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ORIGINAL PAPER

Synergistic degradation of diazo dye Direct Red 5B by *Portulaca grandiflora* and *Pseudomonas putida*

R. V. Khandare · A. N. Kabra · A. V. Awate · S. P. Govindwar

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Abstract Plants and bacterial consortium of *Portulaca grandiflora* and *Pseudomonas putida* showed complete decolorization of a sulfonated diazo dye Direct Red 5B within 72 h, while in vitro cultures of *P. grandiflora* and *P. putida* independently showed 92 and 81 % decolorization within 96 h, respectively. A significant induction in the activities of lignin peroxidase, tyrosinase, 2,6-dichlorophenol indophenol reductase and riboflavin reductase was observed in the roots of *P. grandiflora* during dye decolorization; whereas, the activities of laccase, veratryl alcohol oxidase and 2,6-dichlorophenol indophenol reductase were induced in the cells of *P. putida*. Plant and bacterial enzymes in the consortium gave an enhanced decolorization of Direct Red 5B synergistically. The metabolites formed after dye degradation analyzed by UV–Vis spectroscopy, Fourier transformed infrared spectroscopy and high performance liquid chromatography confirmed the biotransformation of Direct Red 5B. Differential fate of metabolism of Direct Red 5B by *P. grandiflora*, *P. putida* and their consortium were proposed with the help of gas chromatography–mass spectroscopy analysis. *P. grandiflora* metabolized the dye to give 1-(4-diazenylphenyl)-2-phenyldiazene, 7-(benzylamino) naphthalene-2-sulfonic acid, 7-aminonaphthalene-2-sulfonic acid and methylbenzene. *P. putida* gave 4-hydroxybenzenesulfonic acid and 4-hydroxynaphthalene-2-sulfonic acid and benzamide. Consortium showed the formation of benzenesulfonic acid, 4-diazenylphenol, 6-aminonaphthalen-1-ol, methylbenzene and naphthalen-1-ol. Consortium achieved an enhanced and efficient degradation of Direct Red 5B. Phytotoxicity study revealed the nontoxic nature of metabolites formed after parent dye degradation. Use of such combinatorial systems of plant and bacteria could prove to be an effective and efficient strategy for the removal of textile dyes from soil and waterways.

Keywords Consortium · Decolorization · Enzymes · Metabolism · Phytoremediation

R. V. Khandare · A. N. Kabra
Department of Biotechnology, Shivaji University, Kolhapur, India

A. V. Awate · S. P. Govindwar (✉)
Department of Biochemistry, Shivaji University, Kolhapur 416004, India
e-mail: spg_biochem@unishivaji.ac.in

Introduction

Dyes are an important class of chemical compounds that are used for various purposes like processing of food, leather, cosmetics and most importantly textile stuffs. Dye processing and manufacturing industries discharge a huge amount of effluents into the environmental sink per day. The recalcitrant nature of textile wastewater is due to the high content of dyestuffs, surfactants, dispersants, acids, bases, salts, detergents, humectants and oxidants (Gharbani et al. 2008). Treatment of these high volumes of wastewater coming mostly from the dyeing processes becomes essential as the presence of color renders these waters esthetically unacceptable and unusable (Nilratnisakorn et al. 2007). Moreover, this wastewater pollutes our valuable water resources leading to reduction of sunlight penetration, which in turn decreases both photosynthetic activity and dissolution of oxygen concentration causing threat to zoo and phytoplanktons and even the algae. Textile dyes are toxic, mutagenic and carcinogenic posing threats to the environment, human health, and agricultural productivity (Bafana et al. 2009); therefore, the treatment

of dye containing effluent prior to their release into the environment becomes necessary.

Numerous options such as membrane filtration, oxidation, ozonation, physical and biological adsorption and microbial biodegradation are available and have been employed for the remediation of dye containing effluents. These treatment and disposal techniques do not always meet the regulatory standards ending with industrial dyes contaminating soil and water bodies (Saratale et al. 2011). Moreover, all these methods are extremely expensive. This financial burden probably plays a role in slowing down global efforts to eradicate pollution, particularly in developing countries where these techniques are clearly not affordable (Pilon-Smits 2005). As a result, it is highly desirable to develop more cost-effective remediation methods such as phytoremediation (Govindwar and Kagalkar 2010). Phytoremediation also enjoys advantages such as being a passive, solar energy-driven and low-cost method which requires no extra maintenance and nutrient inputs; moreover, it is also esthetically pleasant and can be useful to obtain useful products such as wood and flowers and even bioenergy depending upon the habit of the plant used.

Remediation of textile dyes such as Acid Orange 7, Direct Red 5B (DR5B), Brilliant Blue R, Remazol Red, Green HE4B and Remazol Orange 3R has been independently tried by using some plants, namely, *Phragmites australis*, *Blumea malcolmii*, *Tagetes patula*, *Typhonium flagelliforme*, *Aster amellus*, *Portulaca grandiflora* and *Glandularia pulchella* (Davies et al. 2005; Kabra et al. 2011a, b; Kagalkar et al. 2009, 2010; Khandare et al. 2011a, b; Patil et al. 2009). *Rheum rabarbarum* (rhubarb) and *R. hydrolapatum* are able to treat the sulfonated anthraquinones (Aubert and Schwitzguebel 2004). A number of bacteria, fungi and even yeast have been used for degradation of dyes and textile effluents. Plants have a great potential to degrade organic pollutants directly via their own enzymatic machineries and can enhance biodegradation of organic pollutants by microorganisms in their rhizosphere (Glick 2010). Hence, it is of outstanding interest to find an effective microflora and biochemical approach for the decolorization and detoxification of textile wastewater.

