Biodegradation (2010) 21:283–296
DOI 10.1007/s10532-009-9300-0

ORIGINAL PAPER

Decolorization and detoxification of Congo red and textile industry effluent by an isolated bacterium *Pseudomonas* sp. SU-EBT

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Received: 22 July 2009 / Accepted: 15 September 2009 / Published online: 23 September 2009

Abstract The 16S rRNA sequence and biochemical characteristics revealed the isolated organism as *Pseudomonas* sp. SU-EBT. This strain showed 97 and 90% decolorization of a recalcitrant dye, Congo red (100 mg l^{-1}) and textile industry effluent with 50% reduction in COD within 12 and 60 h, respectively. The optimum pH and temperature for the decolorization was 8.0 and 40°C, respectively. *Pseudomonas* sp. SU-EBT was found to tolerate the dye concentration up to 1.0 g l^{-1}. Significant induction in the activity of intracellular laccase suggested its involvement in the decolorization of Congo red. The metabolites formed after decolorization of Congo red, such as *p*-dihydroxy biphenyl, 8-amino naphthol 3-sulfonic acid and 3-hydroperoxy 8-nitrosonaphthol were characterized using FTIR and GC–MS. Phytotoxicity study revealed nontoxic nature of the degradation metabolites to *Sorghum bicolor*, *Vigna radiata*, *Lens culinaris* and *Oryza sativa* plants as compared to Congo red and textile industry effluent. *Pseudomonas* sp. SU-EBT decolorized several individual textile dyes, dye mixtures and textile industry effluent, thus it is a useful strain for the development of effluent treatment methods in textile processing industries.

Keywords *Pseudomonas* sp. SU-EBT · Congo red · Textile industry effluent · Decolorization · Laccase · Phytotoxicity

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Introduction

Textile industries generate millions of liters of untreated effluents per day which are directly discharged into drinking water resources, such as rivers and lakes. This alters pH, increases biochemical oxygen demand (BOD) and chemical oxygen demand (COD) and gives intense coloration. Several textile processing industries use conventional physiochemical effluent treatment methods. These methods have some disadvantages such as, high cost, formation of hazardous by-products, and high energy requirements. Thus, textile industries need to develop effective biological effluent treatment method as an alternative to the conventional physiochemical effluent treatment methods.

A number of biotechnological approaches have been suggested to overcome the problem of physiochemical treatment methods (Figueroa et al. 2009; Constapel et al. 2009) using microorganisms for the treatment of textile dyes and industry effluent

(Blanquez et al. 2004). The effectiveness of microbial decolorization depends on the adaptability and the activity of selected microorganisms. Many microorganisms are capable of degrading textile dyes, including bacteria (Telke et al. 2008), fungi (Parshetti et al. 2006; Kaushik and Malik 2009), yeast (Meehan et al. 2000; Jadhav and Govindwar 2006), actinomycetes (Mane et al. 2008) and algae (Daneshvar et al. 2007). Fungal treatment of the textile industry effluent has some disadvantages, including low pH requirement for the optimum activity of enzymes, the long hydraulic retention time for complete decolorization and requirement of longer time for decolorization (Swamy and Ramsay 1999). In recent years, there has been an increasing interest in bacteria that have potentiality to degrade or mineralize several textile dyes (Kalyani et al. 2009). Bacterial decolorization was associated with the involvement of oxidoreductive enzymes, including laccase, azoreductases and NADH-DCIP reductase (Kalme et al. 2007).

This study was aimed to isolate efficient bacterial strain, which possesses the ability to decolorize textile dyes and textile industry effluent. The isolated strain could decolorize Congo red that is highly recalcitrant. Very few reports are available for Congo red degradation. Hence, we have studied parameters like enzymatic status during decolorization, effect of increasing dye concentration, identification of metabolites formed after decolorization using analytical techniques and toxicity study of metabolites formed after decolorization using Congo red as model dye. Further, the study was extended for textile dyes mixtures and textile industry effluent for the optimization of the biocatalyst.

Materials and methods

Dyestuff, chemicals and microbiological media

All chemicals were of highest purity and of analytical grade. Congo red was obtained from SRL Chemicals, India. Textile dyes and textile effluent were obtained from local textile industries of Ichalkaranji, India. The *o*-tolidine and other fine chemicals were obtained from SRL Chemicals, India. Peptone, yeast extract and agar powder were obtained from Hi-media Laboratory, India.

Isolation, screening and identification of the microorganism

The microorganisms present in the soil samples from the effluent disposal site of a textile dyeing industry located at Ichalkaranji, India were enriched in nutrient broth containing mixture of textile dyes (each 200 mg l^{-1}; Congo red, Eriochrome black T, Trypan blue, Remazol blue 3R, Reactive red HE3B, Reactive navy blue RX and Reactive navy blue HE2R) under static condition. After 48 h of incubation, 1 ml of cell suspension was transferred to fresh nutrient broth containing mixture of dyes to screen the strains having the color removing ability. The screening procedure in liquid medium was continued until complete decolorization of the broth. A small amount of the decolorized broth was transferred to nutrient agar plates containing individual and mixture of textile dyes (comprising 100 mg l^{-1}; Congo red, Eriochrome black T, Trypan blue, Remazol blue 3R, Reactive red HE3B, Reactive navy blue RX and Reactive navy blue HE2R). The bacterial colonies which tolerated higher concentration of the dye were isolated by streak plate method. Bacterial isolates were then further screened for their color removal ability in liquid medium to select the superior isolate. The isolated organism was further identified on the basis of morphological and biochemical characteristics.

