Biodegradation (2010) 21:453–463
DOI 10.1007/s10532-009-9315-6

ORIGINAL PAPER

Rapid biodegradation and decolorization of Direct Orange 39 (Orange TGLL) by an isolated bacterium *Pseudomonas aeruginosa* strain BCH

Jyoti P. Jadhav · Swapnil S. Phugare · Rhishikesh S. Dhanve · Shekhar B. Jadhav

Received: 13 June 2009 / Accepted: 5 November 2009 / Published online: 24 November 2009

Abstract A newly isolated novel bacterium from sediments contaminated with dyestuff was identified as *Pseudomonas aeruginosa* strain BCH by 16S rRNA gene sequence analysis. The bacterium was extraordinarily active and operative over a wide rage of temperature (10–60°C) and salinity (5–6%), for decolorization of Direct Orange 39 (Orange TGLL) at optimum pH 7. This strain was capable of decolorizing Direct Orange 39; 50 mg l^{-1} within 45 ± 5 min, with 93.06% decolorization, while maximally it could decolorize 1.5 g l^{-1} of dye within 48 h with 60% decolorization. Analytical studies as, UV–Vis spectroscopy, FTIR, HPLC were employed to confirm the biodegradation of dye and formation of new metabolites. Induction in the activities of lignin peroxidases, DCIP reductase as well as tyrosinase was observed, indicating the significant role of these enzymes in biodegradation of Direct Orange 39. Toxicity studies with *Phaseolus mungo* and *Triticum aestivum* revealed the non-toxic nature of degraded metabolites.

Keywords Biodegradation · DCIP reductase · Direct Orange 39 · Salinity · Tyrosinase

J. P. Jadhav (✉) · S. S. Phugare · R. S. Dhanve · S. B. Jadhav
Department of Biochemistry, Shivaji University, Kolhapur 416 004, India
e-mail: jpj_biochem@unishivaji.ac.in

Introduction

Colors gives delightful pleasure to eyesight but at the same time they may act as serious pollutants when their origin is dyes and dyestuffs. Number of studies have been reported for biological treatment of wastewater containing dyestuff (Stolz 2001). The routine use of dyes in day-to-day life is increasing because of rapid industrialization, most widely in textile, rubber, enamel, plastic, cosmetic and many other industries (Raffi et al. 1997). About 100,000 commercially available dyes are known and nearly 1 million tons of dyes are produced throughout the year, whereas, out of the total usage, 10% of dyes are released in environment as dyestuff waste (Selvam et al. 2003; Maguire 1992). The treatment of such dye containing effluent was initially carried by using physical and chemical treatment processes including adsorption, concentration, chemical transformation, but with time, potential hazards and disadvantages of these methods were noted as, formation of toxic sludge and formation of even more toxic metabolites (Davis et al. 1994; Johnson et al. 1978). Alternatively, approach is shifting towards the use of conventional biological methods to treat such effluents and wastewater containing dyes and toxic chemicals (Chen et al. 2003). These methods are gaining more importance nowadays because of their lesser cost, effectiveness and eco-friendly nature. The metabolites produced after biodegradation are mostly non toxic or comparatively less toxic in nature.

Phytotoxicity studies are generally performed to determine the non toxic nature of metabolites (Patil et al. 2008; Kalyani et al. 2008a, b). Most common methods employed to study phytotoxicity are, monitoring of seed germination and plant growth (Kapanen and Itavaara 2001).

Bioremediation is a process in which the natural capacity of microbes is enhanced to degrade toxic chemicals and waste (Senan and Abraham 2004). Several reports are available indicating that a variety of microbes have been involved in the bioremediation and biodegradation of dyes, which includes some bacteria such as, *Aeromonas hydrophila* (Chen et al. 2003); *Pseudomonas* sp SUK 1 (Kalyani et al. 2008a, b), *Exiguobacterium* sp RD 3 (Dhanve et al. 2008); fungi: *Aspergillus niger* (Fu and Viraraghavan 2002); *Penicillin ochrocloron* (Shedbalkar et al. 2008); yeasts: *Saccharomyces cerevisiae* (Jadhav et al. 2007). Azo dyes were found to be difficult to degrade because of their complicated structure (Kim and Shoda 1999). Biodegradation as well as biotransformation of azo dyes has been reported earlier (Maximo et al. 2003; Liu et al. 2006).

The isolated *Pseudomonas* species for dye degradation has shown to provide efficient biological and combinational methods, which are found to be cost effective alternatives (Hu 1994). *Pseudomonas* sp SUK1 could decolorize the dye Red BLI at concentration of 50 mg l^{-1} within 1 h (Kalyani et al. 2008a, b). *Pseudomonas aeruginosa* NBAR12 was capable of decolorizing 12 different dyes with varying decolorization efficiency, amongst which diazo Reactive Blue 172 was found to be decolorized when glucose and yeast extract were supplemented in medium (Bhatt et al. 2005).