Phytoremediation technique mainly involves the plant roots and the rhizosphere microflora for the degradation of recalcitrant organic compounds. The concentration of bacteria in the immediate vicinity of plant roots is typically 10- to 1,000-fold greater than the bacterial concentration found in the bulk soil (Glick 2010). Plant root exudates and extracts provide an appropriate primary carbon sources for growth of a diverse class of microorganisms which may help to improve in situ remediation of a variety of pollutants (Rentz et al. 2005). Therefore, plants particularly with deep, fibrous roots and fast growth are useful in phytoremediation (Brandt et al. 2006). Plant and microbial synergism has been widely studied for the treatment of different pollutants. For instance, polycyclic and other aromatic hydrocarbons, halogenated compounds such as organochlorine herbicide 2,4-dichlorophenoxyacetic acid, polychlorinated biphenyls, petroleum products and crude-oil have been shown to be degraded by bacterially assisted phytoremediation where, some endophytic bacteria and *Pseudomonas* species were involved (Brandt et al. 2006; Glick 2010; Shaffiqu et al. 2002; Sun et al. 2010). *Pseudomonas* species have also been found to be involved in textile dye decolorization (Kalme et al. 2007). Plant–bacterial synergism of *Populus nigra* and *P. putida*, and some other microorganisms have been proved to be more effective for degradation of diesel oil (Tesar et al. 2002). Similarly, phytoremediation of hydrocarbons by *Cyperus laxus* was shown to be improved when inoculated with some bacterial and fungal isolates (Escalante-Espinosa et al. 2005).

Common garden plants like *A. amellus*, *G. pulchella* and *P. grandiflora* have great potential to clean the dye-polluted sites along with achieving extra benefits like harvesting flowers and growing other important ornamental and nonedible plants. Moreover, these wastelands could be brought under public use by maintaining gardens. This communication deals with bacterially assisted phytoremediation of sulfonated azo dye Direct Red 5B (DR5B). *P. putida* that could survive on the root exudates of *P. grandiflora* Hook. and hence the strain name given as *P. putida* strain PgH played independent role in the degradation of DR5B. *P. putida* is cosmopolitan in distribution; moreover, it is not pathogenic to animals or plants, and can therefore be used safely for environmental applications (Timmis 2002). The striking morphological feature of *P. grandiflora* is the massive, finely structured root system and its creeper habit (Khandare et al. 2011b). Furthermore, it is adaptable to a wide range of edaphic and climatic conditions throughout the tropics and subtropics and is one of the most common garden plants. The strategy was to discover a naturally found plant and microbial synergism and explore it to achieve an efficient and enhanced decolorization of DR5B in vitro. The study was carried out during August 1, 2010 to April 14, 2011 at the Department of Biotechnology and Department of Biochemistry, Shivaji University, Kolhapur, India.

Materials and methods

Chemicals

2,2′-Azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS) and riboflavin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Nicotinamide adenine dinucleotide

(di-sodium salt), *n*-propanol, 2,6-dichlorophenol indophenol (DCIP), veratryl alcohol and catechol were purchased from Sisco Research Laboratories, Mumbai, Maharashtra, India. Tartaric acid was obtained from BDH chemicals (Mumbai, Maharashtra, India). All the chemicals used were of the highest purity available and of an analytical grade. DR5B was procured from Mahesh, Dyeing industry, Ichalkaranji, Maharashtra, India.

Isolation and identification of root-associated bacteria

The soil adhering to the wild plant roots was washed off under tap water and exposed to dye solution at a concentration of 20 mg l^{-1}. The dye was found to be decolorized up to 98 % within 40 h, during decolorization process some sort of bacterial growth was observed in the decolorized dye solution. All those mixed bacteria could survive on root exudates and some of them were probably the rhizosphere microorganisms. The bacteria were isolated by streak plate method on nutrient agar plate. It was incubated at 30 °C for 24 h and pure culture was obtained subsequently. A preliminary identification of the culture was done by biochemical tests. The 16S rDNA analyses were carried out by Bangalore Genei, Bangalore, India. The 16S rDNA sequence was initially analyzed at NCBI server (http://www.ncbi.nlm.nih.gov) using BLAST (blastn) tool and corresponding sequences were downloaded. The obtained sequence was deposited at GenBank. Phylogenetic tree was constructed by neighbor-joining method, using the MEGA4 package. The bacterium was identified as *P. putida* and given the strain name PgH.

Organism and culture conditions

The pure culture of *P. putida* was grown in 250-ml Erlenmeyer flask, containing 100 ml nutrient broth [(g l^{-1}): peptone 5, NaCl 5, yeast extract 1.5 and beef extract 1.5] at 30 ± 2 °C for 24 h under static condition.

Tissue culture conditions for the development of plants

Since, bacterial contamination was observed along with decolorization of the dye while studying with wild plants it would be doubtful to claim that plant alone could decolorize the dye; therefore, in vitro-grown cultures of *P. grandiflora* were obtained by seed culture method. The seeds of *P. grandiflora* were collected from the local nursery. The seeds are small and brown. The collected seeds were washed thoroughly with running tap water and air dried on a filter paper, then pooled in an Eppendorf tube and treated for 10 min with 0.2 % Bavistin (Carbendazim 50 % WP BASF, India) containing few drops of detergent (Labolene). This was followed by 3–4 washes with sterile distilled water. Then the seeds were surface sterilized with 0.1 % $HgCl_2$ (w/v) for 10 min. To eliminate the traces of $HgCl_2$, the seeds were rinsed repeatedly with sterile distilled water under aseptic condition. These seeds were cultured and spread in phyta jars containing 30 ml of half-strength Murashige and Skoog's (MS) basal medium (Murashige and Skoog 1962). The pH of the medium was adjusted to 5.8, prior to the addition of solidifying agent Clarigel (0.2 % w/v, Hi Media, India). The medium was autoclaved at 103.43 kPa and 121 °C for 20 min. Cultures were maintained at 25 ± 2 °C with 16 h light and 8 h darkness. The half-strength MS medium was found suitable for 75 % of seed germination. After 2 weeks, seeds started to germinate. The germinated seedlings were aseptically excised and shoot tips were isolated and subcultured on plain MS medium in test tubes. After 4 weeks, shoots started producing roots, which were long and profuse. These plantlets were then used for phytoremediation studies.