16S rRNA sequence analysis

The 16S rRNA sequencing of isolated organism was done in Gene*O*mbio Technologies Pvt. Ltd., Pune, India. The 16S rRNA sequence was initially analyzed at DDJB server (http://www.ddbj.nig.ac.jp) using BLAST (blastn) tool and corresponding sequences were downloaded. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The bootstrap consensus tree inferred from 1,000 replicates was taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. 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. 2004). The clock calibration to convert distance to time was 0.01 (time/node height). The tree is 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 and are in the units of the number of base substitutions per site (Tamura et al. 2004). All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 771 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007).

Microorganism, media and culture condition

Pseudomonas sp. SU-EBT was maintained on nutrient agar medium (g l^{-1}; bacteriological peptone 10, yeast extract 3, NaCl 5, and agar powder 20) and decolorization experiment was carried out in nutrient broth containing (g l^{-1}; NaCl, 5; bacteriological peptone, 10; and yeast extract, 3).

Decolorization of textile dyes

A loopful of *Pseudomonas* sp. SU-EBT culture was inoculated in 250 ml Erlenmeyer flask containing 100 ml sterile nutrient broth and incubated at static and shaking (120 rpm) condition. Congo red (100 mg l^{-1}) was added in each flask after 24 h of incubation and reincubated under static and shaking condition. Aliquots (3 ml) of the culture media were drawn after regular intervals for color measurement. Suspended particles from the culture medium were removed by centrifugation at 7,000 rpm for 20 min. Decolorization was monitored by measuring the absorbance of the supernatant at 490 nm using Hitachi U-2800 Spectrophotometer. The cell concentration was determined using OD of the culture at 620 nm (OD_{620}). The relation between bacterial cell concentration and OD_{660} was 1.0 OD_{660} = 0.148 mg wet weight of cells l^{-1}. The COD was measured by the procedure reported earlier (APHA 1991). The ability of *Pseudomonas* sp. SU-EBT to decolorize Congo red at wide pH (1–14) and temperature (10–50°C) was studied by carrying the decolorization experiment at respective pH and temperature. The effect of dye concentration on decolorization performance of *Pseudomonas* sp. SU-EBT was studied by observing the decolorization of increasing concentration of Congo red (mg l^{-1}; 200, 400, 600, 800 and 1,000). Percent decolorization and the wet cell weight at different time intervals were measured. The decolorization of individual and mixture of textile dyes was studied (Table 1). The intensity of color was measured at maximum absorbance wavelength of respective dyes or mixture of dyes.

Decolorization of textile effluent

The original textile effluent was filtered by using the filter paper. 100 ml of the filtered effluent was taken

Table 1 Decolorization of individual and mixture of textile dyes by *Pseudomonas* sp. SU-EBT

Dyes	Dye class	C.I. No.	CAS No.	λ max	Decolorization (%)	Time (h)
Golden yellow HER	Sulfonated diazo	NA	61951-85-7	430	90 ± 02	12
Remazole blue 3R	NA	NA	12225-45-5	530	97 ± 03	12
Reactive red HE3B	Sulfonated monoazo	25810	61951-82-4	540	92 ± 02	12
Reactive navy blue RX	Vinyl sulfone	NA	NA	560	95 ± 03	12
Reactive navy blue HE2R	Vinyl sulfone	NA	85782-76-9	610	90 ± 04	12
Direct red 2B	NA	NA	NA	530	92 ± 03	18
Direct brown MR	Sulfonated diazo	NA	NA	520	90 ± 02	12
Disperse brown 3RL	Monoazo	11152:2	NA	440	92 ± 03	10
Disperse navy blue 3G	NA	11344	NA	570	90 ± 04	12
Scarlet RR	NA	11131	NA	528	90 ± 02	12
Eriochrome black T	Monoazo	NA	1787-61-7	620	90 ± 03	20
Trypan blue	Diazo	NA	72-57-1	660	95 ± 02	20
Mixture of textile dyes	–	–	–	530	90 ± 04	30

into 250 ml flasks. These flasks were sterilized at 121°C for 20 min. Thereafter, each flask was inoculated with 50 ml of the inoculum and incubated at static condition. Samples were withdrawn after regular intervals and analyzed for color removal. Controls without effluent or inoculum were run under identical conditions. The intensity of color was measured at its maximum absorbance wavelength (490 nm). The physiochemical parameters of the textile effluent were pH, 8.2; electric conductivity, 3.84 μs m^{-1}; total suspended solids, 1,040 mg l^{-1}; total dissolved solids, 8,770 mg l^{-1}; total hardness, 400 mg l^{-1}; COD, 1,000 mg l^{-1}; BOD, 200 mg l^{-1}; total alkalinity, 200 mg l^{-1} (APHA 2005) and mixture of reactive, disperse and direct dyes. Percent decolorization was calculated as follows.

$$\text{Decolorization}\,(\%) = \frac{(\text{Initial absorbance}) - (\text{Observed absorbance})}{(\text{Initial absorbance})} \times 100$$

All decolorization experiments were performed in three sets. Abiotic (without microorganism) controls were included.

Enzyme assays

Preparation of cell free extract

The cells of *Pseudomonas* sp. SU-EBT were harvested by centrifugation of the 24 h grown culture at 7,000 rpm for 20 min. The cell pellets were suspended in 20 mM potassium phosphate buffer (pH 7.5) and sonicated (30 s, 60 amplitude, 7 strokes) at 4°C. The sonicated sample was centrifuged at 7,000 rpm for 20 min under cold condition. The clear supernatant obtained after centrifugation was used as the source for intracellular enzymes. Culture medium without cells was used as a source of extracellular enzyme.

