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**Degradation of Reactive Black 5 dye by a newly isolated
bacterium *Pseudomonas entomophila* BS1**

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1 **Degradation of Reactive Black 5 dye by a newly isolated bacterium *Pseudomonas***
2 ***entomophila* BS1**

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Draft

Abstract

The textile and dye industries are considered as one of the major sources of environmental pollution. The present study was conducted to investigate the degradation of an azo dye Reactive Black 5 (RB 5) using a bacterium isolated from soil samples collected around a textile industry. The bacterial strain BS1 capable of degrading azo dye RB 5 was isolated and identified as *Pseudomonas entomophila* based on 16S rDNA sequencing. Effects of different parameters on the degradation of RB 5 was studied to find out the optimal conditions required for maximum degradation which was 93% after 120 h of incubation. Static conditions with pH in the range of 5-9 and temperature of 37 °C were found to be optimum for degrading RB 5. Enzyme assays demonstrated that *P. entomophila* possessed azoreductase which played important role in degradation. The enzyme was dependent on FMN and NADH for its activity. Furthermore, possible degradation pathway of the dye was proposed through GC-MS analysis, which revealed that the metabolic products were naphthalene-1,2-diamine and 4-(methylsulfonyl) aniline. Thus the ability of this indigenous bacterial isolate for simultaneous decolorization and degradation of the azo dye signifies its potential application for treatment of industrial wastewaters containing azo dyes.

Keywords: Azoreductase, Decolorization, Degradation, Reactive Black 5, Textile industry.

51 Introduction

52 Azo dyes from textile and leather industry are common contaminants of soil and groundwater
53 in several countries. The situation is even worse in developing countries like India, where
54 about 1.5 million litres of dye related effluent, mostly untreated, is discharged by mills every
55 day; leading to chronic and acute toxicity to humans (Saratale et al. 2009). Presently, more
56 than 10,000 commercially available dyes are known. Azo dyes, used in textile industries, are
57 the largest group of dyes and constitutes about 60-70% of all dye stuff production (Ong et al.
58 2010; Lang et al. 2013, Zhao et al. 2014). They possess one or more azo groups ($R_1-N=N-$
59 R_2) that are chromophores; and aromatic rings, the latter are mostly substituted by sulfonate
60 groups thereby making them highly soluble in water and hence difficult to be removed from
61 wastewater (Saratale et al. 2011). The complex structure and xenobiotic nature make the azo
62 dyes and their degradation products recalcitrant to biodegradation and, in many cases, they
63 have been reported to be both mutagenic and carcinogenic (Cartwright 1983; Chung and
64 Cerniglia 1992; Pinheiro et al. 2004). Therefore, effective removal of azo dyes from
65 wastewater effluents before discharge into the environment has been a big challenge. In
66 addition to the environmental concern, the textile industry consumes very large amounts of
67 potable water ($800-1000\text{ m}^3\text{ton}^{-1}$ of finished textile). In many countries where potable water
68 is scarcely available, this large water consumption has become intolerable and therefore, it is
69 recommended to recycle the wastewater in order to decrease the water requirements.

70 For the treatment of textile wastewater, several physico-chemical methods (like adsorption,
71 coagulation, flocculation, membrane filtration, ozonation, electrochemical, and radiolysis
72 etc.) have been used. Although some of these processes have been effective, their application
73 is limited due to the high cost, excess usage of chemicals, and excessive sludge generation
74 with subsequent disposal problem (Saratale et al. 2011). Biological treatment methods are
75 eco-friendly, have been proven to be efficient and more cost effective and hence are gaining

importance in today's situation. Microorganisms such as actinomycetes, fungi, algae, yeast, aerobic and anaerobic bacteria; and their enzymes have been successfully utilised to degrade a wide variety of dyes (Kaushik and Malik 2009; Gupta et al. 2010a; 2010b; Srinivasan et al. 2014). The microbial enzymes are responsible for the degradation of different types of dyes. Under anaerobic conditions, azo bonds of dyes are broken with relative ease and results in the formation of corresponding aromatic amines, which are more toxic than the parent dye.