Development of consortium-PP

After getting promising results during the initial decolorization studies with wild plants of *P. grandiflora* along with the involvement of *P. putida*, it was decided to use this naturally occurring synergism in the form of a consortium to achieve enhanced and efficient decolorization and degradation of the model dye. The log phase growth of *P. putida* (24 h) obtained in nutrient broth was used along with in vitro-grown plantlets of *P. grandiflora* to develop a consortium. A single plantlet in 10 ml distilled water was inoculated with 0.5 ml of log phase growth of *P. putida*. The bacteria were found to survive and thrive well in this built consortium.

Decolorization experiments

Decolorization experiments were initially carried out with a wild plant for the dye DR5B, where one healthy plant (30 g) with 7–8 shoots and a dense root system, suspended in distilled water containing DR5B solution at a concentration of 20 mg l^{-1} and 98 % decolorization within 40 h was achieved. After getting promising results with wild plants, further decolorization experiments were carried out with tissue cultured plants in three independent sets. Plantlets of the same growth stage, equal number of shoots, and almost equivalent dry weights (average wet weight of the entire plant was 9 g, and dry weight was 0.8 g) while the shoot length was about (10 cm) were used for all the experiments to keep as far as possible the same and accurate plant source. Experiment were carried out in 25 × 150 mm test tubes that

contained two plantlets in each tube containing 10 ml of autoclaved distilled water with DR5B (20 mg l^{-1}). Results were obtained in autoclaved distilled water containing dye; therefore, it was used as a medium for our further studies which thus helped to reduce the cost of overall experiment. To the second set, 10 ml of dye solution was inoculated by adding 1 ml of log phase culture of *P. putida* and to the third set, 1 plantlet along with 0.5 ml of microbial growth was exposed to 10 ml of DR5B solution. Absorbance was measured at an interval of 12 h each by removing 0.5 ml of solution and centrifuging at 4,561*g* for 10 min to remove any solid matter if present and the absorbance of the clear solution was measured at the wavelength of 530 nm. Decolorization percentage was calculated as follows:

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

Abiotic controls contained the respective dye solutions which were devoid of plants and bacteria; whereas three biotic controls were kept in which had plants, the other had only bacteria and one with plant and bacteria in consortium, immersed in plain sterilized distilled water.

Preparation of cell-free extract

Roots of the plants were cut from the shoot system, weighed, finely chopped and then suspended in 50 mM potassium phosphate buffer (pH 7.4). The chopped root tissue was then ground in a mortar and pestle followed by homogenization in a glass homogenizer and then centrifugation at 8,481*g* for 20 min. The cell-free extract thus obtained was used as an enzyme source. The supernatant obtained after harvesting the plant roots was used as a source of extracellular enzymes after centrifugation.

There was no significant growth of *P. putida* in plain distilled water; therefore, log phase growth of *P. putida* obtained in nutrient broth was used for preparation of cell-free extract for enzymatic studies. The cells were suspended in 50 mM potassium phosphate buffer, homogenized adequately and then sonicated using SONICS, vibra cell, ultrasonic processor (Germany) giving 10 strokes each of 15 s at an interval of 45 s at the amplitude of 40 to get crude enzyme solution. The test sample had cells after degradation of 100 ml of 20 mg l^{-1} DR5B solution, whereas the abiotic control contained only dye in nutrient broth and the biotic control with microbial growth but no dye was used. The cell-free solution obtained after harvesting cells was used as a source of extracellular enzymes.

Enzyme assays

Activities of the enzymes lignin peroxidase (LiP), veratryl alcohol oxidase, laccase, tyrosinase, DCIP reductase and riboflavin reductase were determined spectrophotometrically at room temperature in case of control and test for both plants and microorganisms. LiP activity was determined by monitoring the formation of propanaldehyde at 300 nm in a reaction mixture of 2.5 ml containing 100 mM *n*-propanol, 250 mM tartaric acid, and 10 mM H_2O_2 (Kalme et al. 2007). Laccase activity was determined in a reaction mixture of 2 ml containing 10 % ABTS in 0.1 M acetate buffer (pH 4.9), and increase in optical density was measured at 420 nm (Telke et al. 2010). Tyrosinase activity was determined as described by Surwase and Jadhav (2010). NADH-DCIP reductase was measured in cell-free extract as per the earlier report (Salokhe and Govindwar 1999). Veratryl alcohol oxidase activity was determined by using veratryl alcohol as a substrate. The reaction mixture contained 1 mM veratryl alcohol in 0.05 M citrate phosphate buffer (pH 3.0), and enzyme in a total volume of 2 ml, which was used for the determination of oxidase activity. Oxidation of the substrate at room temperature was monitored by an absorbance increase at 310 nm due to the formation of veratraldehyde. One unit of enzyme activity was measured as the amount of enzyme that releases 1 μmol product min^{-1} (Tamboli et al. 2011). While riboflavin reductase activity was determined by the earlier reported method of Russ et al. (2000).

