water used in the dyeing processes. It is estimated that 280,000 tonnes of textile dyes are discharged in such industrial effluent every year worldwide [2], out of that azo dyes make up about a half of all known dyestuffs in the world, making them the largest group of synthetic colorants and the most common synthetic dyes released into the environment [3]. The effluents from these industries are complex, containing a wide variety of dyes and other products, such as dispersants, acids, bases, salts, detergents, humectants, oxidants, etc. Discharge of these colored effluents into rivers and lakes results into reduced dissolved oxygen concentration, thus creating anoxic conditions that are lethal to resident organisms. Many reports indicate that textile dyes and effluents have toxic effect on the germination rates and biomass of several plant species, where as plant play many important ecological function such as providing the habitat for a wildlife, protecting soil from erosion, and providing bulk of organic matter that is so significant to soil fertility [4,5]. Therefore, the treatment of industrial effluents containing aromatic compounds becomes necessary prior to their final discharge to the environment. Existing physical/chemical technologies for color removal are very expensive and commercially unattractive [6]. Biological processes provide an alternative to existing technologies because they are more cost-effective, environmentally friendly, and do not produce large quantities of sludge. Many microorganisms belonging to the different taxonomic groups of bacteria, fungi, actinomycetes and algae have been reported for their ability to decolorize azo dyes [7]. Pure fungal cultures have been used to develop bioprocesses for the mineralization of azo dyes, but the long growth cycle and moderate decolorization rate limit the performance of fungal decolorization system [8]. In contrast, bacterial decolorization is normally faster. Previous studies indicated that, bacterial strains like *P. mirabilis*, *P. luteola*, *Pseudomonas* sp. and *K. rosea* have shown very promising results for dye degradation under anoxic conditions [9–12]. The decolorization of sulfonated Reactive Red 2 dye were studied by number of investigator, but most of these studies have emphasized only decolorization/degradation of dye, with no discussion of the degradation by product and their toxicity in the environment [13–16]. Very recently few of investigators have reported degradation and toxicity of products formed after decolorization at static (anoxic) condition [12,17–19] with respect to other dyes.

In order to develop a practical bioprocess for the treatment of dye wastewater, it is necessary to isolate and investigate the microorganisms capable of degrading azo dyes. In the present investigation we have evaluated the decolorization, biodegradation of Reactive Red 2 and mixture of various textile dyes which are

* Corresponding author. Tel.: +91 231 2609365; fax: +91 231 2691533.
E-mail address: jpj_biochem@unishivaji.ac.in (J.P. Jadhav).

0304-3894/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jhazmat.2008.07.020

commonly used in textile industries of Solapur, India by novel isolated bacterium *Pseudomonas* sp. SUK1 in static condition. Enzymes involved in the degradation were assayed and metabolites formed after degradation were analysed. Further, the toxicity potential of the dye on two common plant seed *S. vulgare* and *P. mungo* was compared before and after microbial degradation.

2. Experimental

2.1. Dyes and chemicals

Tartaric acid was obtained from BDH Chemicals, India. *n*-Propanol from SRL Chemicals, India. DCIP (2,6-dichlorophenol indophenol sodium salt) obtained from Sisco Research Laboratories Pvt. Ltd. Mumbai, India and NADH were obtained from Sigma Chemical Company (USA). The dyes Reactive Red 2, Reactive Blue 59, Reactive Red BLI, Reactive Navy Blue HE2R, Reactive Orange 4, Reactive Golden yellow HER, Reactive Red HE8B, Reactive Orange 72, Reactive Red Brown, Reactive Green HE4BD were obtained from Gurudutta Textile Industry, Solapur, India.

2.2. Isolation, screening and identification of dye degrading microorganism

The isolation, screening and identification of novel dye degrading microorganism was done as earlier reported Kalyani et al. [20]. The 16S rDNA sequence was initially analyzed at NCBI server (http://www.ncbi.nlm.nih.gov) using BLAST (blastn) tool and corresponding sequences were downloaded. Phylogenetic tree was constructed by Neighbour-Joining method [21], using the MEGA4 package [22].

2.3. Acclimatization

The acclimatization was done by gradually exposing *Pseudomonas* sp. SUK1 to the increasing concentrations of dye as per earlier report Kalme et al. [23]. The successive transfers of culture into fresh nutrient medium containing 100, 150, 200, 250 and 300 mg l^{-1} of the Reactive Red 2 dye was done at 30 °C in static condition. This acclimatized microorganism was used for all studies.

2.4. Culture media

The composition of decolorizing medium was (g l^{-1}): peptone 10, NaCl 5, yeast extract 2 and beef extract 1.

2.5. Decolorization experiments

A loopful of microbial culture (*Pseudomonas* sp. SUK1) was cultivated in static condition for 24 h at 30 °C in 250 ml Erlenmeyer flask containing 100 ml media, after 24 h the dye was added at concentration 300 mg l^{-1}. Where as, to examine the effect of initial dye decolorization in static condition, the dye was added with different concentration 1, 2, 3, 4, and 5 g l^{-1} after 24 h growth. Aliquots (3 ml) of the culture media was withdrawn at different time intervals, centrifuged at 7000 rpm for 15 min to separate the bacterial cell mass. Decolorization of the dye was analyzed using UV–vis spectrophotometer (Hitachi U 2800, Tokyo, Japan) at 540 nm. Mixture of 10 dyes (Reactive Blue 59, Reactive Red BLI, Reactive Navy Blue HE2R, Reactive Orange 4, Reactive Golden Yellow HER, Reactive Red HE8B, Reactive Orange 72, Reactive Red 2, Reactive Red Brown, Reactive Green HE4BD), each at concentration 100 mg l^{-1} were added in 24 h grown culture medium, decolorization was measured spectrophotometrically at visible wavelength 470 nm. Reduction in chemical oxygen demand (COD) was also studied in the same sample [24]. The chloride interference was removed by using $HgSO_4$; the mixture of diluted sample (before and after treatment) and $K_2Cr_2O_7$ was refluxed (acidic condition and Ag_2SO_4 as catalyst) in a reflux flask equipped with condenser on a hot plate for 3 h. The refluxed sample was titrated against ferrous ammonium sulfate (FAS) as titrant. The NB medium was used as blank and similar condition was used for test. Finally COD was calculated by using following formula:

$$\text{COD (mg l}^{-1}) = \frac{(A-B)\times \text{normality}\times 1000\times 8}{\text{volume of sample (mL)}}$$

where A is the ml of FAS was used for blank, B is the ml of FAS was used for test sample, N is the normality of FAS and 8 is the milliequivalent weight of oxygen.