Metabolism of these colorless aromatic amines varies in different strains of microorganisms (Du et al. 2015). They may be further degraded aerobically or anaerobically by some strains (Chang et al. 2004; Saratale et al. 2011) or remain resistant to degradation in other strains, thereby causing environmental hazards (Murali et al. 2013).

Although decolorization of azo dyes by bacteria has been reported substantially (Chang and Kuo 2000; Chen et al. 2003; Kalyani et al. 2009; Chang et al. 2011; Thakur et al. 2014), information on possible degradation intermediates and enzymes involved in degradation process is scanty. Moreover, in-depth characterization of native bacteria capable of efficient dye decolorization and degradation would be ideal for utilization in wastewater treatment. In the present study, an attempt has been made to isolate and characterize an efficient dye degrading bacterium from wastewater contaminated soil. The nutritional requirements and physico-chemical factors were optimized to enhance its degradation efficiency. Further, an attempt was also made to understand the possible dye degradation pathway of RB 5 based on GC-MS analysis of degradation products and involvement of reductive enzymes (azoreductase).

Material and methods

Dyes and chemicals

Reactive Black 5 is a high molecular weight (992 g mol^{-1}) diazo type reactive dye containing vinyl sulfonate reactive groups, as represented by the chemical structure (Table 1). Reactive Black 5 (Remazol Black B) dye used in this study was purchased from Sigma Aldrich (USA). All other chemicals were of analytical grade, purchased from SRL and Hi-media Laboratories, Mumbai, India.

Sample collection

Kanpur, located on the banks of the river Ganges between the parallels of $26^{\circ}28'N$ and $80^{\circ}24'E$ is one of the important industrial centres of India, housing a large number of textile and leather industries. Industrial effluents arising from these industries are consistently discharged into the river Ganges, flowing across the City. A total of 12 samples have been collected from January 2012 to December 2014. Wastewater was collected in sterile glass bottles as per the method described in APHA (1998), from the surrounding area of the textile industries. Composite soil samples were also collected from surface to depth of about 15 cm as described by Aleem and Malik (2003), around the textile industries.

GC-MS analysis of textile wastewater

Textile wastewater was extracted with dichloromethane (DCM) as described in APHA (1998). Wastewater was filtered through whatman filter paper grade 1 (pore size $11 \mu\text{m}$) and $0.45 \mu\text{m}$ syringe filter (Millipore, USA). The wastewater was then extracted thrice with equal volume of dichloromethane (DCM). Aqueous and organic phases were separated using separating funnel. Organic phase containing the metabolites was collected from acidic and basic fractions. The extracted organic phase was evaporated at 40°C under reduced pressure with the help of vacuum pump and made up to 5 mL. These samples were filtered through $0.45 \mu\text{m}$ membrane filter before they were used for GC-MS analysis.

Screening for dye degrading bacteria

For the isolation of dye degrading bacteria enrichment technique was used. Nutrient broth amended with RB 5 at $100 \mu\text{g mL}^{-1}$ was inoculated with 10% of wastewater (v/v) and soil samples (w/v) and cultured under microaerophilic (static) conditions at 37°C for 48 h. 100 μL of the suspension was spread-plated on the nutrient agar plates containing RB 5 at $100 \mu\text{g mL}^{-1}$, following incubation the colonies with different morphology showing clear zone were purified and taken for identification by sequencing of 16S rDNA.