Enzyme activities

All enzyme activities were assayed in the cell free extract as well as culture media at room temperature (25°C). Laccase activity was determined by using procedure reported earlier (Miller et al. 1997). The reaction mixture (2 ml) contained 1.75 ml sodium acetate buffer (pH 4.0) and 50 μl *o*-tolidine (1 mM). The reaction was initiated by addition of the enzyme. One unit of enzyme activity was defined as the amount of enzyme required for increase in 1.0 ABS unit min^{-1} under assay condition. NADH-DCIP reductase assay was carried out using procedure of Salokhe and Govindwar (1999). The composition of the assay mixture (5.0 ml) was 25 μM DCIP, 4.75 ml of potassium phosphate buffer (20 mM, pH 7.5) and 0.1 ml of enzyme solution. The reaction was initiated by addition of 50 μM NADH. The decrease in color intensity of DCIP was observed at 595 nm. The DCIP reduction was calculated using its molar extinction coefficient (ε) of 19 mM^{-1} cm^{-1}. Azoreductase assay was carried out using procedure of Chen et al. (2005). Composition of the assay mixture (2.0 ml) was 4.45 μM of methyl red (MR), 100 μM NADH, 1.7 ml of potassium phosphate buffer (20 mM, pH 7.5). The reaction mixture was pre-incubated for 4 min followed by the addition of NADH and monitored for the decrease in color absorbance (430 nm) at room temperature. The reaction was initiated by addition of 0.1 ml of the enzyme solution. Methyl red reduction was calculated by using its molar extinction coefficient of 0.023 μM^{-1} cm^{-1}. NADH-DCIP reductase and azoreductase activity was expressed in units. One unit of enzyme activity was defined as amount of enzyme required to reduce 1 μM of substrate min^{-1}.

All the enzyme assays were run in triplicates and average rates were calculated. Protein concentration was determined by using Lowry method with bovine serum albumin as the standard protein (Lowry et al. 1951).

Extraction and analysis of the metabolites formed after biodecolorization

The culture medium was centrifuged at 7,000 rpm for 20 min after total decolorization to remove the cell mass. Culture supernatant containing the metabolites formed after decolorization of Congo red were extracted using equal volume of ethyl acetate; dried over anhydrous Na_2SO_4 and concentrated in a rotary vacuum evaporator. Metabolites formed after decolorization of Congo red were characterized by using the Fourier Transform Infrared Spectroscopy (FTIR). FTIR analysis was done in the mid IR region of 400–4,000 cm^{-1} with 16 scan speed. The pellets prepared using spectroscopic pure KBr (5:95), were fixed in sample holder and analyses were carried out. The

metabolites formed after decolorization of Congo red were identified by using GC–MS. Rotary vacuum concentrated sample was dissolved in methanol and GC–MS analysis of metabolites formed after decolorization were carried out using a QP 5000 mass spectrophotometer (Shimadzu). The ionization voltage was 70 eV. Gas chromatography was conducted in temperature programming mode with a Resteck column (0.25 mm × 30 mm; XTI-5). The initial column temperature was 40°C for 4 min, which was increased linearly at 10°C min^{-1}–270°C and held at 4 min. The temperature of injection port was 275°C and GC–MS interface was maintained at 300°C. The helium was carrier gas; flow rate was 1 ml min^{-1} and 30 min run time. The compounds were identified on the basis of mass spectra and using the NIST library stored in the computer software (version 1.10 beta Shimadzu) of the GC–MS.

Toxicity study

Phytotoxicity test were performed in order to assess the toxicity of Congo red, textile industry effluent and metabolites formed after decolorization of Congo red and textile industry effluent (Parshetti et al. 2006). Tests were carried out on four kinds of plants which are commonly used in Indian agriculture: *Sorghum bicolor*, *Vigna radiata*, *Lens culinaris* and *Oryza sativa*. 15 seeds of each plant were sowed into a plastic sand pot. The sand pot was prepared by adding 20 g of washed sand into the plastic pot. The Congo red and ethyl acetate extracted metabolites (dry) were dissolved separately in distilled water and the final concentration made was of 700 ppm. The filtered textile effluent was directly used to assess its toxicity. Toxicity study was done by watering (5 ml) the seeds of each plant with Congo red sample, textile industry effluent sample and extracted metabolites sample. The control was run by watering the seeds with distilled water. The watering was done two times per day. Germination (%), length of shoot and root was recorded after 13 days. The experiments were carried out at room temperature.

Statistical analysis

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

Results and discussion

Isolation, screening and identification of microorganism

Interest in the bioremediation of pollutants using bacteria has intensified in recent years, as many researches have demonstrated the efficacy of bacterial bioremediation over fungal and actinomycetes. Bacteria are vital in recycling nutrients, with many steps in nutrient cycles depending on these organisms, such as the fixation of nitrogen from the atmosphere and putrefaction. These largely unexplored reservoirs of resources could be useful for innovative applications, which are helpful to human beings. Bacterial isolate which efficiently decolorized various dyes (100 mg l^{-1}; Congo red, Eriochrome black T, Trypans blue, Reactive navy blue RX and Reactive blue HE2R) in the nutrient broth was selected. Selected strain was identified as *Pseudomonas* sp. SU-EBT on the basis of 16S rRNA sequence and biochemical characteristics (Table 2). The phylogenetic position of the strain *Pseudomonas* sp. SU-EBT was determined on basis of the 16S rRNA sequence (771, FJ600377). The phylogenetic relationship between the strain *Pseudomonas* sp. SU-EBT and other related microorganisms is shown in Fig. 1. The homology assay result indicated the strain *Pseudomonas* sp. SU-EBT was in the phylogenetic branch of the *Pseudomonas* genus and showed 99% sequence homology with related microorganism.

Decolorization of dyes and textile industry effluent

Industrial effluent is not stable and it varies often in a wide range depending upon the process practiced. Adaptation of microorganisms to varying pH and temperatures conditions makes them more suitable for the degradation of industrial effluents. *Pseudomonas* sp. SU-EBT decolorized the textile dyes at broad pH and temperature range. The optimum pH and temperature for dye decolorization was 8.0 and 40°C (data not shown). Sulfonated diazo dye Congo red is recalcitrant to biodecolorization (Sugiura et al. 1999). *Pseudomonas* sp. SU-EBT showed 97% decolorization of Congo red in nutrient broth within 12 h with 50% reduction in COD under static condition (Fig. 2). The isolated *Exiguobacterium* sp.