All enzyme assays were carried out at 30 °C with reference blanks that contained all components except the enzyme. The protein contents of all the samples were determined using Lowry's method (Lowry et al. 1951). All enzyme assays were run in triplicate, average rates were calculated and one unit of enzyme activity was defined as a change in absorbance unit min^{-1} mg of $protein^{-1}$.

Extraction of DR5B products

Plants were removed from the decolorized dye solutions and it was centrifuged to remove any solid matter if present like root hairs and/or microbial cells. Extraction of products from the supernatant was carried out using equal volume of ethyl acetate and the extract was then evaporated over anhydrous Na_2SO_4 and dried. The solid residue obtained was dissolved in a small volume of HPLC-grade methanol and the sample was then used for analytical studies. This was the test sample. A dye solution of 20 mg l^{-1} was used as abiotic control, three different sets of biotic control contained plantlets of *P. grandiflora*, *P. putida* and, consortium-PP in distilled water, separately. Extraction of the supernatant was carried out by the above-mentioned procedure.

Analytical procedures for the detection of biotransformation products

Decolorization of all the dyes was monitored using UV–Vis spectroscopic analysis (Hitachi U-2800; Hitachi, Tokyo, Japan) using supernatants, whereas biotransformation was monitored using high performance liquid chromatography (HPLC) and Fourier transformed infrared spectroscopy (FTIR). Identification of the metabolites produced was carried out using gas chromatography–mass spectroscopy (GC–MS). HPLC analysis was carried out (Waters model no. 2690; Waters Corp., Milford, MA) on C18 column (symmetry, 4.6 mm × 250 mm) by using methanol with flow rate of 1 ml min^{-1} for 10 min and UV detector at 254 nm. The phytotransformed dye DR5B was characterized by FTIR (Perkin Elmer, Spectrum one B; Shelton, WA) and compared with the control sample. The FTIR analysis was performed in the mid-IR region of 450–4,000 cm^{-1} with 16 scan speed. The samples were mixed with spectroscopically pure KBr in the ratio of 5:95, pellets were fixed in sample holder, and the analyses were carried out. GC–MS analysis of the metabolites was carried out using a Shimadzu 2010 MS Engine, equipped with integrated gas chromatograph with a HP1 column (60 m long, 0.25 mm i.d., nonpolar). Helium was used as carrier gas at a flow rate of 1 ml min^{-1}. The injector temperature was maintained at 280 °C with oven conditions as: 80 °C kept constant for 2 min—increased up to 200 °C with 10 °C min^{-1}, raised up to 280 °C with 20 °C min^{-1} rate. The compounds were identified on the basis of mass spectra and using the National Institute of Structure and Technology (NIST) library.

Phytotoxicity study

A dye solution of 2,000 ppm in distilled water, was prepared with ethyl acetate extracted metabolites from all the three sets (*P. putida*, *P. grandiflora* and consortium-PP) and applied for the toxicity testing on seeds of *Sorghum vulgare and Phaseolus mungo* at room temperature. These two crop plants are commonly cultivated in India. Five ml distilled water was used as a control, the seeds were put in 5 ml solution of dye, and degraded metabolites separately at room temperature. The shoot length (plumule) and root length (radicle) were measured after 7 days and seed germination percentage was calculated.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparisons test. Readings were considered significant when P was ≤ 0.05.

Results and discussion

Identification of bacterial isolate and phylogenetic analysis

Partial sequencing of the 1,374 bp of 16 s rDNA of the bacterial strain and its phylogenetic analysis (Fig. 1)

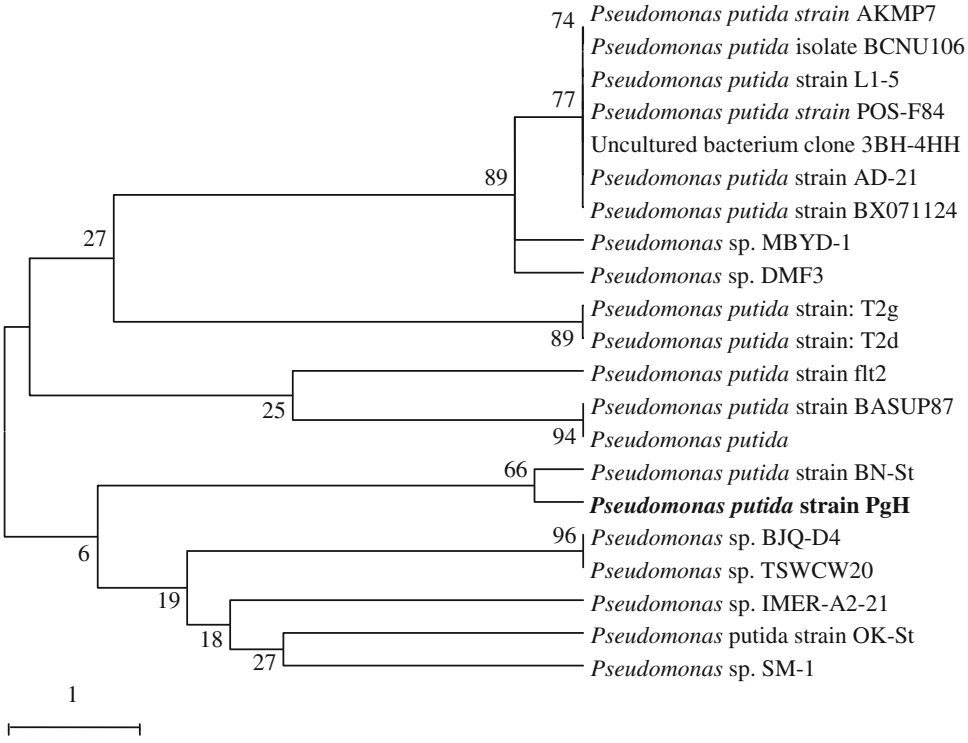

Fig. 1 Phylogenetic analysis of 16 s rDNA sequence of *Pseudomonas putida* stain PgH. The percent numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analyses. The *scale bar* (*1*) indicates the genetic distance