Present study was aimed to isolate efficient bacterial strain, which possesses the ability to decolorize various textile dyes. The isolated strain *Pseudomonas aeruginosa* strain BCH could decolorize Direct Orange 39 (Orange TGLL) efficiently. Previously photo-catalytic degradation of Direct Orange 39 was reported with the help of semiconductor photo-catalytic systems (Afarani 2008). Similarly, application of high voltage pulsed electrical discharge was reported for the removal of Direct Orange 39 from model waste water (Vujevic et al. 2004). To the best of our knowledge, this is the first report on biodegradation of Direct Orange 39. Enzymatic status before and after biodegradation were monitored to access whether any induction of the enzyme systems occurs. Analysis of samples extracted after biodegradation were performed with UV–Vis spectroscopy, HPLC and FTIR. Particularly dye wastes are harmful to agricultural and marine habitat; hence to determine the toxic nature of the dye as well as its degraded metabolites, phytotoxicity testing was carried out on two common plant seeds, *Sorghum vulgare* and *Phaseolus mungo.*

Materials and methods

Dyes and chemicals

Tartaric acid was obtained from BDH Chemicals, India, veratryl alcohol from SRL Chemicals, India. DCIP (2,6-dichlorophenol indophenol sodium salt) was obtained from Sisco Research Laboratories Pvt. Ltd. Mumbai, India. NADH was obtained from Sigma Chemical Company; (USA). The dye Direct Orange 39 was obtained from local textile industry, Ichalkaranji (India).

Isolation and identification of microorganism

Isolation of the microbial strain was carried out from the soil contaminated with effluent discharge of textile processing and dye manufacturing unit in Ichalkaranji (India), by enrichment culture technique. 100 ml yeast extract medium, with 100 mg l^{-1} of Direct Orange 39, was inoculated with 2 g of soil sample contaminated with textile waste. After 48 h of incubation, a loopful of sample from the broth was streaked on yeast extract medium agar plate containing 100 mg l^{-1} of Direct Orange 39 and colonies showing decolorization zone were selected. Morphologically distinct bacterial strains were selected for screening of dye decolorization. The most efficient strain was then selected for further studies. Identification of the isolate as *Pseudomonas aeruginosa,* was done by 16S rRNA analysis at geneOmbio Technologies, Pune, and the sequence is deposited in the Gene Bank.

Phylogenetic analysis and sequence alignment

Initially the 16S rRNA gene sequence was analyzed at NCBI server (http://www.ncbi.nlm.nih.gov) using BLAST (blastn) tool and corresponding sequences of

homologous species were downloaded and used for phylogenetic analysis. The evolutionary history was inferred using neighbor joining method (Saitou and Nei 1987). 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. 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. Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007).

Organism and culture condition

Pure culture of the isolate was maintained at 4°C and frequently cultured on yeast extract medium slants having composition (g l^{-1}); yeast extract (5.0), NaCl (5.0), agar (25.0). The culture was used for decolorization studies after inoculating in yeast extract medium having same composition as stated above except agar. The decolorization experiments were carried out at 30°C, under static conditions unless otherwise stated.

Decolorization studies

All the decolorization experiments were performed in triplicates. Decolorization study of Direct Orange 39 was carried out by addition of 50 mg l^{-1} of dye in 250 ml Erlenmeyer flask containing 100 ml of yeast extract medium containing 24 h old culture of *Pseudomonas aeruginosa* strain BCH. All the studies were carried at 30°C and at pH 7 unless stated otherwise. Samples (3 ml) were withdrawn after various time intervals to monitor decolorization rate. Aliquots withdrawn were centrifuged at 6,000 rpm for 10 min, and residual dye concentration in supernatant was measured at 410 nm. Decolorization rate was expressed as percentage decolorization and calculated using the formula % Decolorization = $A - B/A \times 100$; where A is initial absorbance and B is observed absorbance. Above mentioned protocol was followed during the study of effect of shaking and static conditions, effect of different carbon and nitrogen sources, effect of physicochemical parameters and the effect of increasing dye concentrations on decolorization. The isolate was also checked for its ability to decolorize various textile dyes.

Effect of physico-chemical parameters

Various physicochemical parameters like temperature and pH were monitored to study their effect on decolorization of Direct Orange 39. Yeast extract medium (100 ml each) with 24 h old culture was inoculated with dye (50 mg l^{-1}) and incubated at 4, 10, 20, 30, 40, 50 and 60°C. Similarly broths with different pH as 3, 5, 7, 9, and 11 were inoculated with strain and after 24 h of incubation; dye (50 mg l^{-1}) was added to observe the effect on decolorization.

Decolorization with increasing dye concentration

Various dye concentrations ranging from 50 to 1500 mg l^{-1} were added to yeast extract medium in order to examine the effect of varying initial dye concentrations on decolorization under static condition.

Fed batch decolorization

In this study 50 mg l^{-1} of dye was added to 24 h grown culture (100 ml yeast extract broth) of isolated bacterial strain. After optimum decolorization, 50 mg l^{-1} of dye was added without supplement of any external nutrients. Dye was added continuously until the culture lost its decolorization efficiency to the significant extent.

Effect of salinity on decolorization

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, 5 and 6% in the yeast extract medium.

Effect of carbon and nitrogen sources

During this study organism was supplemented with additional carbon and nitrogen sources along with

yeast extract medium. Different carbon sources like glucose, lactose, sucrose, maltose, xylose, starch, citric acid, oxalic acid, fructose and pyruvate were added as a supplement individually, to the yeast extract medium at 0.3% concentration. Similarly, in the next set, different nitrogen sources like ammonium nitrate, potassium nitrate, urea, ammonium chloride, and sodium nitrate were added at a concentration of 0.3% to the medium.