Table 2 Morphological, biochemical and physiological test for the identification of strain *Pseudomonas* sp. SU-EBT

Test	Obs.
Colony morphology	
Configuration	Circular
Gram's reaction	Negative
Cell shape	Small rod
Motility	+
Biochemical tests	
Growth in VP broth	
pH < 6	−
pH > 7	+
Voges posker test	−
Catalase test	+
Oxidase test	+
Lysine decarboxylase	+
Ornithine decarboxylase	+
Phenylalanine deaminase	−
Urease	−
Citrate utilization	+
H_2S production	−
Esculin hydrolysis	−
Starch hydrolysis	−
Gelatin hydrolysis	−
Nitrate reduction	−
Acetate utilization	+
L-Arginine utilization	+
Malonate utilization	+
Sugars fermentation	
D-Glucose	−
Lactose	−
D-Arabinose	−
Maltose	−
Utilization of carbohydrates	
D-glucose	−
L-Arabinose	−
Galactose	−
Lactose	−
Trehalose	−
Maltose	−
D-Fructose	−
Mannitol	+
Galactose	−
Raffinose	−
Trehalose	−
Sucrose	−
Mellibiose	−
Antibiotic sensitivity	
Chloramphenicol	I
Erythromycin	I
Fusidic acid	I
Methicillin	I
Novobiocin	I
Penicillin-G	I
Streptomycin	I
Ceftazidine	NI
Ciprofloxacin	I
Cephotaxic acid	I
Nitrofurantrin	I
Nalidixic acid	I
Netillin	I
Physiological tests	
Anaerobic growth	−
Growth in Nacl	
1, 3, and 5%,	+
7, and 10%	−
Growth at temperature	
10, 20, 30, and 40°C	+
50°C	−

Obs. Observations; +ve sign indicate positive test; −ve sign indicate negative test; *I* Inhibition; *NI* No inhibition

RD3 required 48 h for decolorization of sulfonated dizao dye Navy blue HE2R (Dhanve et al. 2009). This suggests the potential of *Pseudomonas* sp. SU-EBT to degrade the sulfonated azo dyes. *Pseudomonas* sp. SU-EBT was unable to decolorize the textile dyes at shaking condition. This may be due to repressed activity of oxidases (laccase) involved in the decolorization. *Bacillus* sp. ADR also showed maximum decolorization at the static condition but less decolorization at shaking condition due to the decreased activity of phenol oxidase at shaking condition. The decolorization of azo dyes using *Bacillus* sp. ADR was associated with phenol oxidase and NADH-DCIP reductase enzyme. However, the growth of microorganism was higher in shaking condition compared to static (Telke et al. 2009). The *Pseudomonas* sp. SU-EBT efficiently decolorized (90%) the high concentration of Congo red (1 g l^{-1}) within 60 h. The decolorization efficacy of

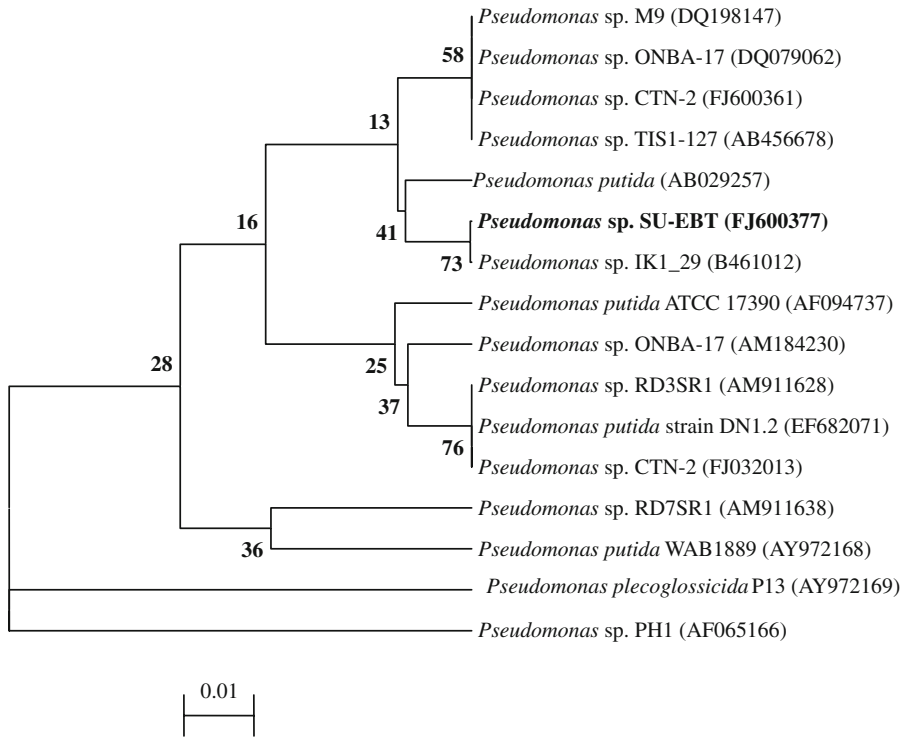


Fig. 1 Phylogenetic analysis of 16 s rRNA sequence of *Pseudomonas* sp. SU-EBT. The *per cent numbers* at the nodes indicate the levels of bootstrap support based on neighbor-joining analyses of 1,000 replicates. The *scale bar* (0.01) indicates the genetic distance. *Brackets* represents sequence accession numbers