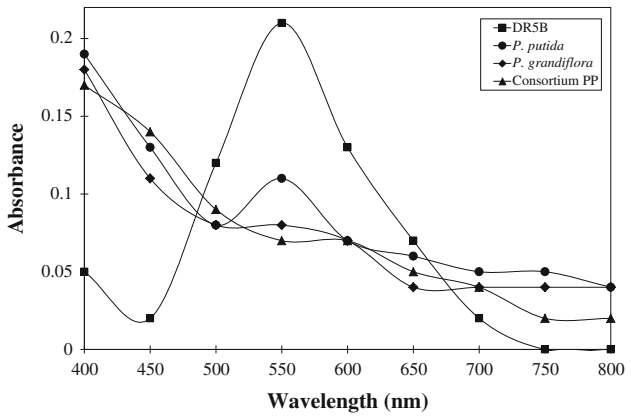

Fig. 2 UV–Vis spectrophotometric analysis of DR5B and metabolites formed by *Pseudomonas putida*, *Portulaca grandiflora* and consortium-PP

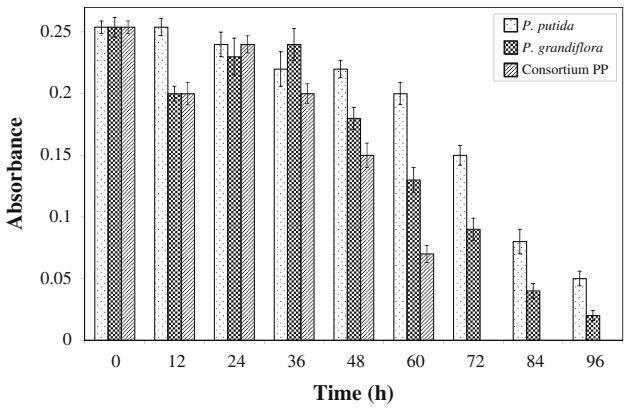

Fig. 3 Decrease in absorbance of DR5B after treatment by *Pseudomonas putida*, *Portulaca grandiflora* and consortium-PP in 96 h

showed that it was closely related to *P. putida* hence the strain was identified as *P. putida* and given the strain name PgH. The obtained sequence was deposited at GenBank under the accession number JF827293.

Decolorization of DR5B by *P. grandiflora*, *P. putida* and consortium-PP

UV–visible scan (400–800 nm) of medium supernatants withdrawn after 96 h of exposure to tissue cultured plants of *P. grandiflora*, *P. putida* and consortium-PP indicated decolorization and decrease in the concentration of the DR5B dye (Fig. 2).

Two tissue cultured plantlets of *P. grandiflora* alone could decolorize 10 ml solution of DR5B up to 92 % within 96 h. One ml log phase growth culture of *P. putida* could decolorize the same volume of dye solution up to 80 % within 96 h, whereas the plant and microbial consortium-PP was found more efficient in decolorization and was able to decolorize the dye up to 100 % within 72 h (Fig. 3). The initial decrease in absorbance in case of plants and the consortium was because of the adsorption of the dye on the roots of *P. grandiflora* at 12 h. This adsorption was found to be removed from the roots later from 24 to 36 h onwards and the dye was degraded subsequently. Similar observation was also made in case of degradation of Navy Blue HE2R dye by *P. grandiflora* (Khandare et al. 2011b). The bacteria could survive on root exudates of the plant and brought about the decolorization of DR5B more efficiently in consortium-PP as compared to individual plant and bacteria. The bacteria exposed to dye solution alone had no nutrient inputs, therefore, the decolorization was inefficient and slower. The in vitro cultures *P. grandiflora* were devoid of any contamination and therefore, the decolorization in these control conditions was only because of the plant. The increased pollutants degradation potential is the result of higher microbial densities and metabolic activities in the rhizosphere due to microbial growth on root exudates and cell debris originating from the plant roots (Weyens et al. 2009). Plant and microbial combinations have been proved to be more effective for the degradation of diesel oils as compared to individual plants of black poplar and associated microbial species (Tesar et al. 2002). Inoculation with a microbial consortium was found to improve phytoremediation of hydrocarbons by *Cyperus laxus* Lam. (Escalante-Espinosa et al. 2005). In this study, consortium-PP was found to be more potent and thus gave more efficient and enhanced decolorization of DR5B than individual *P. putida* and *P. grandiflora* cultures.

Enzymatic analysis

In order to have an additional insight into the decolorization mechanism, the activities of LiP, laccase, veratryl alcohol oxidase, tyrosinase, DCIP reductase and riboflavin reductase were assayed. Significant induction in the activities of LiP (630 %), tyrosinase (438 %), DCIP reductase (283 %) and riboflavin reductase [625 %] enzymes was observed in the roots of *P. grandiflora* during dye decolorization, whereas, enzyme activities of laccase (252 %), veratryl alcohol oxidase (72 %) and DCIP reductase (162 %) were found to be induced in *P. putida*, which are summarized in Table 1. The inductions in the enzyme activities suggest their presumable involvement in the transformation dye into simpler metabolites. The idea behind using this consortium was to have all the enzymes together to degrade the parent dye and therefore, synergistic degradation of DR5B by consortium-PP was observed to be faster and efficient.