All decolorization experiments were performed in the three sets and the decolorization activity is expressed in terms of the percentage decolorization as follows:

$$\text{decolorization activity (\%)} = \frac{[(\text{initial absorbance}) - (\text{observed absorbance})]\times 100}{\text{initial absorbance}}$$

Bacterial growth was measured by estimating the intracellular protein content. Cell pellet was washed in distilled water to remove adhere dye particles and then boiled in 1 M NaOH for 15 min, protein content was measured by Lowry method [25]. The relation between protein concentration and OD_{620} was 1.0 OD_{620} = 462 mg of protein.

2.6. Preparation of cell free extract

The bacterial cells were grown in the nutrient broth at 30 °C for 24 h consider being control, centrifuged at 7000 rpm for 20 min. These cells (75 mg ml^{-1}) were suspended in a potassium phosphate buffer (50 mM, pH 7.4) and sonicated (Sonics-vibracell ultrasonic processor) keeping sonifier output at 50 amp and giving 7 strokes each of 30 s, with 3 min interval at 4 °C. The homogenate was centrifuged at 8000 rpm for 20 min and supernatant was used as a source of crude enzyme. Similar procedure was used to quantify enzyme activities during the dye decolorization at different time intervals (2 h, 4 h and 6 h).

2.7. Enzyme assays

Activity of lignin peroxidase was assayed by using procedure reported earlier [20]. NADH-DCIP reductase activity was determined using a procedure reported earlier [26]. Azoreductase enzyme activity was assayed by modifying earlier method [27]; the assay mixture (2 ml) contained 50 mM phosphate buffer (pH 7.4), 1 mM NADH, 0.25 μM Reactive Red 2 and 0.2 ml of crude enzyme. The reaction mixture was pre-incubated for 4 min followed by the addition of NADH and monitored for the decrease in color intensity at 540 nm at room temperature. Enzyme activity was calculated by using molar absorption coefficient of Reactive Red 2 (2.250 mM^{-1} cm^{-1}) at 540 nm.

2.8. Analytical procedure

The decolorization was monitored by using UV–vis spectroscopy analysis (Hitachi U 2800). Where as, the metabolites produced during biodegradation of Reactive Red 2, were extracted with equal volumes of ethyl acetate. The extract was dried over anhydrous Na_2SO_4 and evaporated to dryness in rotary evaporator. The crystals obtained were dissolved in small volumes of HPLC grade methanol and the same sample was used for FTIR, HPLC and GC–mass spectral

analysis. HPLC analysis was carried out (Waters model no. 2690) on RP-C18 guard column. The mobile phase was methanol with flow rate was 1 ml min^{-1}. FTIR analysis of biodegraded Reactive Red 2 was carried out using Perkin Elmer 783 Spectrophotometer by compared with control dye. The FTIR analysis was done in the mid IR region of 400–4000 cm^{-1} with 16 scan speed. The samples were mixed with spectroscopically pure KBr, pellets formed were fixed in sample holder, and the analysis carried out. The identification of metabolites formed after degradation was carried using a QP2010 gas chromatography coupled with mass spectroscopy (Shimadzu). The ionization voltage was 70 eV. Gas chromatography was conducted in the temperature programming mode with a Restek column (0.25 mm, 60 m; XTI-5). The initial column temperature was 80 °C for 2 min, then increased linearly at 10 °C min^{-1} to 280 °C, and held for 7 min. The temperature of the injection port was 280 °C and the GC/MS interface was maintained at 290 °C. The helium carrier gas flow rate was 1.0 ml min^{-1}. Degradation products were identified by comparison of retention time and fragmentation pattern, as well as with mass spectra in the NIST spectral library stored in the computer software (version 1.10 beta, Shimadzu) of the GC–MS.

2.9. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparisons test.

2.10. Phytotoxicity study

Phytotoxicity of the Reactive Red 2 was performed in order to assess the toxicity of textile industry effluent. The obtained product was dissolved in the water to form a final concentration of 5000 ppm. The study was carried out (at room temperature) using *S. vulgare* and *P. mungo* (10 seeds of each) by adding separately 10 ml sample of the Reactive Red 2 (5000 ppm) and its degradation products (5000 ppm) per day. Control set was carried out using water at the same time. Germination (%) and length of plumule and radicle was recorded after 8 days.

3. Results and discussion

3.1. Phylogenetic position of isolates

To analyze the phylogenetic position, the 16 S rDNA sequence of the strain SUK1 (996 bp, EF541140) was determined. Fig. 1 showed the phylogenetic relationship between the strain SUK1 and other related microorganisms found in the GenBank database. The homology assay result indicated that the strain SUK1 was in the phylogenetic branch of the *Pseudomonas*. SUK1 exhibited a maximum identities (97%) to *P. pseudoalcaligens* AB276372, *P. mendocina* strain 147 AY870673. The earlier report shows other species of *P. mendocina* have role in biodegradation of dye [28], where as there was no literature available on *P. pseudoalcaligens* in dye degradation, but, this microorganism has highly developed for the degradation of other toxic compounds like nitrobenzene and cyanide [29,30].