Preparation of cell free extract

Bacterial cells, 24 h cultivated were harvested by centrifugation at 7,000 rpm, for 15 min, at 4°C and suspended in 50 mM phosphate buffer pH 7.4. These cells (100 mg ml^{-1}) were chilled properly and sonicated (Sonics-vibracell ultrasonic processor), keeping sonifier output at 40 amp and giving 7 strokes each of 30 s, with time interval of 2 min at 4°C. The homogenate was then centrifuged at 8,000 rpm for 15 min and the supernatant obtained was used as crude enzyme source. Similar procedure was followed for determination of enzyme activity after dye decolorization.

Enzyme assay

Variety of biotransformation enzymes were studied viz lignin peroxides, NADH- dichlorophenol indophenol reductase (NADH-DCIP reductase), and tyrosinase. Activities of lignin peroxidases, tyrosinase were assayed spectrophotometrically in the cell free extract at room temperature where blank contained all the components except enzyme. All experiments were carried out in triplicates and the average rates were calculated. Lignin peroxidase activity was assayed by modifying the procedure reported by (Shanmugam et al. 1999) in which veratryl alcohol (2 mM) was used as substrate, enzyme activities were calculated using the extinction coefficients of veratraldehyde (9,300 M^{-1} cm^{-1}) at 310 nm (Dhanve et al. 2009). NADH-DCIP reductase activity was determined by procedure reported earlier (Salokhe and Govindwar 1999). The reduction of DCIP was calculated using extinction coefficient of 19 mM cm^{-1} (Patil et al. 2008). Tyrosinase activity was calculated by procedure reported earlier (Ali et al. 2007).

UV–Vis spectral analysis, HPLC and FTIR

Supernatant of the decolorized medium was extracted with equal volume of ethyl acetate and concentrated by evaporation. The dried residue was dissolved in HPLC grade methanol and further used for analytical studies like HPLC, FTIR. UV–Vis spectral analysis was carried by using Hitachi UV–Vis spectrophotometer (UV 2800) and changes in its absorption spectrum (200–800 nm) were recorded. 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 mobile phase. FTIR analysis was carried out using Shimadzu FTIR-8400S spectrophotometer.

Toxicity study

The degradation metabolites of Direct Orange 39 were extracted with ethyl acetate and dried residues obtained were dissolved in water to obtain final concentration of 1,000 ppm. These samples were then used for toxicity study. The phytotoxicity study was carried out at room temperature using *Phaseolus mungo* and *Triticum aestivum* seeds by irrigating separately 5 ml samples of Direct Orange 39 and its degradation metabolites (1,000 ppm) per day. Control set was carried out using plain water at the same time. Length of plumule (shoot), radical (root) and germination (%) was recorded after 7 days.

Statistical analysis

Data were analyzed by One-way analysis of variance with Tukey–Kramer multiple comparisons test. Values are mean of three experiments. Readings were considered significant when P was <0.05.

Results and discussion

Phylogenetic position of isolates

Phylogenetic position of *Pseudomonas aeruginosa* strain BCH in relation to the species of this genus is as illustrated (Fig. 1). The figure showed phylogenetic relationship between the *Pseudomonas aeruginosa* strain BCH and other related microorganisms found in the GenBank database. The digits adjacent

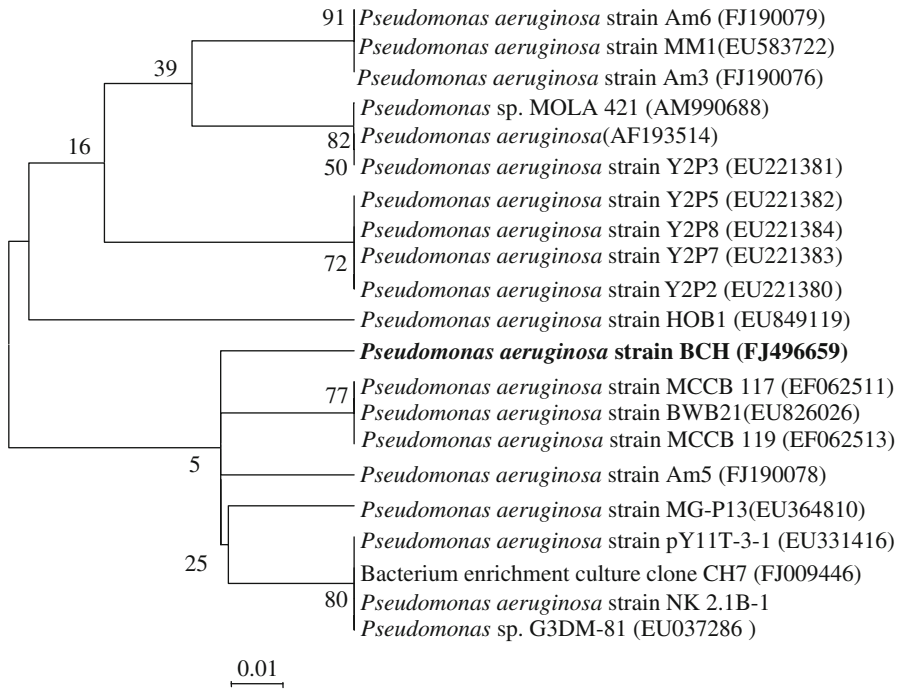

Fig. 1 Phylogenic tree of *Pesudomonas aeruginosa* strain BCH. Phylogenetic analysis of 16 s rRNA gene sequence of *Pesudomonas aeruginosa* strain BCH. The percent numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analyses of 1,000 replicates. The *scale bar* (0.01) indicates the genetic distance. *Brackets* represents sequence accession numbers