Some organic compounds can be directly degraded and completely mineralized by plant and microbial enzymes (Wild et al. 2005). Plant, microbial and even combinatorial

Table 1 Enzyme activities in *P. putida* cells and in the roots of *P. grandiflora* at 0 and 96 h of dye addition

Enzyme	*P. putida* strain PgH		*P. grandiflora* Hook.	
	0th hour	96th hour	0th hour	96th hour
Lignin peroxidase[a]	0.23 ± 0.03	0.33 ± 0.01	0.23 ± 0.01	1.68 ± 0.28*
Veratryl alcohol oxidase[a]	0.46 ± 0.01	0.79 ± 0.01**	0.03 ± 0.02	0.20 ± 0.017
Laccase[a]	0.25 ± 0.01	0.88 ± 0.02*	0.11 ± 0.025	0.08 ± 0.18
DCIP reductase[b]	13.20 ± 0.058	34.58 ± 0.15*	58.5 ± 15.6	223.9 ± 23.20*
Tyrosinase[a]	NA	NA	1.32 ± 0.42	7.10 ± 1.56***
Riboflavin reductase[c]	NA	NA	0.16 ± 0.41	1.16 ± 2.08**

Values are a mean of three experiments ± SEM. Significantly different from control (0 h) at * $P < 0.001$, ** $P < 0.01$ and *** $P < 0.05$ by one-way ANOVA with Tukey–Kramer comparison test

NA no activity

[a] Activity in units min^{-1} mg^{-1}

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

[c] μg of Riboflavin reduced min^{-1} mg $protein^{-1}$

systems for enzymatic decolorization and degradation of azo dyes has significant potential to address the problem of dye containing effluent (Govindwar and Kagalkar 2010). Peroxidases from *Ipomoea palmata* and *Saccharum spontaneum* and *Phragmites australis* have been used for dye decolorization (Carias et al. 2007; Peralta-Zamora et al. 1998; Shaffiqu et al. 2002). LiP, tyrosinase, veratryl alcohol oxidase, DCIP reductase and laccase activity have been shown to be induced in root system of *T. patula*, *B. malcolmii, T. flagelliforme, A. amellus* and *G. pulchella* during the decolorization of DR5B, Brilliant Blue R, Remazol Red and Green HE4B (Kabra et al. 2011a, b; Kagalkar et al. 2009, 2010; Khandare et al. 2011a, b; Patil et al. 2009). The activities of similar enzymes have been observed to be induced in the roots of *Brassica juncea* during the degradation of synthetic mixture of dyes and even textile effluents (Ghodake et al. 2009). Inductions in the activities of all these enzymes during dye degradation have been reported in a number of bacteria (Olukanni et al. 2010). Plants and microorganisms, including bacteria and fungi, contain many similar enzymes for detoxification or transformation of xenobiotics (Arthur et al. 2005). Enzymatic treatments present no risk of biological contaminations and have less impact on the ecosystem.

Analysis of the decolorized product

FTIR spectral comparison between DR5B and its products formed after decolorization by *P. putida*, *P. grandiflora* and consortium-PP within 96 h of dye decolorization experiments confirmed biotransformation of the dye into different metabolites. FTIR spectrum of the dye DR5B (Fig. 4a) shows the presence of different peaks at 1,754.7 cm^{-1} for C=O stretching, at 1,619.3 cm^{-1} for N=N stretching indicating the presence of azo bond in dye structure. Peaks at 1,546.7 and 1,486 cm^{-1} shows N–O stretching, while peaks at 1,286.8, 1,134.7, 1,045.1 cm^{-1} represent S=O stretching indicating the presence of sulfo group in dye structure. All other peaks show C–H deformations. The products of the dye formed after decolorization by *P. putida* (Fig. 4b) shows few peaks of C–H deformation indicating cleavage of the dye molecule. Peak at 2,318.2 cm^{-1} represents NH_3 indicating formation of amines. The peak at 1,703.4 cm^{-1} represents C=O stretching. The peaks at 1,229.5 and 1,023.2 cm^{-1} shows the presence of sulfo groups in the products, but decrease in the number of these peaks indicates loss of sulfo groups. The azo group also seemed to be disappearing from dye. The FTIR spectrum of the products formed after decolorization by *P. grandiflora* (Fig. 4c) showed peaks at 1,669.0 and 1,107.3 cm^{-1} representing C=O, which indicates the appearance of these groups in the products. The peaks at 1,443.5 and 1,298.3 cm^{-1} represent N–O stretching indicating the presence of azo-like groups in the products. The only peak at 1,225.7 cm^{-1} stands for S=O stretching, this also supports the desulfonation of dye molecule by plant enzymes. The products formed after decolorization by consortium-PP (Fig. 4d) showed a peak at 1,451.2 cm^{-1} which shows C–C stretching and the peak at 1,512.3 cm^{-1} represents N–O stretching, the peaks at 1,298.3 and 1,229.5 cm^{-1} represents S=O stretching indicating presence of sulfo groups in the products but decrease in their number indicates more deamination and desulfonation of DR5B. The azo bond was found to be disappearing from the parent dye. All these products formed by *P. putida*, *P. grandiflora* and consortium-PP were further confirmed by GC–MS analysis and supports degradation of DR5B. Significant disappearance of major peaks and formation of new peaks in the FTIR spectra of metabolites obtained after dye degradation suggests the biotransformation of dye. The