3.2. Effect of static and shaking conditions

The acclimatized culture of the *Pseudomonas* sp. SUK1, under agitation conditions demonstrated a better growth (296 ± 2.4 mg l^{-1}) than that under static conditions (214 ± 2.8 mg l^{-1}). As for decolorization, agitated culture showed almost no decolorization in 24 h, while the static culture decolorized more than 96% of the initial dye concentration (300 mg l^{-1}) in 6 h. To conform whether this decolorization was due to the microbial action or change in pH, the change in pH was recorded, which was in the range of 6.2–7.5 at static condition. Confirms the decolorization of dye was due to microbial action. Anaerobic or static conditions were necessary for bacterial decolorization, though the cell growth was poorer than that under aerobic condition. Therefore, static conditions were adopted to investigate bacterial decolorization in the following experiments. The results

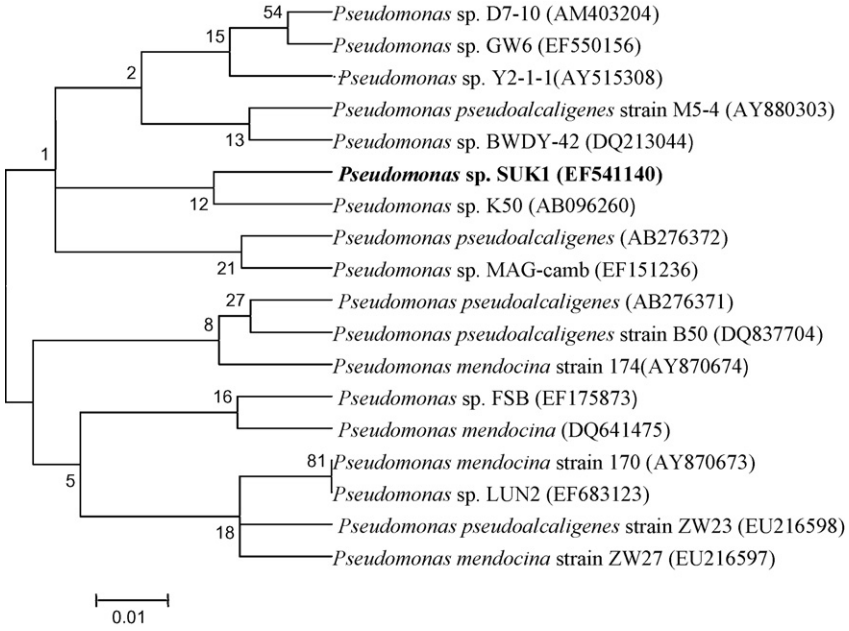

Fig. 1. The evolutionary history was inferred using the Neighbor-Joining method; the sequences have been retrieved from NCBI database, showing the phylogenetic relationships of *Pseudomonas* sp. SUK1 and other species of genus *Pseudomonas*. Numbers at nodes shows the level of bootstrap support based on data for 1000 replication. Bar, 0.01 substitutions per nucleotide position and numbers in parenthesis represent GenBank accession numbers.

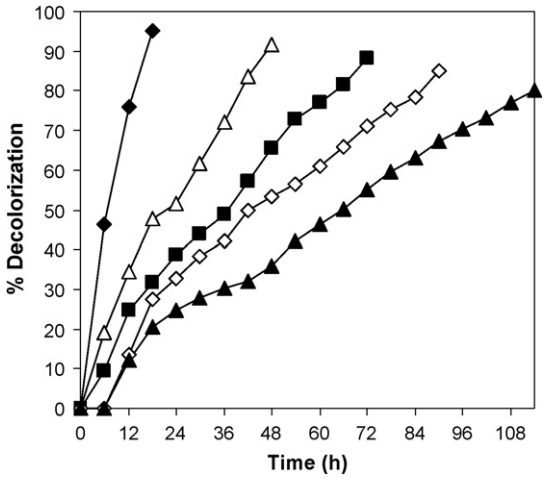

Fig. 2. Percentage decolorization of Reactive Red 2 at various dye concentrations, i.e. 1 g l^{-1} (♦), 2 g l^{-1} (△), 3 g l^{-1}(■), 4 g l^{-1} (◊) and 5 g l^{-1} (▲) by *Pseudomonas* sp. SUK1.

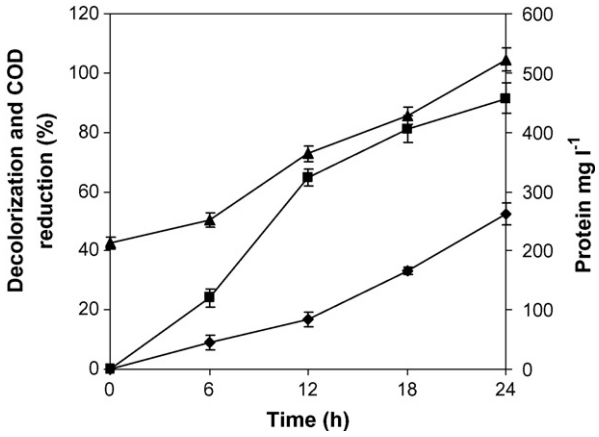

Fig. 3. Mixture of dye decolorization and COD reduction at static condition. Cell biomass (▲), % dye decolorization (■) and % COD reduction (♦).

were similar to those of studies on pure bacterial strains such as, *P. mirabilis*, *P. luteola*, *P. desmolyticum* and *S. marcescens* [9,10,19,31]. Under aerobic conditions azo dyes are generally resistant to attack by bacteria [32]. Azo dye decolorization by bacterial species if often initiated by enzymatic reduction of azo bonds, the presence of oxygen normally inhibits the azo bond reduction activity since aerobic respiration may dominate utilization of NADH; thus impeding the electron transfer from NADH to azo bonds [33].

3.3. Effect of different concentrations of dyes on decolorization

To assess the maximum decolorization ability of *Pseudomonas* sp. SUK1 on Reactive Red 2, strain was tested against different concentrations of the dye (Fig. 2). At 1–2 g l^{-1} dye concentration 95–91% dye removal was observed; 88–84% decolorization was observed at concentration of 3–4 g l^{-1} and 80% of dye was removed at concentration 5 g l^{-1}. The time required for decolorization of 1 g l^{-1} dye was 18 h, whereas it was 48 h for 2 g l^{-1} dye concentration. As concentration was increased (3–5 g l^{-1}), the time required for decolorization varied from 72 to 114 h. When the dye concentration was as high as 5 g l^{-1}, almost 80% of the dye was removed after 114 h. This means that an acceptable high color removal can be achieved by the *Pseudomonas* sp. SUK1 strain in an extensive range of azo dye concentrations. Similarly, the earlier report shows the decolorization of dye at various concentrations [7,15,23] but, the decolorization was not prolonged at certain concentration of dye as compare to *Pseudomonas* sp. SUK1. In addition, a substrate inhibition effect was observed as further concentration of dye increases. Reduction in color removal may result from the toxicity of dyes to bacteria through the inhibition of metabolic activities. Azo dyes generally contain one or more sulphonic-acid groups on aromatic rings, which might act as detergents to inhibit the growth of microorganisms [34].