to nodes are the statistical frequency of the indicated species. The numbers shown in parentheses are accession numbers of different species. The homology assay results indicated that the strain BCH in phylogenetic branch exhibited maximum similarity (99%) to *Pseudomonas aeruginosa* strain Am3 (FJ190076), *Pseudomonas aeruginosa* strain Am6 (FJ190079), and *Pseudomonas aeruginosa* strain Am5 (FJ190078). *Pseudomonas aeruginosa* strain BCH, is deposited in gene bank under the accession number FJ 496659.

Effect of physico-chemical parameters

It was found that change in pH significantly affects the decolorization rate. *Pseudomonas aeruginosa* strain BCH showed significant growth at pH 5, 7 and 9, but highest decolorization (93.06%) was observed at pH 7. Decolorization was found to be up to 43 and 61% at pH 5 and 9 respectively, while pH 3 and 11 were found to be inhibitory for the growth. Studies with different temperature showed that the strain was effective over wide range of temperatures i.e. from 10 to 60°C; it shows optimum decolorization up to 93% with more or less similar time required for decolorization. When the cultures of *Pseudomonas aeruginosa* strain BCH were grown under shaking conditions, significantly better growth was observed as compared to the static condition. As far as decolorization was concerned, agitated culture showed no decolorization even after 24 h, while static culture showed maximum decolorization (93.06%) within just 45 ± 5 min. Anaerobic or static conditions were necessary for bacterial decolorization, though the cell growth was poorer than that under aerobic condition. Similar results were reported earlier by studies on pure bacterial strains such as, *Proteus mirabilis, Pseudomonas luteola*, *Pseudomonas desmolyticum* and *Serratia marcescens* (Chen et al.1999; Chang et al. 2001; Kalme et al. 2006; Verma and Madamwar 2003). Under aerobic conditions azo dyes are generally resistant to attack by bacteria (Hu 1998). 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 (Stolz 2001).

Effect of initial dye concentration

The decolorization efficiency varied from 93.06 to 60% for dye concentration of 50–1,500 mg l^{-1} respectively (Fig. 2). Increase in dye concentration resulted in a significant change in the percentage decolorization as well as the time required for decolorization. Optimum decolorization for 50 mg l^{-1} dye was obtained within 45 ± 5 min, which gradually increased up to 1080 ± 5 min for 1,500 mg l^{-1}. Further more increase in dye concentration up to 2,000 mg l^{-1} resulted in drastic decrease in decolorization up to 21%. These studies indicate that increase in dye concentration significantly affects the enzyme systems involved in decolorization of Direct Orange 39, ultimately resulting into the decrease in percentage decolorization, whereas time required for decolorization increased. Similar studies were reported earlier for biodegradation of reactive yellow 84A where *Exiguobacterium* sp. RD3 could decolorize the dye maximally up to 1 g l^{-1} with 21.05% decolorization efficiency (Dhanve et al. 2008).

Effect of repeated addition of dye aliquots

Prime intention of this study was to check the decolorization efficiency of the bacterial isolate to decolorize Direct Orange 39, by repeated addition of dye under fed batch process (Fig. 3). The *Pseudomonas aeruginosa* strain BCH was able to decolorize Direct Orange 39 up to 11 cycles with gradual reduction in the decolorization efficiency ranging from 93 to 72%, from first to last cycle respectively. In first cycle 93.06% reduction in initial dye concentration was observed within 45 ± 5 min, while this relation remains steady up to 5th cycle. From 6th cycle up to last cycle the decolorization efficiency gradually decreased, while more time was required for decolorization. Repeated addition of dye to the medium reduces the decolorization efficiency; this might be due to the culture entering into the stationary phase and subsequently into the death phase, resulting in the inhibition of enzyme systems gradually. Reports are available for the decolorization of Reactive Red 2 by *Pseudomonas* sp SUK1 up to 12 successive cycles (Kalyani et al. 2008a, b).

Effect of salinity on decolorization

Exposure of strain to increasing salt concentration in yeast extract medium showed significant growth and decolorization, up to 5.5% of salt concentration. Decolorization efficiency was found to be reduced as the salt concentration increases gradually (Fig. 4). Salt concentration above 5.5% was found to inhibit the growth. Generally, sodium concentration above 3 g l^{-1} can cause moderate inhibition of most bacterial activities (De Baere et al. 1984). Inhibition to microorganisms by high salt concentration may cause plasmolysis or loss of activity of cells (Panswad and Anan 1999). The ability of the strain to tolerate salt concentration up to 5.5% makes it significant, as it can decolorize Direct Orange 39, at high salinity levels. Similar studies, showing the effect of salinity on the decolorization were reported earlier (Salah-Uddin et al. 2007; Dhanve et al. 2008).