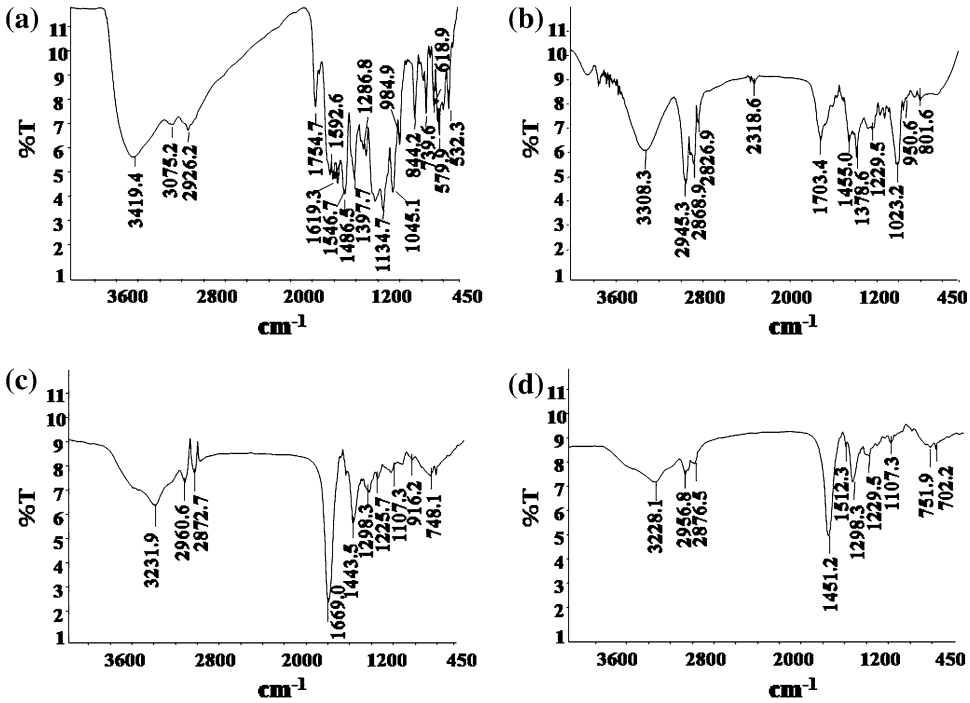

Fig. 4 FTIR analysis of **a** DR5B and its metabolites formed by **b** *Pseudomonas putida*, **c** *Portulaca grandiflora* and **d** consortium-PP

FTIR profiles also helped further to predict the metabolism of the parent dye differentially by *P. putida*, *P. grandiflora* and consortium-PP independently.

HPLC analysis of DR5B showed a peak at the retention time of 1.77 min (Fig. 5a), while the products formed after dye degradation by *P. putida* showed peaks at the retention times 1.21, 1.64, 1.93, 2.75, and 3.08 min (Fig. 5b). Dye metabolites formed by *P. grandiflora* showed peaks at retention times 2.89, 3.15, 3.38 and 4.21 min (Fig. 5c). Products of the dye metabolized by consortium-PP show peaks at retention times 3.02, 3.38, 3.76 min and 7.45 (Fig. 5d). A common peak at 3.38 min can be seen in both plants and the consortium-PP metabolites. Significant disappearance of the peaks found in the dye sample and the appearance of new peaks in the different metabolites samples with new retention times support the biotransformation of parent dye into new chemical species.

GC–MS analysis of metabolites formed after dye degradation also confirmed the degradation of parent dye into different metabolites. Based on different enzyme activities that were induced in the plants and bacteria, pathways of dye degradation and differential fates of metabolism have been proposed which clearly indicate the particular action of enzymes on the complex structure of dye molecule to metabolize into simpler chemical species. Veratryl alcohol oxidase from *P. putida* brings about the cleavage of DR5B into benzamide and intermediate (a). Intermediate (a) is then cleaved into intermediate (b) and 4-hydroxynaphthalene-2-sulfonic acid. Intermediate (b) undergoes asymmetric cleavage to form intermediate (c) and intermediate (d). Intermediate (c) after oxidative cleavage by laccase gives 4-hydroxybenzenesulfonic acid (Fig. 6a). In case of *P. grandiflora* the dye molecule is first cleaved asymmetrically by LiP into intermediates (A) and (B). Intermediate (A) after desulfonation gives 1-(4-diazenylphenyl)-2-phenyldiazene and intermediate (B) after reduction and hydroxylation gives 7-(benzylamino) naphthalene-2-sulfonic acid. This is again asymmetrically cleaved into 7-aminonaphthalene-2-sulfonic acid and methylbenzene (Fig. 6b). In case of consortium-PP, enzymes from plants and bacteria synergistically facilitate the dye metabolism; DR5B is first cleaved into benzenesulfonic acid and intermediate I by LiP and/or veratryl alcohol oxidase to give intermediate I and benzenesulfonic acid. Intermediate I is asymmetrically cleaved into intermediate II and III. Intermediate II undergoes oxidative cleavage by laccase to form 4-diazenylphenol, whereas, intermediate III undergoes asymmetric cleavage to give 6-aminonaphthalen-1-ol and methylbenzene. 6-Aminonaphthalen-1-ol after deamination forms naphthalen-1-ol (Fig. 6c). LiP and veratryl alcohol oxidase are known to cleave the dye molecules symmetrically or asymmetrically (Jadhav et al. 2009; Kalme et al. 2007). Azo linkages have been shown to be oxidatively cleaved by laccase (Chivukula and Renganathan 1995). The proposed pathways show the initial steps involved in the dye metabolism by *P. grandiflora*, *P. putida* and consortium-PP, independently; further, these metabolites could be treated by other soil microflora and the vegetation thriving at the site of pollution. Research aimed at better understanding of the interactive roles among plants roots and