3.4. Decolorization of the dye mixture and COD reduction

Dyes of different structures are often used in the textile processing industry, and, therefore, the effluents from the industry are markedly variable in composition. The observed decolorization was very fast, i.e. 91% and the COD removal was always lower than percent decolorization at given concentration. Observed COD reduction of 52% showed a partial mineralization of mixture of dyes (Fig. 3). COD reduction had occurred in the solution, which is indicative of the fact that the color of the dyes mixture was removed through degradation.

3.5. Enzyme analysis

The lignin peroxidase, azoreductase and DCIP reductase enzyme activities were recorded during the time course of Reactive Red 2 degradation (Table 1). Induction in the lignin peroxidase and azo reductase was observed during the time course of dye decolorization, where as, DCIP reductase activity remains constant under the conditions specified. Lignolytic enzymes of fungi are immensely studied for their applications in the degradation of polymeric dyes from textile industry and paper whitening. Among them most frequently studied fungus for these applications is basidiomycete *P. chrysosporium* and *B. adusta*, whose secreted enzymes have an enormous potential for decolorizing textile dyes [35–37]. Similarly, recent report shows that bacteria also able to produce the lignin peroxidase, which has ability to degradation of polymeric dyes from the textile industry and paper whitening through oxidative cleavage [12,19,20,23,38]. Where as, azoreductase is the key enzyme expressed in azo dye degrading bacteria that catalyze the reductive cleavage of the azo bond, which has been identified in several

Table 1
Intracellular enzyme activities in of *Pseudomonas* sp. SUK1 cells in induced state (at 2 h, 4 h and 6 h during decolorization) compared to cells in control

Enzyme assay	Control	2 h	4 h	6 h
Lignin peroxidase[a]	0.935 ± 0.21	1.219 ± 0.16***	1.219 ± 0.17***	1.80 ± 0.20***
Azoreductase[b]	0.078 ± 0.012	0.087 ± 0.037	0.15 ± 0.06***	0.10 ± 0.05*
DCIP-reductase[c]	29.24 ± 1.86	30.45 ± 2.14	31.54 ± 1.41	30.32 ± 1.75

Values are mean of three experiments ± S.E.M. Significantly different from control cells at *$P < 0.05$, ***$P < 0.001$ by one-way ANOVA with Tukey–Kramer comparison test.

[a] Enzyme unit's min^{-1} mg protein^{-1}.

[b] μM of Reactive Red 2 reduced min^{-1} mg protein^{-1}.

[c] μg of DCIP reduced min^{-1} mg protein^{-1}.

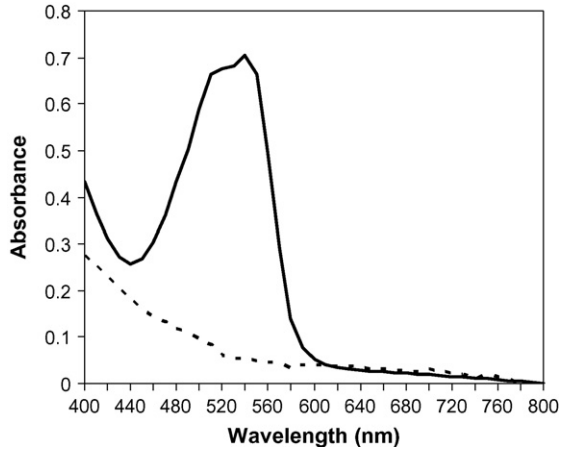

Fig. 4. UV–vis spectral scans from batch culture of *Pseudomonas* sp. SUK1 containing 300 mg l^{-1} of Reactive Red 2 at incubation time: 0 h (—) and 6 h (- - -).

species of bacteria; such as, *Pseudomonas* KF46, *P. luteola*, *P. aeruginosa*, *Bacillus* sp. strain OY1-2, *S. aureus* [27,32,39–41]. All these azo reductases demonstrated better abilities at degrading azo dyes than whole cells, which possessed new potentials for the degradation of azo dyes with enzyme. Our investigations using crude enzyme preparation on the involvement of any azoreductase using Reactive Red 2 showed the presence of intracellular azoreductase activity, which may be responsible for the reduction of azo bonds in the dye decolorization process.

3.6. Biodecolorization and biodegradation analysis

UV–vis scan (400–800 nm) of supernatants at different time intervals showed decolorization and decrease in the dye concentration from batch culture (Fig. 4). Peak observed at 542 nm (control) was decreased without any shift in λ_{max} up to complete decolorization of the dye in the medium (6 h). To investigate the azo dye degrading mechanism further FTIR, HPLC and GC–MS analysis were carried. The FTIR spectrum of a control dye compared with extracted metabolites (6 h) was shown in Fig. 5 Peak in the control dye spectrum represented the stretching vibrations of S=O at 1048 cm^{-1}, symmetric stretching at 2924 cm^{-1} and asymmetric stretching at 2853 cm^{-1} for C–N. 3422 cm^{-1} represented the presence of free NH group from parent dye structure. The stretching vibrations between C–Cl showed a band at 677 cm^{-1}. The stretching between C–H was reported at 2924 cm^{-1}, whereas peak at 1578 cm^{-1} represented –N=N– stretching of azo group. The FTIR spectrum of 6 h extracted metabolites showed significant change

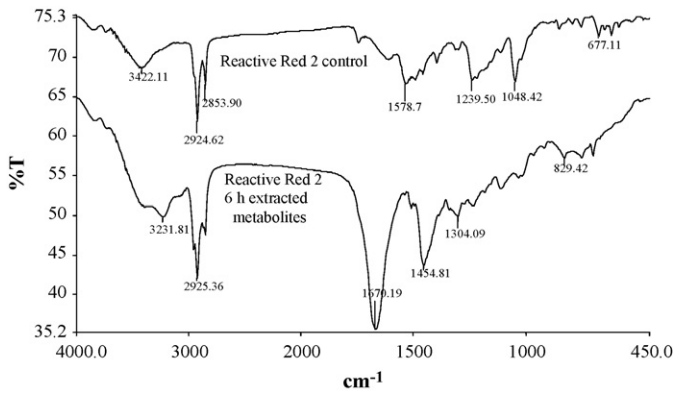

Fig. 5. FTIR spectra of Reactive Red 2 and its degradation metabolites.