16S rDNA sequencing and phylogenetic analysis

Bacterial isolates were characterized by sequencing of 16S rDNA. Total cell DNA was extracted from overnight grown cultures using Bacterial Genomic Miniprep Kit (Sigma, USA) as per manufacturer's instructions. PCR amplification of 16S rRNA gene from the selected isolates was performed using the universal primers 27f and 1492r (Weisburg et al. 1991). PCR amplifications were carried out in a 25 μl reaction volume containing 100 ng of template DNA; at 0.5 μM each primers; 10 mM Tris-chloride (pH 8.3); 3.0 mM MgCl_2 ; dNTP mix (Fermentas, USA) at 0.6 mM; and 1.25 U of *Taq* DNA polymerase (Fermentas, USA). The reaction mixture was heated at 95°C for 2 min followed by amplification in DNA thermal cycler (Biorad, USA) programmed for 30 cycles at 95°C for 30 s, 57°C for 60 s and 72°C for 2 min and final extension at 72°C for 10 min. An aliquot of the each reaction mixture (5 μl) was analyzed by agarose gel electrophoresis (1.5% agarose) to check for the expected amplicon of 1500 bp. The electrophoresed products were stained with ethidium bromide and visualized under UV light. The remaining amplified products were purified (QIAGEN Inc., Valencia, USA) and sequenced bi-directionally (Big Dye, Applied Biosystems). The homology of the sequences obtained were determined by NCBI BLAST (Altschul et al. 1990). Sequences were aligned, verified and edited using Bioedit 7.2.0 (Hall 1999) and Molecular Evolutionary Genetics Analysis (MEGA v6.0) (Tamura et al. 2013) software programs. The evolutionary history was inferred using the Neighbor-Joining method

(Saitou and Nei 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004). The analysis involved 47 nucleotide sequences. Evolutionary analyses were conducted in MEGA v6.0 (Tamura et al. 2013).

Dye decolorization

Based on the 16S rDNA sequence analysis, one bacterium each from different genera were tested for their dye decolorizing ability in LB broth (amended with 50 µgmL⁻¹ of the dye) and incubated at 37 °C in a static condition. After every 24 h, the culture was withdrawn upto 120 h and centrifuged for 20 min at 8000×g. The supernatant was used for reading absorbance at 597 nm (λ_{max} of RB 5). The percent decolorization was calculated spectrophotometrically, by determining the initial absorbance (before decolorization) and final absorbance (after decolorization).

$$\% \text{ Decolorization} = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100$$

Half-life (t_{1/2}) of the RB 5 degradation was also calculated using initial concentration (N₀) and remaining concentration (N_t) of RB 5 after 120 h (T) of incubation as follows:

$$N_t = N_0 \times \left(\frac{1}{2}\right)^n$$

$$T = t_{1/2} \times n$$

Based on decolorization efficiency, one bacterial strain identified as *Pseudomonas entomophila* BS1 exhibiting the highest RB 5 decolorizing ability was taken for further studies.

Mode of decolorization (physical adsorption v/s biodegradation) and effects of different parameters on decolorization process

In order to determine the possible way of decolorization, the heat killed and the living cells of the bacterium were used for calculating the percent decolorization. For all of the decolorization studies, each experiment was performed in triplicate. Effects of different parameters on decolorization of RB 5 by growing cells of *P. entomophila* BS1 including dye

concentration (50, 100, 200, 300, 400 and 500 $\mu\text{g mL}^{-1}$), temperature (25, 30 and 37 $^{\circ}\text{C}$), initial pH (4, 5, 6, 7, 8 and 9), salt concentration (0, 5, 10, 20, 40, 60, 80, and 100 g L^{-1}) and shaker speed (50, 100, 150 and 200 rpm) were investigated. Overnight bacterial culture was inoculated in LB broth, amended with 50 $\mu\text{g mL}^{-1}$ of RB 5 dye (except for when using different dye concentrations). Inoculated medium was incubated at 37 $^{\circ}\text{C}$ (except for when at different temperatures) under static condition (except for when at different rpm). The extent of decolorization was calculated and recorded.