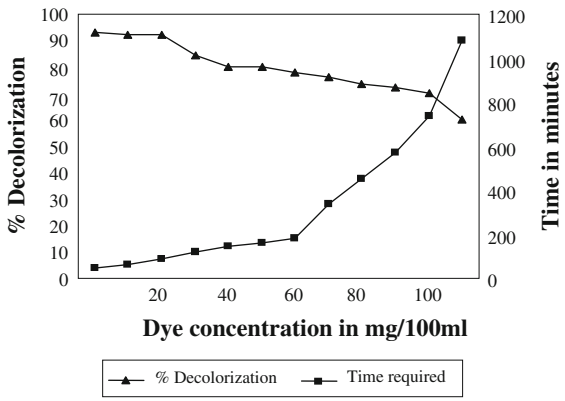

Fig. 2 Effect of initial dye concentration on decolorization

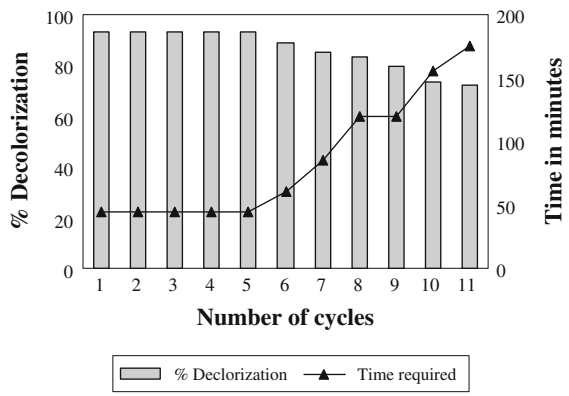

Fig. 3 Effect of repeated addition of dye on decolorization

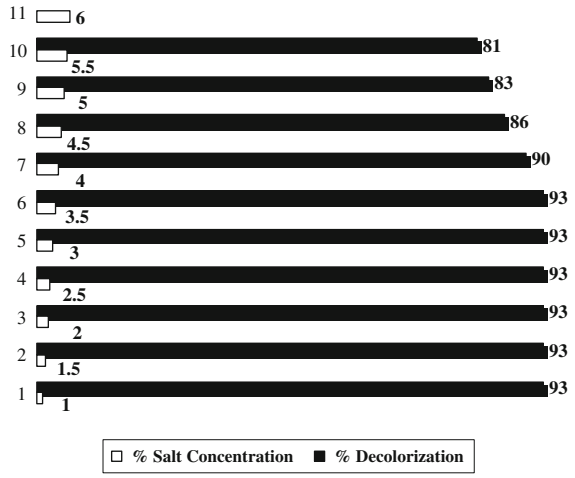

Fig. 4 Effect of salinity, on Direct Orange 39 decolorization

Effect of carbon and nitrogen sources

It was found that with glucose, sucrose, maltose time required for decolorization (45 ± 5 min) remained steady while decolorization percentage varied from 91 to 93%, which remains similar for fructose, starch, pyruvate and lactose but time required for decolorization was significantly increased from 60 to 150 min. Whereas citric acid, oxalic acid, xylose were found to be complete inhibitory for the decolorization. In case of nitrogen sources as ammonium nitrate, sodium nitrate and potassium nitrate, the decolorization was found to be 92–93%, while time required varied from 60 to 90 min. When ammonium chloride and urea were used as nitrogen sources, the time span for decolorization was found to be extended up to 120–130 min. Studies regarding the effect of various carbon and nitrogen sources on dye decolorization have been reported earlier (Telke et al. 2008). Presence of various carbon and nitrogen sources in medium might be having stimulatory or inhibitory effect on induction of enzyme systems involved in the decolorization of Direct Orange 39; resulting in the variation in time required for decolorization as well as percentage decolorization.

Various textile dyes decolorization

Pseudomonas aeruginosa strain BCH was tested for its ability to decolorize various textile dyes (Table 1), since the waste water from the textile industry contains mixture of dyes from various classes as well as with varying concentrations. It was found that; strain BCH could decolorize a variety of different dyes within short period of time with significant decolorization efficiency. The time required for decolorization and total percentage decolorization was varied for different dyes. Variation in decolorization efficiency and time required for decolorization may be due to structural differences of dyes (Paszczynski et al. 1992).

Enzymatic analysis

Enzymatic studies of microbial decolorization of Direct Orange 39 showed, increase in the activities of lignin peroxidase, tyrosinase and DCIP reductase. Significant increase in the activities of these enzymes, after decolorization, indicates involvement of these enzymes for degradation of Direct Orange 39 (Table 2). The combined action of these oxidoreductive enzymes might be responsible for biodegradation and decolorization of Direct Orange 39. Similar reports of involvement of oxidoreductive enzymes for decolorization of Navy blue HE2R have been previously quoted (Dhanve et al. 2008).