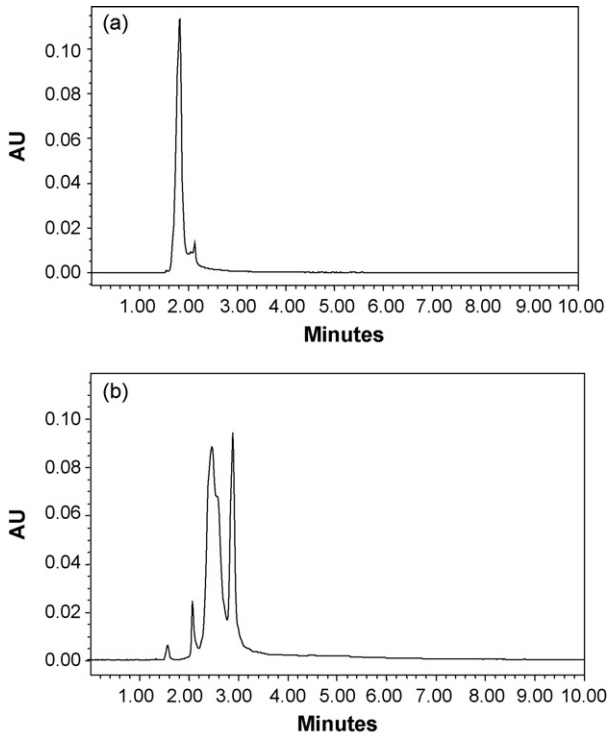

Fig. 6. HPLC elution profile of Reactive Red 2 (a) and its degradation metabolites (b).

in the positions of peaks when compared to control dye spectrum. A new peak 1304 cm^{-1} represented formation of secondary aromatic amines with C–N vibration, peak at 3231 cm^{-1} represented N–H stretching in amide. A new peak at 1454 cm^{-1} represented C–H deformation of acyclic CH_2. The C–H deformation showed at 829 cm^{-1} pointed towards formation of benzene ring with C=O stretching at 1670 cm^{-1}. The HPLC analysis of dye sample collected at the beginning showed one major peak at retention time 1.80 and one minor peak at retention time 2.13 (Fig. 6a). As, the decolorization progressed the emergence of additional peaks were observed due to degradation of parent dye (after 6 h) with two minor peaks at retention time 1.63 min, 2.13 min and two major peaks at retention time 2.51 min and 2.93 min (Fig. 6b). In order to verify degradation products formed during dye decolorization by *Pseudomonas* sp. SUK1 the GC–MS analysis was carried out, which reveals presence of several peaks. The low molecular weight aromatic compound were produced from the degradation of Reactive Red 2 by *Pseudomonas* sp. SUK1. The pathway is proposed in degradation of Reactive Red 2, Fig. 7, showing various steps involved in the degradation. However very little is known about the nature of degradation products formed in these reactions and mechanism by which peroxidase oxidise and reductase reduce the dye molecules. According to our proposal, the H_2O_2 forms of peroxidase catalyzed initially the asymmetric cleavage, resulted in the intermediate product which was identified as phenylazo-2-naphthol 5,8 disulfonic acid with retention time 28.76 min and a mass peak of 407 (Fig. 8a), 1,3,5 triazine 2,4 diol with retention time 26.56 min and a mass peak 113 (Fig. 8b) supports the oxidative asymmetric cleavage of Reactive Red 2. The reduction of phenylazo-2-naphthol 5,8 disulfonic acid by azoreductase giving rise the reactive intermediate product in this reaction as 1-amino-8-naphthol 2,5 disulfonic acid at retention time 27.36 min and a mass peak 317 (Fig. 8c), and aniline at retention time 26.14 min and a mass peak 93 (Fig. 8d). While, the degradation of naphthalene moiety substituted with hydroxyl, an amino and sulfonic group (intermediate product of reductive cleavage) was observed