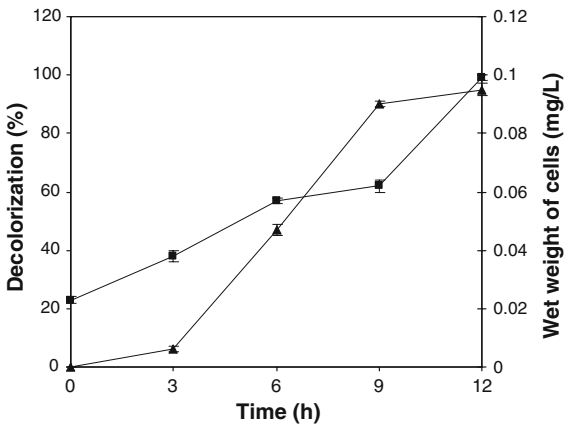


Fig. 2 Decolorization of Congo red at static condition. Percentage decolorization (*filled triangle*) and wet weight of cells in mg l^{-1} (*filled diamond*). Data points represent the mean of three independent replicates, standard error of the mean (SE) is indicated by *error bars*

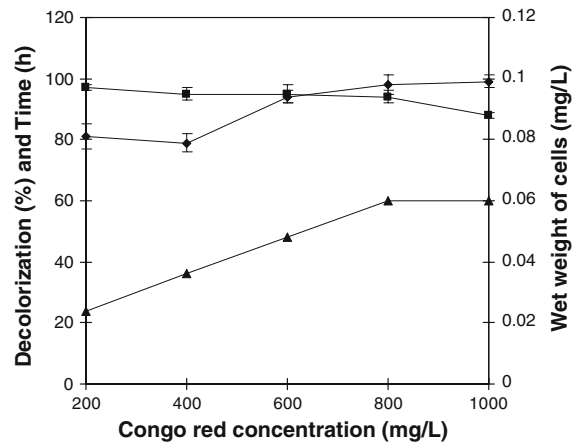


Fig. 3 Effect of dye concentration on decolorization performance of *Pseudomonas* sp. SU-EBT. Percentage decolorization (*filled triangle*), wet weight of cells in mg l^{-1} (*filled diamond*) and time required for decolorization in h (*filled square*). Data points represent the mean of three independent replicates, standard error of the mean (SE) is indicated by *error bars*

Pseudomonas leutola was decreased with increasing dye concentration (Hsueh and Chen 2007). The time required for decolorization was increased with increasing dye concentration (Fig. 3). Industrial effluent is a mixture of various textile dyes. The isolation of bacteria, which are capable of decolorization of mixture of textile dyes, is important for the effective decolorization of textile effluent. *Pseudomonas* sp. SU-EBT decolorized various individual and mixture of reactive, disperse and direct dyes with decolorization efficiency varying from 90 to 97% (Table 1).

Table 3 The environmental parameters for untreated and treated textile industry effluent

Environmental parameters	Untreated textile effluent	Treated textile effluent
COD (mg l^{-1})	1,000 ± 10	500 ± 10
BOD (mg l^{-1})	200 ± 05	110 ± 05
Total alkalinity (mg l^{-1})	200 ± 05	70 ± 06
Total hardness (mg l^{-1})	400 ± 06	280 ± 07
Color (%)	100 ± 03	10 ± 03

Pseudomonas sp. SU-EBT showed 90% decolorization of the textile effluent within 60 h with 50 and 45% reduction in COD and BOD, respectively (Table 3). *Citrobacter* sp. strain KCTC 18061P strain removed 70% of effluent color within 5 days of incubation. Decolorization was due to the adsorption of color to cells (Jang et al. 2007). These observations suggested that *Pseudomonas* sp. SU-EBT might be the potential strain for the degradation of textile dyes and textile industry effluent.

Enzymatic analysis

The involvement of oxidoreductive enzymes (phenol oxidase and NADH-DCIP reductase) in decolorization of azo dyes was described earlier (Telke et al. 2009). Intracellular laccase activity was significantly increased after 6 h (145%) and 12 h (160%) of incubation as compared to 0 h (24 h grown culture). Extracellular laccase activity was significantly decreased after 12 h (50%) of incubation as compared to 0 h. No significant change was seen in an intracellular NADH-DCIP reductase activity after 6 and 12 h of incubations as compared to 0 h. Azoreductase activity was significantly decreased after 6 h (26%) and 12 h (26%) of incubation as compared to 0 h (Table 4). The above observations demonstrated that Congo red was decolorized by the involvement of intracellular laccase of *Pseudomonas* sp. SU-EBT. Laccase is the key enzyme, responsible for the decolorization of textile dyestuff and textile industry effluent (Faraco et al. 2009).

Analysis of metabolites obtained after decolorization

Congo red and textile industry effluent showed the maximum absorbance at 490 nm. Significant decrease in the absorbance at 490 nm (Fig. 4) suggested the decolorization of Congo red and textile industry effluent. The difference in FTIR spectrum of Congo red and metabolites obtained after its decolorization resulted in biodegradation. The FTIR spectrum of Congo red showed specific peaks in fingerprint region for unsubstituted and multisubstituted naphthalene or benzene rings. This was supported by the peaks at 644 cm^{-1} for C–C bending vibrations, 695 cm^{-1} for C–H stretching vibrations for disubstituted aromatic compound, 833 cm^{-1} for *p*-disubstituted ring vibrations, 1,062 cm^{-1} for S=O stretching vibrations of sulfonic acid, 1,356 cm^{-1} for C–N bending vibrations, 1,446 cm^{-1} for aromatic C=C stretching vibrations, 1,587 cm^{-1} for N=N stretching vibrations, 3,070 cm^{-1} for O–H stretching vibrations and 3,441 cm^{-1} for N–H stretching vibrations of primary amine (Fig. 5a). The FTIR spectrum of metabolites obtained after decolorization of Congo red showed peaks at 522 cm^{-1} for ring bending vibrations, 819 cm^{-1} for *p*-disubstituted benzene ring vibrations, 1,444 cm^{-1} aromatic C=C stretching vibrations, 1,664 cm^{-1} C–O stretching vibrations, 3,419 cm^{-1} for N–H stretching vibrations of primary amide (Fig. 5b). The absence of peak at 1,587 cm^{-1} for N=N stretching vibrations in the FTIR spectrum of metabolites obtained after decolorization of Congo red was because of the cleavage of azo bond.