UV–Vis spectrophotometric analysis

Samples were analyzed by UV–Vis spectroscopy to confirm that decolorization was due to biodegradation and not merely visual decolorization. Control (Direct

Table 1 Various dye decolorization by *Pseudomonas aeruginosa strain* BCH

Sr. No.	Dye	Time required for decolorization (h)	% Decolorization
1	Red HE8B	1.5	96
2	Navy Blue HE2R	4	94.33
3	Navy blue 3G	8	91.03
4	Dark red 2B	20	89.32
5	Cotton blue	9	88
6	Reactive Orange M2R	11	90.13
7	Orange HE2R	8	92.01
8	Golden yellowHB4G	13	89

Table 2 Enzyme activities

Enzyme activity	Control cells	C ells obtained after decolorization
Lignin peroxidase[a]	3.670 ± 0.31	7.332 ± 0.35*
DCIP reductase[b]	28.918 ± 1.224	58.785 ± 1.323*
Tyrosinase[c]	0.73 ± 0.049	1.408 ± 0.073*

[a] Micromoles of product formed min^{-1} mg $enzyme^{-1}$

[b] Microgram DCIP reduced min^{-1} mg $protein^{-1}$

[c] Enzyme unit min^{-1} mg $protein^{-1}$

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

Orange 39) scanned from 200 to 800 nm showed the maximum absorbance at 410 nm. Samples from the inoculated broth were also scanned similarly, which showed a significant decrease in the absorption at 410 nm. Disappearance of peak indicates decolorization (Fig. 5).

HPLC analysis

High performance liquid chromatographic analysis of Direct Orange 39 and metabolites obtained after its decolorization by isolated bacterium *Pseudomonas aeruginosa* strain BCH showed the peaks at different retention times. Direct Orange 39 showed peak at 1.430 min (Fig. 6a) while metabolites extracted after decolorization showed peaks at 2.734 and 2.967 min (Fig. 6b). Conversion of single peak of Direct Orange 39 to two different peaks having altered retention time indicates the biodegradation of the dye Direct Orange 39, into different metabolites.

FTIR analysis

The FTIR spectrum of the control dye compared with extracted metabolites (45 ± 5 min) is as shown (Fig. 7a). Peaks in the control dye spectrum represented the C=O stretching of amide at 1627.97 cm^{-1}, C–H deformation of alkanes at 1471.74 cm^{-1}, S=O stretching of sulphur compound at 1207.48 cm^{-1}, whereas peaks at 844.85 and 717.54 cm^{-1} represent benzene ring containing two adjacent H atoms, presence of sulphur compound was indicated by stretching at 634.60 cm^{-1}, similarly stretching at 559.38 cm^{-1} showed presence of halogenated

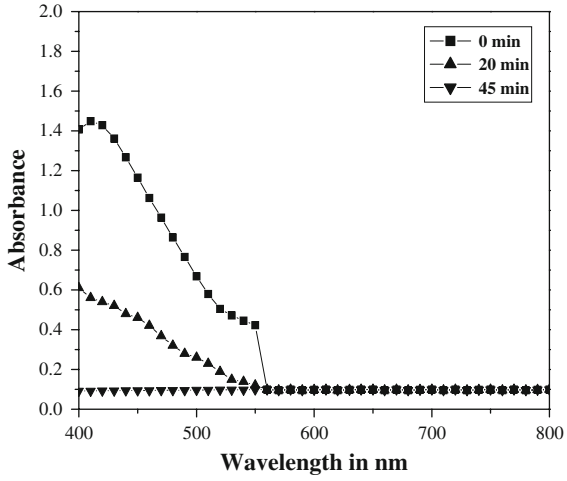

Fig. 5 UV–Vis spectral analysis of Direct Orange 39 decolorization

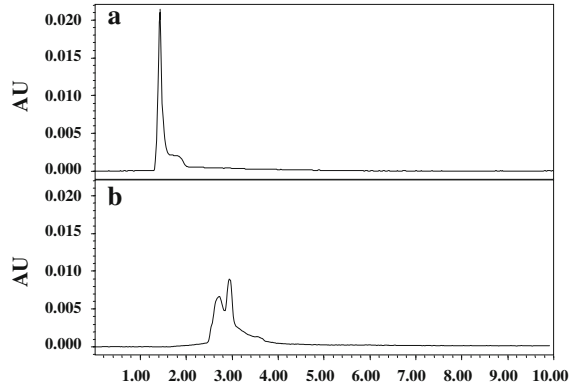

Fig. 6 HPLC elution profile of the Direct Orange 39 (**a**) and its degradation metabolites (**b**)

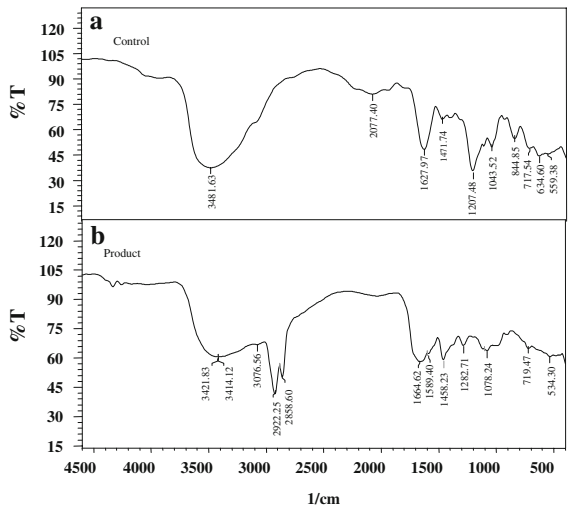

Fig. 7 FTIR spectra of Direct Orange 39 (**a**) and its degradation metabolites (**b**)