Enzyme assay

Azoreductase activity was assayed by the method of Nachiyar and Rajakumar (2005). In order to assay the enzyme responsible for degradation of the dye in *P. entomophila* BS1, bacterial cells were harvested (8000 $\times$ g, 20 min), washed by 10 mM sodium phosphate buffer (pH 7.1) and suspended in an equal volume of 50 mM sodium phosphate buffer (pH 7.1). Following the addition of DNase and cell beads, the pellet was used for the preparation of cell-free extract using cell mill. Enzyme assay was started with a reaction mixture containing potassium phosphate buffer (25mM, pH 7.1), FMN (10 μM), Dithiothreitol (5mM), NADH (0.1 mM), substrate (RB 5 dye, 30 μM), ddH₂O and enzyme (cell-free extract). All the reaction mixture, except NADH, was pre-incubated at room temperature for 4 min and the reaction was started by adding NADH, and the mixture was monitored spectrophotometrically at 597 nm. The slope of the initial linear decrease in absorption was taken for calculating the azoreductase activity based on the molar absorption coefficient of RB 5 ($\epsilon=21.329 \text{ mmol}^{-1}\text{cm}^{-1}$). One unit of enzyme activity was defined as the amount of enzyme required to decolorize 1 nmol of dye per min under the assay conditions. The assay was also done with different controlled conditions in order to determine the factors on which the enzyme activity was dependent. Protein concentration of the cell-free extract was determined by Bradford assay. All the assays were conducted in triplicate.

197 Analysis of the degradation products

198 For determining the possible changes in the absorption spectra of the dye during the

199 degradation of RB 5 by *P. entomophila* BS1, the culture supernatant from the decolorized

200 media was scanned from 350-750 nm using a UV-vis spectrophotometer against a baseline

201 defined by the absorbance of clarified samples from dye free media. For biodegradation

202 analysis, nutrient broth amended with 50 $\mu\text{g mL}^{-1}$ of the dye, was incubated first in static and

203 then in shaking conditions. After decolorization of the broth, metabolites were extracted

204 thrice with equal volume of dichloromethane and were identified using gas chromatography

205 along with mass spectrometry with a VARIAN GC-MS-4000 gas chromatograph, a VARIAN

206 CP-8410 auto sampler and an ION TRAP mass spectrometer. The system was controlled by a

207 0 VARIAN STAR MS WORK STATION v6.9.1. The chromatographic column was a

208 ZEBRON ZB-1701 (30 m 0.25 mm i.d.; 0.15 mm film thickness). The carrier gas was helium

209 at a pressure of 8.7 psi. The sample injection was made in split mode using a BRUKER-

210 GLASS liner. The compounds were identified on the basis of mass spectra and using the

211 NIST library (National Institute of Standards and Technology).

212 Results

213 The results of the present study showed that the bacterium *P. entomophila* BS1 is endowed

214 with a high ability of metabolizing the textile dye RB 5 at temperature of 37 °C and in the pH

215 range of 5-9. It was found that the decolorization was mainly due to the degradation of the

216 dye by the action of azoreductase enzyme. The metabolic intermediates of the degradation

217 were naphthalene-1,2-diamine and 4-(methylsulfonyl) aniline, as analysed by GC-MS.

218 GC-MS analysis of wastewater

219 The mass spectra of fragments for the major peaks in the gas chromatograms of textile

220 wastewater at the particular retention time were compared with the mass spectra of the NIST

library. GC-MS analysis revealed the presence of different organic compounds, mainly esters, amines, carboxylic acids and other aliphatic and aromatic hydrocarbons (Table 2).

Screening of dye degrading bacteria

Forty one bacteria (15 and 26 each from textile industry wastewater and contaminated soil) with colonies surrounded by a nearly decolorized zone were successfully isolated and then acclimatized to high concentration of RB 5.