We are proposing the biodegradation pathway of Congo red on the basis of GC–MS data and the action of enzymes involved in decolorization. The first step

Table 4 Enzymatic status during decolorization of Congo red

Enzymes	0 h	6 h	12 h
Extracellular laccase[a]	50 ± 7.0	50 ± 6.0	40 ± 3.0**
Intracellular laccase[b]	0.33 ± 0.07	0.58 ± 0.06*	0.64 ± 0.07**
Azo reductase[b]	0.89 ± 0.01	0.23 ± 0.01**	0.23 ± 0.01**
DCIP reductase[b]	27.4 ± 0.5	29.3 ± 0.53	26 ± 0.44

All enzyme activities checked after different time interval (0, 6 and 12 h of incubation)

Values are mean of three experiments ± SEM (Standard error of mean), significantly different from control cells at $*P < 0.05$ and $**P < 0.001$ by one way analysis of variance (ANOVA) with Tukey–Kramer multiple comparisons test

[a] Enzyme activity—U l^{-1} of culture supernatant

[b] Enzyme activity—U mg^{-1} of protein min^{-1}

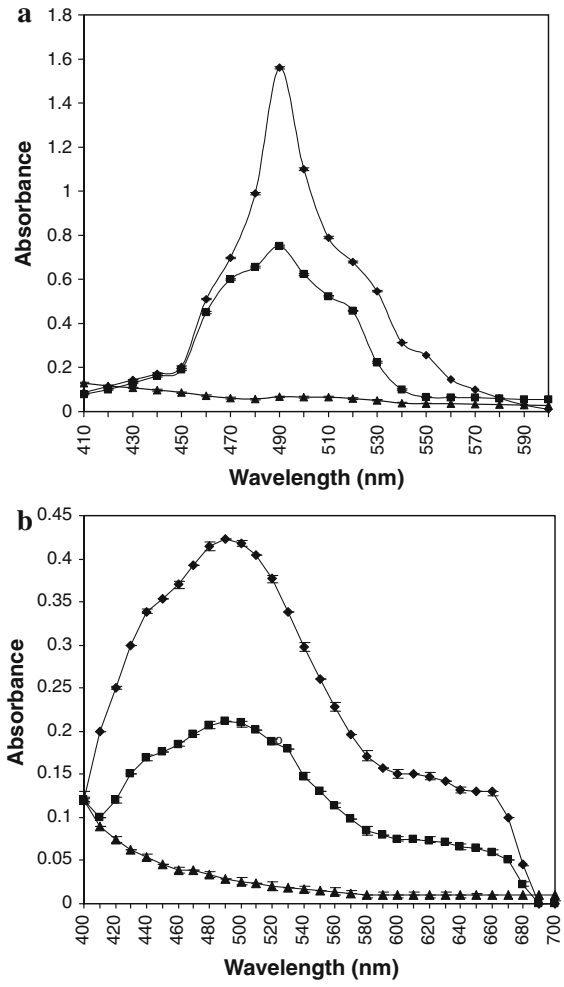


Fig. 4 The spectral characteristic of untreated and treated sample of Congo red and textile industry effluent. Spectrum of initial sample (*filled diamond*), spectrum of half decolorized sample (*filled square*) and spectrum of complete decolorized sample (*filled triangle*). Data points represent the mean of three independent replicates, standard error of the mean (SE) is indicated by *error bars*

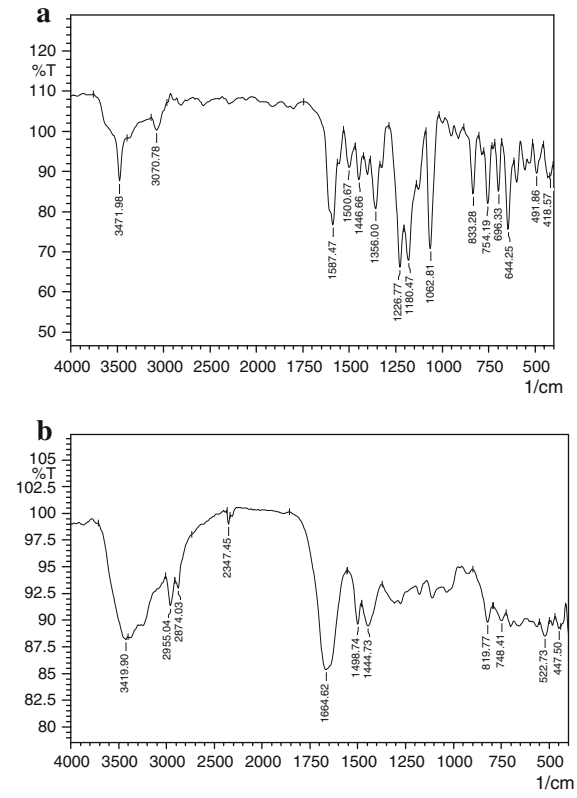


Fig. 5 FTIR spectrum of control dye Congo red (**a**) and metabolites obtained after complete decolorization of Congo red (**b**)

in the decolorization of azo dyes using oxidoreductive enzyme (laccase) is the formation of electron deficient reaction center (carbocation ion). This carbocation ion is highly reactive intermediate and often the target of nucleophillic attack by nucleophiles like –OH or $–SO_3$ or halogen ions, which caused asymmetric cleavage of azo bond (Chivukula and Renganathan 1995). The first step in biodegradation of Congo red comprises asymmetric cleavage of azo bond leading to the formation of *p*-dihydroxybiphenyl and postulated diazine intermediate. The diazine intermediate was presumably unstable in the presence of molecular oxygen. Further, it looses molecular nitrogen to produce sulfonaphthalene radical, which was scavenged by –OH nucleophile to yield 8-amino naphthol 3-sulfonic acid intermediate (Chivukula and Renganathan 1995). 3-hydroperoxy 8-nitrosonaphthol was formed by amine oxidation followed by desulfonation activity of laccase as reported earlier (Amitaia et al. 1998) confirming that Congo red was cleaved into nontoxic hydroxyl and hydroperoxy metabolites rather than toxic aromatic amines (Fig. 6; Table 5).