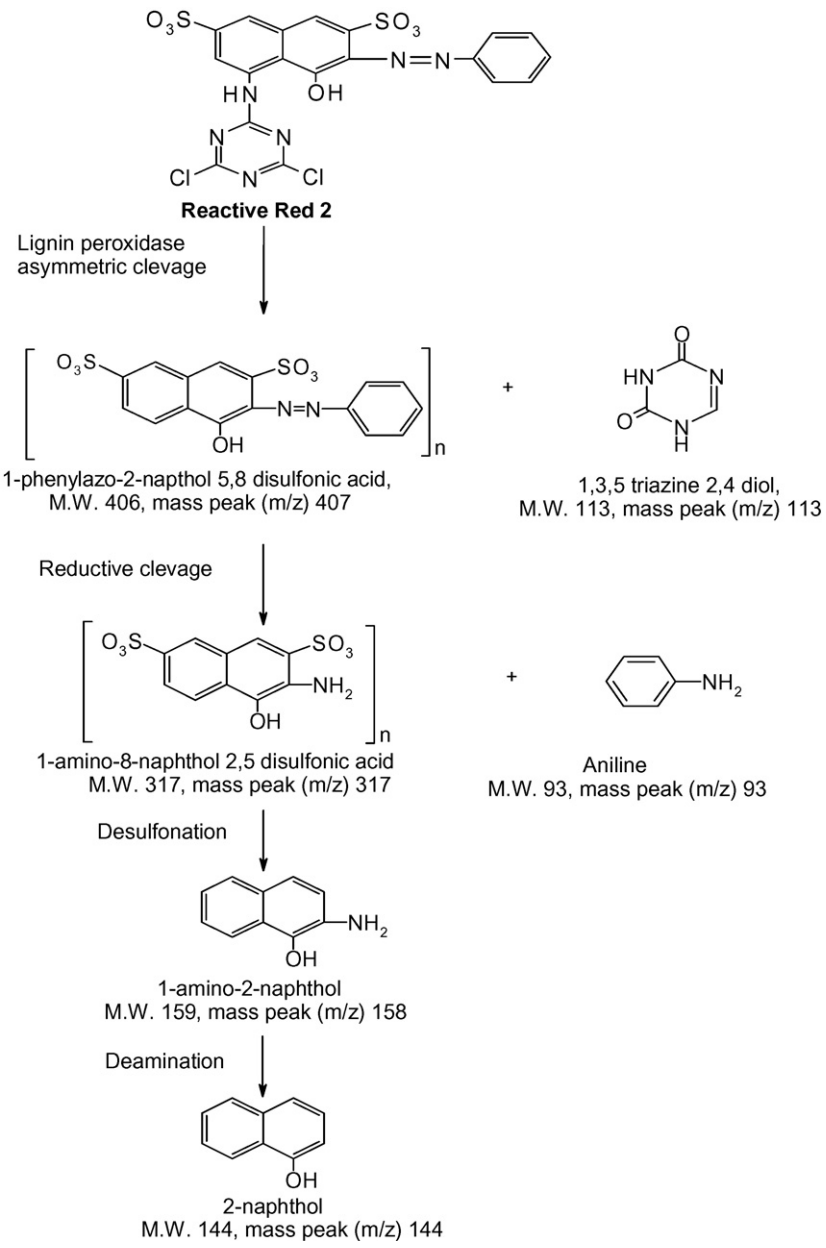

Fig. 7. Proposed pathway for biodegradation of Reactive Red 2 by *Pseudomonas* sp. SUK1.

in the form of low molecular weight compound such as 1-amino-2-naphthol with retention time 28.98 min and a mass peak 158 (Fig. 8e). Further, deamination reaction might be followed giving rise the final identified product 2-naphthol in this pathway, which was recognized from the *m*/*z* value obtained, having molecular weight 144 with retention time 27.12 min (Fig. 8f). Thus salient features of this mechanistic proposal, are the release of azo linkage and formation of phenyl radicals as intermediates, which indicates that the *Pseudomonas* sp. SUK1 have potential to degrade the dye Reactive Red 2.

Table 2
Phytotoxicity study of Reactive Red 2 and its degradation product

Parameters	*Sorghum vulgare*			*Phaseolus mungo*		
	Water	Reactive Red 2[a]	Extracted metabolite[a]	Water	Reactive Red 2[a]	Extracted metabolite[a]
Germination (%)	100	0	80	100	80	100
Plumule (cm)	7.12 ± 1.82	0.0 ± 0.0***	6.35 ± 0.88	11.88 ± 0.80	1.75 ± 0.40***	10.20 ± 1.10
Radicle (cm)	7.01 ± 1.59	0.0 ± 0.0***	2.50 ± 0.92*	3.67 ± 0.82	0.29 ± 0.09***	3.05 ± 0.33

Values are mean of three experiments, S.E.M. (±), significantly different from the control (seeds germinated in water) at $^{*}P < 0.05$ and $^{***}P < 0.001$ by one-way analysis of variance (ANOVA) with Tukey–Kramer comparison test.

[a] 5000 ppm concentration.

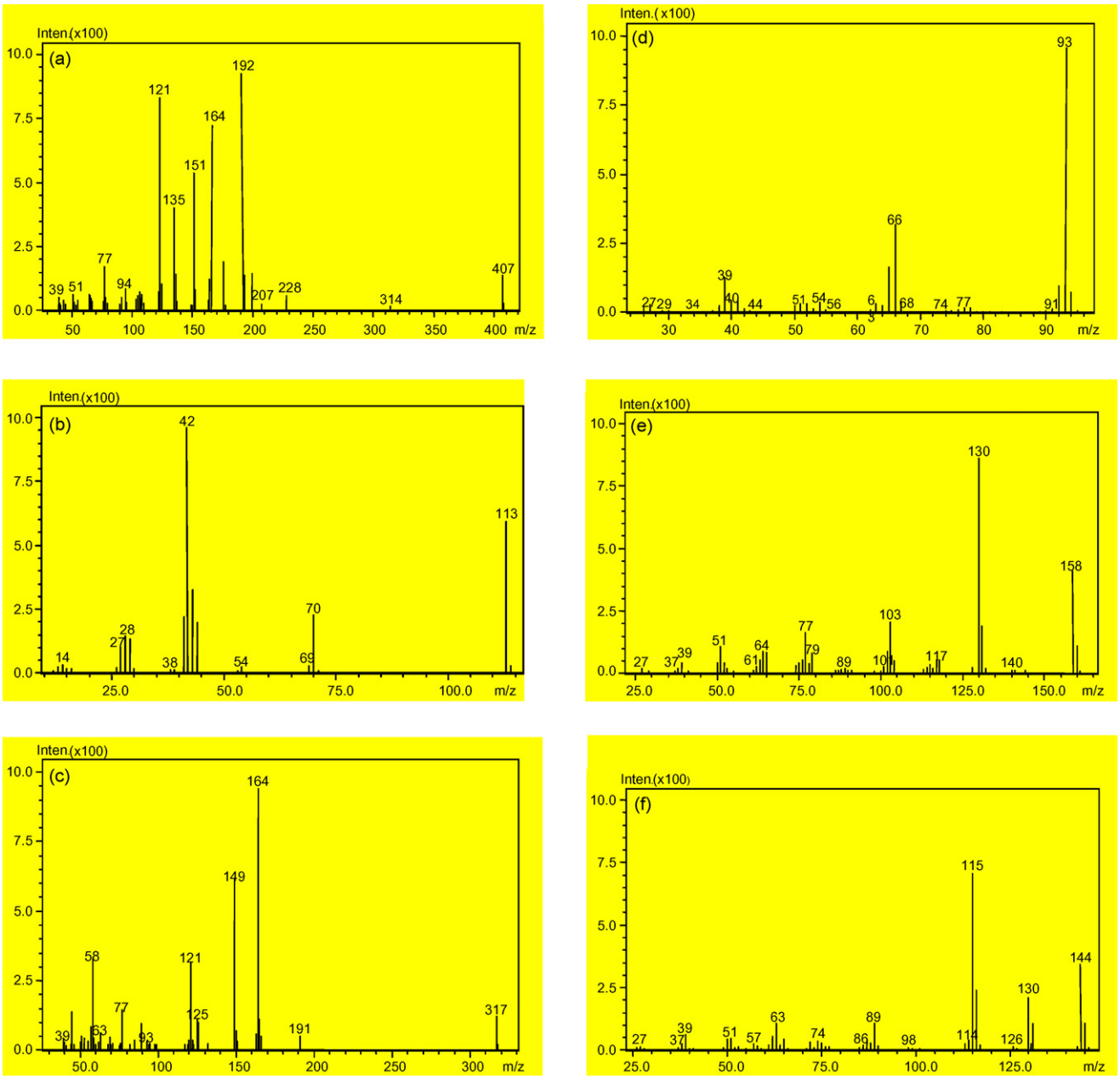

Fig. 8. Identification of metabolites of Reactive Red 2 by GC–MS. (a) Phenylazo-2-naphthol 5,8 disulfonic acid, (b) 1,3,5 triazine 2,4 diol, (c) 1-amino-8-naphthol 2,5 disulfonic acid, (d) aniline, (e) 1-amino-2-naphthol and (f) 2-naphthol.