16S rDNA sequencing and Phylogenetic analysis

To determine the phylogenetic relationship among the selected isolates, a 1500 bp amplicon corresponding to 16S rDNA was successfully amplified and sequenced in all the 41 isolates. The partial sequences were aligned with the 16S rDNA sequences of the corresponding sequences in GenBank (Accession no. NR102865, NR111998, NR114154, NR113812, NR044338, NR113615) and compared using Bioedit (Version 7.2.0). It was observed that the obtained sequences showed 97-100% identity with the reported sequences on BLAST analysis. The partial 16S rDNA sequences were submitted to GenBank. Of the 41 isolates tested, 27 were found to be most similar to *Pseudomonas sp.*, 8 with *Enterobacter sp.*, 3 with *Arthrobacter sp.* and the remaining 3 isolates showed maximum identity with *Pantoea sp.*, *Leclercia sp.*, and *Ochrobactrum sp.* Among all the selected isolates, *Pseudomonas sp.* was the most abundant (27) followed by *Enterobacter sp.* (8), *Arthrobacter* (3), *Pantoea sp.* (1), *Leclercia sp.* (1), and *Ochrobactrum sp.* (1). A tree representing the phylogenetic relationship among the 16S rDNA was constructed using Neighbor-joining method (Fig. 1) by MEGA (version 6.0).

The accession numbers of the partial 16S rDNA sequences (KP684060- KP684100) obtained in this study are available at NCBI (<http://www.ncbi.nlm.nih.gov/BLAST>).

Dye decolorization

Out of the 41 bacterial isolates, screened by 16S rDNA sequence analysis, one bacterium each from different genera were tested for their dye decolorizing ability and they showed different levels of percent decolorization ranging from 65-93% after 120 h of incubation (data not shown). Based on decolorization efficiency, one bacterial isolate BS1 showed maximum decolorization potential, the bacterium was identified as *P. entomophila* based on the BLAST analysis of 16S rRNA gene sequence (Fig. S1). *P. entomophila* BS1 was found to exhibit significant decolorization of 77% after 48 h of incubation, and maximum value (93%) was achieved after 120 h of incubation. Half-life ($t_{1/2}$) of the RB 5 degradation was found to be 33.38 h. Other soil bacterial isolates like *Ps. monteilii* BS7, *Ps. fulva* BS30, *Enterobacter cloacae* BS10 and *Enterobacter aerogenes* BS24 showed 74%, 89%, 77% and 81% of the dye decolorization after 120 h of incubation, respectively.

Mode of decolorization (Physical adsorption v/s biodegradation) and effects of different parameters on decolorization process

When heat-killed bacterial cells were added in the culture medium, only 4% decolorization was observed after 120 h of incubation. In contrast, 88% was achieved in the culture inoculated with live bacterial cells (Fig. 2a). *P. entomophila* BS1 showed higher decolorizing capability at the temperatures of 30 °C and 37 °C (88 and 93% respectively) after 120 h of incubation while at room temperature (25 °C) only 67% of decolorization was observed (Fig. 2b). The bacterial isolate has shown good decolorization at the pH range of 5-9 (about~94% decolorization) (Fig. 2c) and at pH 4 very insignificant reduction was observed. The isolate grew well up to the concentration of 500 $\mu\text{g mL}^{-1}$ of the dye and has shown significant amount of decolorization (80% at 500 $\mu\text{g mL}^{-1}$) (Fig. 2d). It was also found that with increasing dye concentration, there is a gradual decrease in percentage decolorization (93% at 50 $\mu\text{g mL}^{-1}$ and 80% at 500 $\mu\text{g mL}^{-1}$). The decolorization process appeared to be O_2 -sensitive, since percent decolorization decreased with increasing the speed of the shaker incubator (Fig. 2e).

Decolorization of the dye was about 93% under static condition, and significantly decreased in aerobic condition (19% and 14% at 150 and 200 rpm, respectively). Furthermore, the bacterium was found to tolerate significantly high concentration of salt and simultaneously decolorizing the dye as well. At salt concentration of 20 gL^{-1} , decolorization was observable (51% after 120 h of incubation). Decolorization process started to decrease when salt concentration exceeded 40 gL^{-1} and only 12% decolorization occurred at 100 gL^{-1} salt concentration (Fig. 2f).

Enzyme assay

Enzyme activity in the cell-free extract was found to be 0.0012 Umg^{-1} . When in the reaction mixture either FMN or NADH was not added, the activity was found to be zero, which showed that the enzyme was dependent on FMN as prosthetic group and NADH as co-factor for the activity. Moreover the enzyme was appeared to be constitutively expressed, since the activity was also recorded when no dye was added in the bacterial growth medium for the preparation of cell-free extract (Table 3).