compound, N=N stretching of azides and N–H stretching of amides at 2077.40 cm^{-1} confirms the azo nature of dye. The FTIR spectrum of extracted metabolites (Fig. 7b) after decolorization of dye showed significant change in the positions of peaks when compared to control dye spectrum. Peak in extracted metabolites spectrum represented N–H stretching of amides at 3421.83, 3414.12, 3076.56 cm^{-1}, whereas two newly formed peaks at 2922.25 and 2858.60 cm^{-1} represented C–H stretching of alkanes, C=N stretching of oximes; oxazines was represented at 1664.62 cm^{-1}, presence of aromatic compounds were indicated by stretching at 1589.40 cm^{-1}, N=O stretching of nitrosamines was represented at 1458.23 cm^{-1}, peak at 1282.71 cm^{-1} represents C–N vibrations of aromatic secondary amines, similarly peak at 1078.24 cm^{-1} represents S=O stretching of sylfoxids whereas peak at 534.30 cm^{-1} represents presence of halogenated compounds.

Phytotoxicity study

Most common methods employed to study phytotoxicity are monitoring of seed germination and plant growth. As dye waste from industries are most commonly discharged to the near by agricultural area and water bodies, it was important to reveal the toxicity of Direct Orange 39 and its degraded metabolites on plant systems. Germination (%) of both *Triticum aestivum* and *Phaseolus mungo* seeds was found to be less with Direct Orange 39 as compared with its degraded metabolites. The phytotoxicity study showed that length of plumule and radical was affected in case of the dye Direct Orange 39 whereas with degraded metabolites it showed significant growth, compared to control (Table 3), which indicate that, the isolated *Pseudomonas aeruginosa* strain BCH was not only able to decolorize Direct Orange 39 but was also able to detoxify it.

Conclusions

The newly isolated *Pesudomonas aeruginosa* strain BCH was found to be most effective and having enormous potential of dye degradation under versatile environmental conditions, amongst the rest of the strains that are described up till now in the literature. The decolorization and degradation of Direct Orange 39 by *Pesudomonas aeruginosa* strain BCH might be because of lignin peroxidase, tyrosinase and NADH-DCIP reductase. Since this strain decolorizes a number of dyes, it can be used for textile waste water treatment.

Table 3 Phytotoxicity studies of Direct Orange 39 and its degradation product

Parameters	*Triticum aestivum*			*Phaseolus mungo*		
	Water	Direct Orange 39	Extracted metabolite	Water	Direct Orange 39	Extracted metabolite
Germination (%)	100	60	100	100	80	100
Root (cm)	9.47 ± 0.12	3.64 ± 0.12**	8.9 ± 0.078*	10.40 ± 0.15	3.75 ± 0.22**	9.50 ± 0.23*
Shoot (cm)	10.180 ± 0.25	3.90 ± 0.38**	8.80 ± 0.2*	8.60 ± 0.13	2.52 ± 0.15**	8.02 ± 0.068*

Values are mean of three experiments, SEM (±), significantly different from the control (seeds germinated in water) at * $P < 0.05$, ** $P < 0.001$, by One-way analysis of variance (ANOVA) with Tukey–Kramer comparison test

References

Afarani HRE (2008) Preparation of metal catalysts on granule glass for degradation of textile dyes as environmental contaminants. World Appl Sci J 3:738–741

Ali S, Shultz JL, Haq I (2007) High performance microbiological transformation of L-tyrosine to L-dopa by *Yarrowia lipolytica* NRRL-143. BMC Biotechnol. doi:10.1186/1472-6750-7-50

Bhatt N, Patel KC, Keharia H, Madamwar D (2005) Decolorization of diazo dye Reactive Blue 172 by *Pseudomonas aeruginosa*. J Basic Microbiol 45:407–418

Chang JS, Chou C, Lin YC, Lin PJ, Ho JY, Hu TL (2001) Kinetic characteristics of bacterial azo dye decolorization by *Pseudomonas luteola*. Water Res 35:2841–2850

Chen KC, Huang WT, Wu JY, Houng JY (1999) Microbial decolorization of azo dyes by *Proteus mirabilis*. J Ind Microbiol Biotechnol 23:686–690

Chen KC, Wu JY, Liou DJ, Hwang SCJ (2003) Decolorization of the textile dyes by newly isolated bacterial strains. J Biotechnol 101:57–68

Davis RJ, Gainer JL, O'Neal G, Wu IW (1994) Photocatalytic decolorization of waste water dyes. Water Environ Res 66:50–53

De Baere LA, Devocht M, Assche PV, Verstraete W (1984) Influence of high NaCl and NH4Cl salt levels on methanogenic associations. Water Res 18:543–548

Dhanve RS, Shedbalkar UU, Jadhav JP (2008) Biodegradation of diazo reactive dye navy blue HE2R (Reactive blue 172) by an isolated *Exiguobacterium* sp. RD3. Biotechnol Bioprocess Eng 13:53–60

Dhanve RS, Kalyani DC, Phugare SS, Jadhav JP (2009) Coordinate action of exiguobacterial oxidoreductive enzymes in biodegradation of reactive yellow 84A dye. Biodegradation 20:245–255

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Fu Y, Viraraghavan T (2002) Dye biosorption sites in *Aspergillus niger*. Bioresour Technol 82:139–145

Hu TL (1994) Decolorization of reactive azo dyes by transformation 436 with *Pseudomonas luteola*. Bioresour Technol 49:47–51