3.7. *Toxicity study*

Previously, it was found that many sulfonated aromatic amines accumulate in the environment as evidenced by their occurrence in the surface water, where they are substantial polluting factor [42]. Other dye metabolites such as unsulfonated aromatic amines are relatively stable in aquatic conditions and are poorly degraded under anaerobic or aerobic wastewater treatment conditions [43]. Thus both sulfonated and unsulfonated aromatic amines are important groups of environmental pollutants formed during reduction of (sulfonated) azo dyes, that can be potentially pass through biological treatment system [44]. Therefore, it was of be concern to assess the toxicity of the dye before and after degradation, results shown in Table 2 indicated that the germination (%) and length of plumule and radicle of the both *S. vulgare* and *P. mungo* seeds was less with Reactive Red 2 treatments as compared to its extracted degradation product and water. This study reveals the metabolites generated after the biodegradation of Reactive Red 2 is less toxic compare to original dye.

4. Conclusions

The acclimated bacterial *Pseudomonas* sp. SUK1 was capable of decolorization, biotransformation and detoxification of the toxic sulfonated azo dye. As per our knowledge this seems to be the first report showing that a single microorganism can tolerate and decolorize higher concentrations of the dye. This strain also showed decolorization of dyes mixture, with significant reduction in COD, indicating the biodegradation of complex oxidizable dyes, showing applicability of the strain to wide variety of individual dye and mixture of dyes. Induction of lignin peroxidase and azoreductase enzymes during the biodegradation of

Reactive Red 2 suggests its involvement in the degradation process.

Acknowledgements

Mr. D.C. Kalyani is thankful to Shivaji University, Kolhapur for awarding with Departmental Research Fellowship.