Analysis of degradation products

In order to propose the possible mechanism of degradation of the dye, UV-vis spectroscopy and GC-MS analysis were carried out. The main visible absorption peak of Reactive Black 5 dye is shown (Fig. 3). The UV-vis scanning spectra (350-750 nm) of supernatants at different time intervals exhibited that the intensity at 597 nm (λ_{max} of RB 5) obviously decreased and reached virtually zero after 48 h of incubation which suggested that during the reaction, the azo bond was cleaved, most likely mediated by azoreductase enzyme, with the primary chromophore being destroyed.

Gas chromatogram of the degraded dye, extracted from decolorized medium (using dichloromethane) showed seven different peaks (Fig. 4) which corresponded to different product molecules. These product molecules then entered into mass spectrometer, which

made each product molecule charged. These charged molecules were accelerated and broken into smaller charged fragments which were displayed as spectral plots (Fig. S2). From the molecular mass and the mass of the fragments, two intermediary products were identified as naphthalene-1,2-diamine (retention time 17.260, m/z 158.20) and 4-(methylsulfonyl)aniline (retention time 22.785, m/z 171.21) respectively.

Discussion

Textile processing industries use a variety of chemicals, therefore, the effluent compositions vary greatly depending on the different processes, used fabrics and machinery (Bisschops and Spanjers 2003). The textile effluent is characterized by high alkalinity, suspended solids, biochemical oxygen demand, chemical oxygen demand, heat, color, acidity and trace metals such as Cr, As, Cu and Zn, with dye concentrations generally below 1 gL⁻¹ (Kaushik and Malik 2009). The removal of color from textile and dyes manufacturing industrial wastewaters has become a real challenge in recent years, since the persistent release of toxic pollutants from these industries have contaminated the surrounding soil and water bodies (Moosvi et al. 2005). In order to overcome this problem, different bacterial strains were isolated from the textile effluents and contaminated soils and screened for their ability to decolorize the textile azo dye. The isolation and screening of azo dye degrading microorganisms from sludge samples collected from the dye wastewater treatment sites have been reported previously (Chen et al. 2003; Senan and Abraham 2004; Khadijah et al. 2009), that indicated the natural adaptation of these microorganisms to survive in the presence of the toxic dyes (Khadijah et al. 2009). As per 16S rDNA sequence analysis, the sequences obtained showed 97-100% identity with the reported sequences on BLAST analysis. They were found to belong to six different genera indicating broad distribution of dye degrading enzymes across bacterial species/genera. The phylogenetic tree revealed that the identical sequences grouped together. Phylogenetic analysis of *P. entomophila* BS1

revealed that it grouped with other reported sequences of *P. entomophila* and other related *Pseudomonas* sp. (Fig.1; Fig. S1).

The decolorization efficiency of *P. entomophila* BS1 is comparable to those reported by Wang et al. (2009) and (2013), where in *Enterobacter* sp. EC3 and *Bacillus* sp. YZU1 showed 92.6% (after 108 h of incubation) and 95% decolorization (after 120 h of incubation), respectively. Similarly, Garg et al. (2012) have also reported that *Pseudomonas putida* SKG-1 removed more than 90% of orange II dye within 96 h of incubation. Under anoxic conditions, many bacterial strains like *P. mirabilis*, *P. luteola*, *Pseudomonas* sp. and *K. rosea*, showing significant reduction of dyes, have also been reported (Chen et al. 1999; Chang et al. 2001). Since only 4% of decolorization occurred through adsorption, therefore, it can be suggested that process is mainly due to degradation of the dye (Fig. 2a). Heteropolysaccharide and lipid components of the cell wall contribute to the biosorption capacity of a microorganism that in turn leads to strong force of attraction between the azo dye and the cell wall (Solís et al. 2012). Adsorption of the dye has been mainly reported in fungal isolates due to their large surface area and only in few bacteria where extra polysaccharide production was found (Binupriya et al. 2010). The optimal temperature for the decolorization of the dye was found to be 37 °C (Fig. 2b). Temperature is an important parameter, as bacteria require optimal temperature for growth and enzyme production. At optimal temperature, there is an optimal microbial growth and greater production of enzymes. Dye decolorization process is inhibited at other temperatures (very high or low) due to the loss of cell viability or deactivation of enzymes involved in the degradation (Cetin and Donmez 2006). It has been previously reported that 37 °C is an optimal temperature for dye decolorization in many bacterial isolates (Kolekar et al. 2008) and in bacterial consortia (Saratale et al. 2010). pH plays an important role in microbial growth and azo dye degradation and optimal pH for color removal have been found to be 6-10 (Chen et al. 2003;