Toxicity study

A number of dyes have been tested for mutagenicity using Ame's bioassay. Several of them have been found to be carcinogenic and mutagenic (Mathur et al. 2005). Phytotoxicity study revealed the toxic nature of Congo red and textile industry effluent to

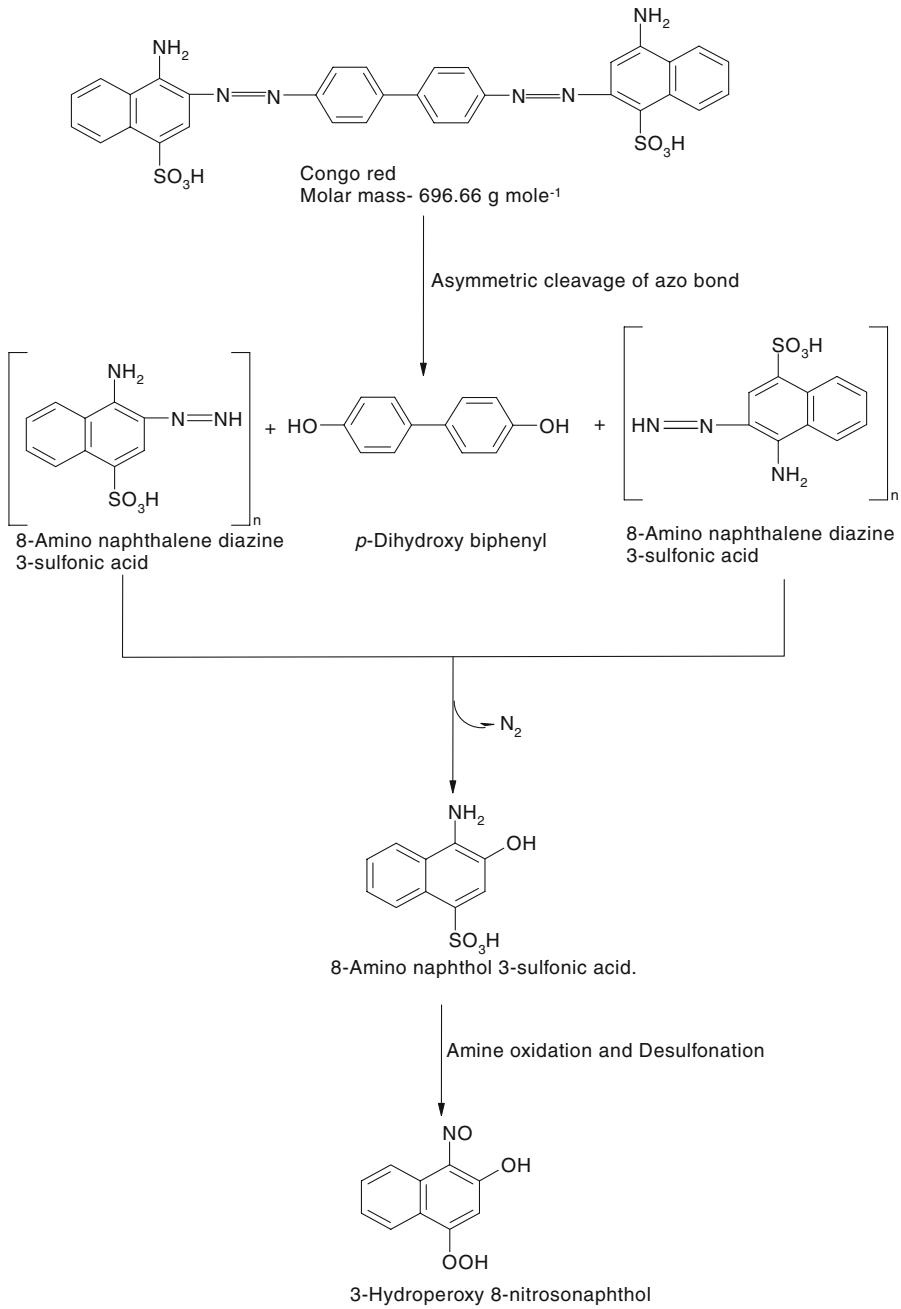
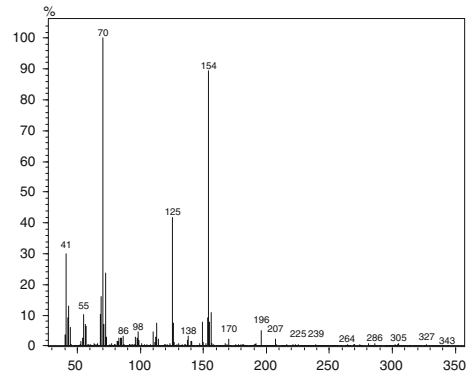
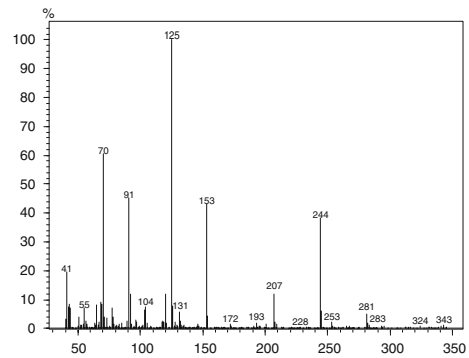
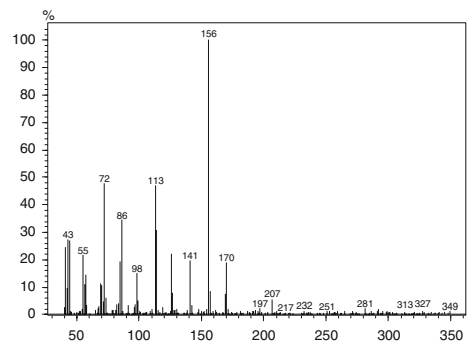