References

[1] A. Pandey, P. Singh, L. Iyengar, Bacterial decolorization and degradation of azo dyes, Int. Biodeter. Biodegrad. 59 (2007) 73–84.
[2] X. Jin, G. Liu, Z. Xu, W. Yao, Decolorization of a dye industry effluent by *Aspergillus fumigatus* XC6, Appl. Microbiol. Biotechnol. 74 (2007) 239–243.
[3] X. Zhao, I. Hardin, HPLC and spectrophotometric analysis of biodegradation of azo dyes by *Pleurotus ostreatus*, Dyes Pigments 73 (2007) 322–325.
[4] W. Wang, Toxicity assessment of pretreated industrial effluent using higher plant, Res. J. Water Pollut. Control Fed. 62 (1991) 853–860.
[5] L.A. Kapustka, M. Reporter, Terrestrial primary producers, in: P. Calow (Ed.), Handbook of Ecotoxicology, vol. 1, Blackwell Scientific Publications, Oxford, 1993, pp. 278–297.
[6] H. Kariminiaae-Hamedaani, A. Sakurai, M. Sakakibara, Decolorization of synthetic dyes by a new manganese peroxidase-producing white rot fungus, Dyes Pigments 72 (2007) 157–162.
[7] S. Asad, M.A. Amoozegar, A.A. Pourbabaee, M.N. Sarbolouki, S.M.M. Dastgheib, Decolorization of textile azo dyes by newly isolated halophilic and halotolerant bacteria, Bioresource Technol. 98 (2007) 2082–2088.
[8] S. Moosvi, X. Kher, D. Madamwar, Isolation, characterization and decolorization of textile dyes by a mixed bacterial consortium JW-2, Dyes Pigments 74 (2007) 723–729.
[9] K.C. Chen, W.T. Huang, J.Y. Wu, J.Y. Houng, Microbial decolorization of azo dyes by *Proteus mirabilis*, J. Ind. Microbiol. Biotechnol. 23 (1999) 686–690.
[10] J.S. Chang, C. Chou, Y. Lin, J. Ho, T.L. Hu, Kinetic characteristics of bacterial azo-dye decolorization by *Pseudomonas luteola*, Water Res. 35 (2001) 2041–2850.
[11] J. Yu, X. Wang, P.L. Yue, Optimal decolorization and kinetic modeling of synthetic dyes by *Pseudomonas* strains, Water Res. 35 (2001) 3579–3586.
[12] G. Parshetti, S. Kalme, G. Saratale, S. Govindwar, Biodegradation of malachite green by *Kocuria rosea* MTCC 1532, Acta Chim. Slov. 53 (2006) 492–498.
[13] J. Axelsson, U. Nilsson, E. Terrazas, T.A. Aliaga, U. Welander, Decolorization of the textile dyes Reactive Red 2 and Reactive Blue 4 using *Bjerkandera* sp. strain BOL 13 in a continuous rotating biological contactor reactor, Enzyme Microb. Technol. 39 (2006) 32–37.
[14] R. Mass, S. Chaudhari, Adsorption and biological decolorization of azo dye Reactive Red 2 in semicontinuous anaerobic reactors, Process Biochem. 40 (2005) 699–705.
[15] K.M. Kodam, I. Soojhawon, P.D. Lokhande, K.R. Gawai, Microbial decolorization of reactive azo dyes under aerobic conditions, World J. Microbiol. Biotechnol. 21 (2005) 367–370.
[16] M.I. Beydilli, S.G. Pavlostathis, Decolorization kinetics of the azo dye Reactive red 2 under methanogenic condition: effect of long-term culture acclimation, Biodegradation 16 (2005) 135–146.
[17] K. Kumar, S.S. Devi, K. Krishnamurthi, D. Dutta, T. Chakrabarti, Decolorization and detoxification of Direct Blue-15 by a bacterial consortium, Bioresource Technol. 98 (2007) 3168–3171.
[18] K. Kumar, S.S. Devi, K. Krishnamurthi, S. Gampawar, N. Mishra, G.H. Pandya, T. Chakrabarti, Decolorization, biodegradation and detoxification of benzidine based azo dye, Bioresource Technol. 97 (2006) 407–413.
[19] S. Kalme, G. Ghodake, S. Govindwar, Red HE7B degradation being desulfonation by *Pseudomonas desmolyticum* NCIM 2112, Int. Biodeter. Biodegrad. 60 (2007) 327–333.
[20] D.C. Kalyani, P.S. Patil, J.P. Jadhav, S.P. Govindwar, Biodegradation of reactive textile dye Red BLI by an isolated *bacterium Pseudomonas* sp. SUK1, Bioresource Technol. 99 (2008) 4635–4841.
[21] N. Saitou, M. Nei, The neighbor-joining method: a new method for reconstructing phylogenetic trees, Mol. Biol. Evol. 4 (1987) 406–425.
[22] K. Tamura, J. Dudley, M. Nei, S. Kumar, MEGA4: molecular evolutionary genetics analysis (MEGA) software (version 4.0), Mol. Biol. Evol. 24 (2007) 1596–1599.
[23] S.D. Kalme, G.K. Parshetti, S.U. Jadhav, S.P. Govindwar, Biodegradation of benzidine based dye Direct Blue-6 by *Pseudomonas desmolyticum* NCIM 2112, Bioresource Technol. 98 (2006) 1405–1410.
[24] American Public Health Association, Standard Methods for Examination of Water and Wastewater APHA, AWWA and WPCF, ninetieth ed., Washington, DC, USA, 1995.
[25] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[26] M.D. Salokhe, S.P. Govindwar, Effect of carbon source on the biotransformation enzyme in *Serratia marcescens*, World J. Microbiol. Biotechnol. 15 (1999) 229–232.
[27] T. Zimmermann, H.G. Kulla, T. Leisinger, Properties of purified orange II azoreductase, the enzyme initiating azo dye degradation by *Pseudomonas* KF46, Eur. J. Biochem. 129 (1982) 197–203.
[28] S. Sarnaik, P. Kanekar, Biodegradation of methyl violet by *Pseudomonas mendocina* MCM B-402, Appl. Microbiol. Biotechnol. 52 (1999) 251–254.
[29] R.U. Halden, S.M. Tepp, B.G. Halden, D.F. Dwyer, Degradation of 3-phenoxybenzoic acid in soil by *Pseudomonas pseudoalcaligenes* POB310 (pPOB) and two modified *Pseudomonas* strains, Appl. Environ. Microbiol. 65 (1999) 3354–3359.
[30] V.M. Luque-Almagro, R. Blasco, M.J. Huertas, M. Martınez-Luque, C. Moreno-Vivian, F. Castillo, M.D. Roldan, Alkaline cyanide biodegradation by *Pseudomonas pseudoalcaligenes* CECT5344, Biochem. Soc. Trans. 33 (2005) 168–169.
[31] P. Verma, D. Madamwar, Decolourization of synthetic dyes by a newly isolated strain of *Serratia marcescens*, World J. Microbiol. Biotechnol. 19 (2003) 615–618.
[32] T.L. Hu, Degradation of azo dye RP2B by *Pseudomonas luteola*, Water Sci. Technol. 38 (1998) 299–306.
[33] A. Stolz, Basic and applied aspects in the microbial degradation of azo dyes, Appl. Microbiol. Biotechnol. 56 (2001) 69–80.
[34] K.C. Chen, J.Y. Wu, D.J. Liou, S.C.J. Hwang, Decolorization of the textile dyes by newly isolated bacterial strains, J. Biotechnol. 101 (2003) 57–68.
[35] W.A. Jarosz, R.J. Kochmanska, E. Malarczyk, W. Wardas, A. Leonowicz, Fungi and their ability to decolorize azo and anthraquinonic dyes, Enzyme Microb. Technol. 30 (2002) 566–572.
[36] D. Wesenberg, I. Kyriakides, S.N. Agathos, White-rot fungi and their enzymes for the treatment of industrial dye effluents, Biotechnol. Adv. 22 (2003) 161–187.
[37] A. Heinfling, M. Bergbauer, U. Szewzyk, Biodegradation of azo and phthalocyanine dyes by *Trametes versicolor* and *Bjerkandera adusta*, Appl. Microbiol. Biotechnol. 48 (1997) 261–266.
[38] A. Raj, R. Chandra, M.M.K. Reddy, H.J. Purohit, A. Kapley, Biodegradation of kraft lignin by a newly isolated bacterial strain, *Aneurinibacillus aneurinilyticus* from the sludge of a pulp paper mill, World J. Microb. Biotechnol. 23 (2007) 793–799.
[39] C.V. Nachiyar, G.S. Rajakumar, Purification and characterization of an oxygen insensitive azoreductase from *Pseudomonas aeruginosa*, Enzyme Microb. Technol. 36 (2005) 503–509.
[40] Y. Suzuki, T. Yoda, A. Ruhul, W. Sagiura, Molecular cloning and characterization of the gene encoding azoreductase from *Bacillus* sp. OY 1-2 isolated from soil, J. Biol. Chem. 246 (2001) 9059–9065.
[41] H. Chen, S.L. Hopper, C.E. Cerniglia, Biochemical and molecular characterization of an azoreductase from *Staphylococcus aereus*. A tetrameric NADPH-dependent flavoprotein, Microbiology 151 (2005) 1433–1441.
[42] F.T. Lange, M. Wenz, H.J. Brauch, Trace-level determination of aromatic sulfonates in water by on-line ion-pair extraction/ion-pair chromatography and their behavior in the aquatic environment, J. High Resolut. Chromotogr. 189 (1995) 243–252.
[43] H.M. Pinheiro, E. Touraud, O. Thomas, Aromatic amines from azo dye reduction: status review with emphasis on direct UV spectrophotometric detection in textile industry wastewaters, Dyes Pigments 61 (2004) 121–139.
[44] N.C.G. Tan, A. van Leeuwen, E.M. van Voorthuizen, P. Slenders, F.X. Prenafeta-Boldu, H. Temmink, G. Lettinga, J.A. Field, Fate and biodegradability of sulfonated aromatic amines, Biodegradation 16 (2005) 527–537.