Kilic et al. 2007). pH of the textile effluent generally varies between 8-9. *P. entomophila* BS1 exhibited decolorizing ability at the pH range of 5-9 (about~94% decolorization), that makes it a promising strain for the effective bio-treatment of dye wastewater. Kalyani et al. (2008) found that bacterial isolates like *Bacillus subtilis*, *Brevibacillus laterosporus*, *Enterbacter* sp, *E. coli* and *Pseudomonas luteola* have decolorized different dyes at a wide pH range, generally in the alkaline region. Percentage decolorization was found to be decreased with increasing dye concentrations. This can be due to the toxicity of the dyes to bacteria that inhibit the metabolic activities at higher dye concentrations. Similar results have also been reported by Gopinath et al. (2009) that in *Bacillus* sp. dye decolorization decreases with increasing initial dye concentration. Since our isolate grew well up to the concentration of 500 μgmL^{-1} of the dye and has shown good decolorization, therefore it can be successfully utilised for the treatment of colored wastewater. The process of decolorization was oxygen sensitive, as maximum decolorization occurred when the bacterium was incubated statically (Fig. 2e). Since in the presence of oxygen, instead of azo groups in the dyes, oxygen is preferred as an electron acceptor (Pearce et al. 2003). Similarly, this has been reported previously that biodegradation of dyes was inhibited in aerobic condition (Moosvi et al. 2005). The bacterium was found to grow and decolorize the dye at significantly high salt concentration (20 gL^{-1}). As salt concentration was further increased, both the bacterial growth and consequently decolorization decreased drastically, because high salt concentration affects osmotic pressure in the bacterial isolate that may even cause cell death (Kolekar et al. 2008; Wang et al. 2013).

Enzymes that cause reduction of azo groups are termed as azoreductases. Azoreductase is the key enzyme expressed in the dye degrading bacteria and can catalyse the reductive cleavage of azo bonds. Azoreductase enzymes have been reported in many organisms (Blumel and Stolz 2003; Chen et al. 2005). These enzymes are having broad specificity, may be tolerant

or sensitive to oxygen, and may be FMN-dependent or independent. In the present study, the bacterial azoreductase was found to be dependent on FMN and NADH for its activity and was constitutively expressed (Table 3). In contrast, azoreductases isolated from several bacteria have been shown to be inducible flavoproteins and able to use both NADH and NADPH as electron donors (Russ et al. 2000). The initial step in azo dye degradation involved reductive cleavage of azo bond with the help of azoreductase, leading to generation of secondary aromatic amines. These amines were further converted into lower molecular weight compounds via the action of other enzymes.

To detect the possible occurrence of biodegradation, control and treated dye samples were analysed by UV-vis spectrophotometer and change in the peaks were recorded. The result showed that the biodegradation played a more important role, as the major peak decreased and reached virtually zero after 48 h. Thus it can be concluded that azo bonds were cleaved by azoreductase enzyme during the process leading to damage in the primary chromophore, as also demonstrated by Oturkar et al. (2011) and Qu et al. (2012). Possible pathway for RB 5 dye degradation was proposed by GC-MS analysis. For the biological decolorization of azo dyes, azo groups were first cleaved reductively by the bacterial azoreductase enzyme. The corresponding amines thus produced might be further attacked by other enzymes.