Fig. 6 Proposed pathway for biodegradation of Congo red. The compounds in *square bracket* are the postulated intermediates

the *Sorghum bicolor*, *Vigna radiata*, *Lens culinaris* and *Oryza sativa* plants. Germination (%) of these plants was less with Congo red and textile industry effluent treatment as compared to the metabolites obtained after its decolorization and distilled water (Tables 6, 7). The Congo red and textile industry effluent were significantly affected the shoot and root growth than the metabolites obtained after its decolorization. This proved the lesser toxicity of these metabolites obtained after decolorization of Congo red and textile industry effluents. Similar results were reported in case of Reactive yellow 84A and metabolites obtained after its decolorization (Dhanve et al. 2009).

Table 5 GC–mass spectral data of metabolites obtained after decolorization of Congo red

Metabolites	Rt. time (min)	Mw (*m*/z)	Mass spectrum
p-Dihydroxy biphenyl	22.11	196	
8-Amino naphthol 3-sulfonic acid	26.43	244	
3-Hydroperoxy 8-nitrosonaphthol	21.17	207	

Conclusions

The present study demonstrated that the isolated bacterium *Pseudomonas* sp. SU-EBT was able to degrade and detoxify the azo dye Congo red and textile industry effluent with significant reduction in COD. The *Pseudomonas* sp. SU-EBT could be potential strain for the treatment of textile dyestuffs

Table 6 Phytotoxicity study of Congo red and metabolites obtained after its decolorization

Observations	*Sorghum bicolor*			*Vigna radiata*			*Lens culinaris*		
	I	II	III	I	II	III	I	II	III
Germination (%)	70	40	70	70	50	70	70	30	70
Shoot length (cm)	4.45 ± 0.42	1.90 ± 0.70***	5.80 ± 0.70$^{\$\$}$	8.22 ± 0.60	3.8 ± 0.38***	8.20 ± 0.52$^{\$\$}$	4.80 ± 0.54	0.00 ± 0.00***	3.80 ± 0.57$^{\$\$\$}$
Root length (cm)	5.0 ± 0.59	2.50 ± 0.86**	4.09 ± 0.60$^{\$}$	6.06 ± 0.47	3.07 ± 0.66***	5.83 ± 0.50$^{\$\$}$	3.10 ± 0.55	0.12 ± 0.05***	1.65 ± 0.22

I = Seeds treated with distilled water

II = Seeds treated with Congo red (700 ppm)

III = Seeds treated with metabolites obtained after complete decolorization of Congo red (700 ppm)

Values are mean of germinated seeds of three experiments, SEM, significantly different from the control (seeds germinated in water) at $^{**}P < 0.01$ and $^{***}P < 0.001$ and significantly different from dye treated sample at $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ and $^{\$\$\$}P < 0.001$ by one way analysis of variance (ANOVA) with Tukey–Kramer multiple comparison test

Table 7 Phytotoxicity study of textile industry effluent and metabolites obtained after its decolorization

Observations	*Sorghum bicolor*			*Vigna radiata*			*Lens culinaris*			*Oryza sativa*		
	I	II	III	I	II	III	I	II	III	I	II	III
Germination (%)	70	20	60	70	20	60	70	00	50	100	30	80
Shoot length (cm)	4.45 ± 0.42	0.62 ± 0.20***	3.40 ± 0.40$^{\$\$\$}$	8.22 ± 0.60	1.11 ± 0.61***	5.05 ± 0.56$^{\$\$\$}$	4.80 ± 0.54	0.0 ± 0.0***	2.95 ± 0.47$^{*\$\$\$}$	4.11 ± 0.29	0.00 ± 0.00***	2.76 ± 0.210$^{\$\$\$}$
Root length (cm)	5.0 ± 0.59	0.58 ± 0.25***	2.50 ± 0.25$^{*\$\$\$}$	6.06 ± 0.47	1.0 ± 0.60***	3.0 ± 0.45$^{*\$\$\$}$	3.10 ± 0.55	0.0 ± 0.0***	0.93 ± 0.11$^{**\$\$\$}$	3.33 ± 0.71	00 ± 0.04***	1.00 ± 0.27$^{*\$\$}$

I = Seeds treated with distilled water

II = seeds treated with textile industry effluent

III = Seeds treated with metabolites obtained after complete decolorization of textile industry effluent

Values are mean of germinated seeds of three experiments, SEM, significantly different from the control (seeds germinated in water) at $^{*}P < 0.05$, $^{**}P < 0.001$ and $^{***}P < 0.001$ and significantly different from dye treated sample at $^{\$\$}P < 0.01$ and $^{\$\$\$}P < 0.001$ by one way analysis of variance (ANOVA) with Tukey–Kramer multiple comparison test

and textile industry effluent as compared to other biological sources.

Acknowledgments One of the authors (Mr. Amar A. Telke) is thankful to the Council of Scientific and Industrial Research, New Delhi for financial assistance. Authors also thank the Common Facility Center, Shivaji University, Kolhapur, India for GC–MS facility.

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