Hu TL (1998) Degradation of Azo Dye RP2B by *Pseudomonas luteola*. Water Sci Technol 38:299–306

Jadhav JP, Parshetti GK, Kalme SD, Govindwar SP (2007) Decolourization of azo dye methyl red by *Saccharomyces cerevisiae* MTCC 463. Chemosphere 68:394–400

Johnson RF, Zenhausen A, Zollinger H (1978) In: Mark HF, Mcketta JJ, Othmer DF Jr, Standen A (eds) Krik-Othmer, 2nd edn. Encyclopedia of chemical technology, vol 2. Wiley, Hoboken, pp 868–910

Kalme SD, Parshetti GK, Jadhav SU, Govindwar SP (2006) Biodegradation of benzidine based dye Direct Blue-6 by *Pseudomonas desmolyticum* NCIM 2112. Bioresour Technol 98:1405–1410

Kalyani DC, Patil PS, Jadhav JP, Govindwar SP (2008a) Biodegradation of reactive textile dye Red BLI by an isolated bacterium *Pseudomonas* sp. SUK1. Bioresour Technol 99:4635–4641

Kalyani DC, Telke AA, Dhanve RS, Jadhav JP (2008b) Ecofriendly biodegradation and detoxification of Reactive Red 2 textile dye by newly isolated *Pseudomonas* sp. SUK1. J Hazard Mater 163:735–742

Kapanen A, Itavaara M (2001) Ecotoxicity tests for compost applications. Ecotoxicol Environ Safety 49:1–16

Kim SJ, Shoda M (1999) Purification and characterisation of a novel peroxidase from *Geotrichum candidum*. Dec. 1 involved in decolourisation of dyes. Appl Environ Microbiol 65:1029–1035

Liu GF, Zhou J, Wang JT, Song ZY, Qv YY (2006) Bacterial decolorization of azo dyes by *Rhodopseudomonas palustris*. World J Microbiol Biotechnol 22:1069–1074

Maguire RJ (1992) Occurrence and persistence of dyes in a Canadian river. Water Sci Technol 25:265–270

Maximo C, Pessoa AMT, Costa-Ferreira M (2003) Biotransformation of industrial reactive azo dyes by *Geotrichum* sp. CCMI 1019. Enzyme Microb Technol 32:145–151

Panswad T, Anan C (1999) Specific oxygen, ammonia, and nitrate uptake rates of a biological nutrient removal process treating elevated salinity wastewater. Bioresour Technol 70:237–243

Paszczynski A, Pastri-Grisby M, Goszeeynski S, Crawford R, Crawford DL (1992) Mineralization of sulfonated azo dyes and sulfanilic acid by *Phanerochaete chrysosporium* and *Streptomyces chromofuscus*. Appl Environ Microbiol 58:3598–3604

Patil PS, Shedbalkar UU, Kalyani DC, Jadhav JP (2008) Biodegradation of Reactive Blue 59 by isolated bacterial consortium PMB11. J Ind Microbiol Biotechnol 35:1181–1190

Raffi F, Hall JD, Cerniglia CE (1997) Mutagenicity of azo dyes used in foods, drugs and cosmetics before and after reduction by Clostridium species from the human intestinal tract. Food Chem Toxicol 35:897–901

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Salah-Uddin M, Zhou J, Qu Y, Guo J, Wang P, Zhao LH (2007) Biodecolorization of azo dye acid red B under high salinity condition. Bull Environ Contam Toxicol 79:440–444

Salokhe MD, Govindwar SP (1999) Effect of carbon source on the biotransformation enzymes in *Serratia marcescens*. World J Microbiol Biotechnol 15:229–232

Selvam K, Swaminathan K, Keo-Sang C (2003) Microbial decolorization of azo dyes and dye industry effluent by *Fomes lividus*. World J Microbiol Biotechnol 19:591–593

Senan RC, Abraham TE (2004) Bioremediation of textile azo dyes by aerobic bacterial consortium. Biodegradation 15:275–280

Shanmugam V, Kumari M, Yadav KD (1999) n-Propanol as a substrate for assaying the lignin peroxidase activity of *Phaenerochaete chrysosporium*. Indian J Biochem Biophys 36:39–43

Shedbalkar UU, Dhanve RS, Jadhav JP (2008) Biodegradation of triphenylmethane dye cotton blue by *Penicillium ochrochloron* MTCC 517. J Hazard Mater 157:472–479

Stolz A (2001) Basic and applied aspects in the microbial degradation of azo dyes. Appl Microbiol Biotechnol 56:69–80

Tamura K, Nei M, Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci USA 101:11030–11035

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Telke AA, Kalyani DC, Jadhav JP, Govindwar SP (2008) Kinetics and mechanism of Reactive Red 141 degradation by a bacterial isolate *Rhizobium radiobacter* MTCC 8161. Acta Chim Slov 55:320–329

Verma P, Madamwar D (2003) Decolourization of synthetic dyes by a newly isolated strain of *Serratia marcescens*. World J Microbiol Biotechnol 19:615–618

Vujevic D, Koprivanac N, Loncaric Bozic A, Locke BR (2004) The removal of Direct orange 39 by pulsed corona discharge from model wastewater. Environ Technol 25:791–800