Conclusion

The removal of dyes from aqueous effluent has received considerable attention within environmental research. In this study, an effective RB 5 decolorizing bacterial strain, *P. entomophila* BS1 was isolated. Decolorization is mainly due to degradation of RB 5 by azoreductase enzyme, not simply a physical surface adsorption. For degradation, *P. entomophila* simply needs a mild condition, which showed remarkable tolerance to high concentrations of RB 5 (up to 500 mgL⁻¹). The ability of the strain to tolerate and degrade azo

dye at a high concentration gives it an advantage for treatment of textile industry wastewaters.

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Legends to Figures

Fig. 1. 16S rDNA sequence based phylogenetic tree of bacterial strains used in this study and related sequences obtained through BLAST analysis

Fig. 2. Decolorization of Reactive Black 5 dye by *Pseudomonas entomophila* BS1 (a) using live and dead cells (heat-killed) (b) temperatures (c) pH (d) initial dye concentrations (e) Different rpm, and (f) salt concentrations

Fig. 3. UV-vis spectra of Reactive Black 5 dye degradation by *Pseudomonas entomophila* BS1 at different time intervals

Fig. 4. Gas chromatogram of degraded products of Reactive Black 5 dye

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136x173mm (300 x 300 DPI)

164x78mm (300 x 300 DPI)

121x85mm (300 x 300 DPI)

147x147mm (300 x 300 DPI)

Table 1. Characteristics of Reactive Black 5 (Remazol Black B) dye used in this study.

Azo dye	Molecular structure	Wavelength (nm)
Reactive Black 5 (Remazol Black B)		597

Source:<http://www.sigmaaldrich.com/catalog/product/sial/306452?lang=en®ion=IN#>

Table 2. Compounds identified in textile wastewaters using GC-MS.

Sample	Compounds identified
Site 1 (acidic DCM fraction)	Cyclic octaatomic sulphur 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester 1,2-Benzenedicarboxylic acid, diisooctyl ester Urs-9(11)-en-12-one-28-oic acid, 3-acetoxy-, methyl ester 17-Pentatriacontene
Site 1 (basic DCM fraction)	2,4-bis(1,1-dimethylethyl)-phenol (1,1'-Biphenyl)-2-amine Azobenzene (1,1'-Biphenyl)-4-amine Benzidine 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester 1,2-Benzenedicarboxylic acid, diisooctyl ester N-phenyl-4-(phenylazo)-benzenamine

Table 3. Determination of enzyme activity in cell-free extract.

Condition 1: When bacterial culture was grown in LB broth (without Reactive Black 5 dye)				
Cell-free extract* (100µl)+	FMN (10 µM)	NADH (0.1 mM)	DDT (5 mM)	Activity (nmol-min ⁻¹ -mg proteins ⁻¹)
+	+	+	+	1.2 ± 0.07
+	–	+	+	0.22 ± 0.02
+	+	–	+	0.12 ± 0.007
Condition 2: When bacterial culture was grown in LB broth (with Reactive Black 5 dye)				
+	+	+	+	0.60 ± 0.03
+	–	+	+	0.14 ± 0.01
+	+	–	+	0.00
+	+	+	–	0.38 ± 0.02
+	–	–	–	0.00
+	–	–	+	0.00
+	–	+	–	0.00
+	+	–	–	0.00
Condition 3: When cell-free extract was dialyzed (bacterial culture was grown in LB broth with Reactive Black 5 dye)				
+	+	+	+	0.38 ± 0.025
+	–	+	+	0.00
+	+	–	+	0.00
+	+	+	–	0.00
+	–	–	–	0.00
+	–	–	+	0.00
+	–	+	–	0.00
+	+	–	–	0.00

* no activity was recorded in the absence of cell free-extract.

‘+’ indicates that the particular substance was in the reaction mixture and ‘–’ indicates that it was not added in the reaction mixture.