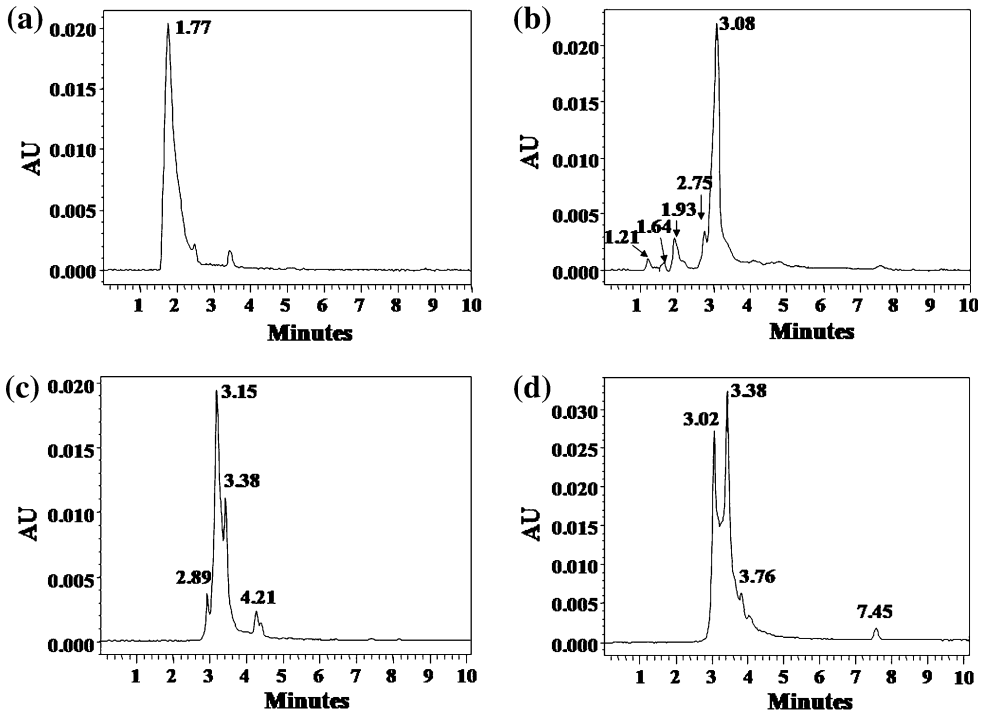

Fig. 5 HPLC profile of **a** DR5B and its metabolites formed by **b** *Pseudomonas putida*, **c** *Portulaca grandiflora* and **d** consortium-PP

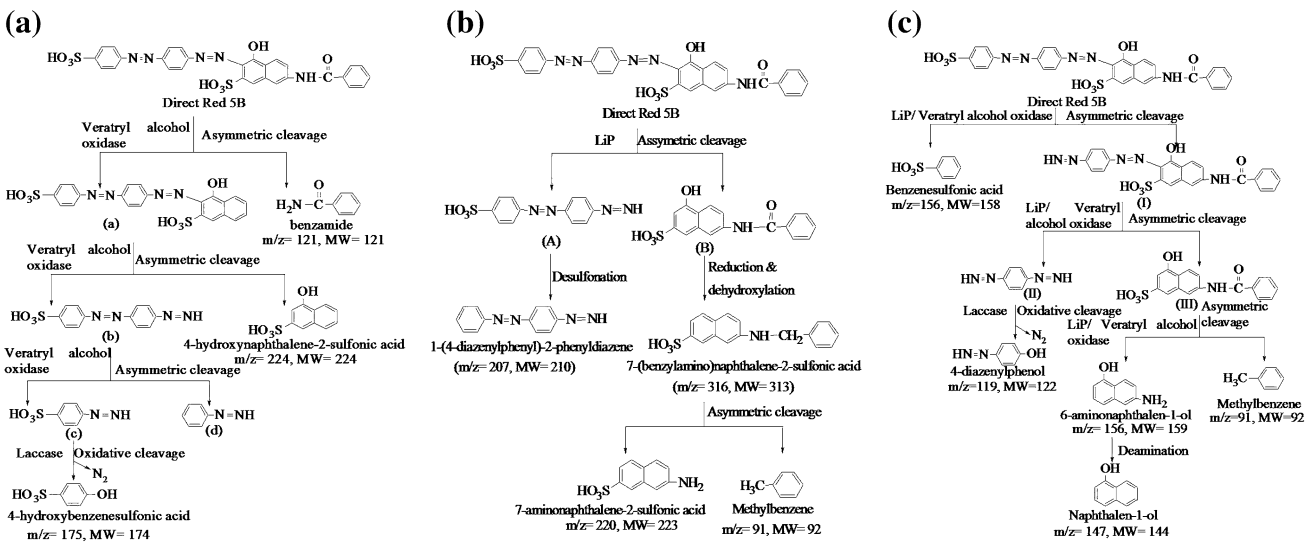

Fig. 6 Pathways of degradation of DR5B by **a** *Pseudomonas putida*, **b** *Portulaca grandiflora* and **c** consortium-PP

microbes could help to utilize their integrative capacity for degradation of toxic dyes (Govindwar and Kagalkar 2010).

Phytotoxicity studies

Textile dyes and effluents have toxic effect on the germination rates and biomass of several plant species. The textile wastewater is used for irrigation of croplands in most of the developing countries like India, where the toxicity analysis of these dyes becomes very important (Kabra et al. 2011b). Table 2 represents the toxicity analysis of the DR5B and its metabolites obtained after decolorization. The dye (2,000 ppm) showed higher inhibitory effect on shoots and root length of plants as compared to metabolite obtained after its degradation. These results indicate the independent detoxification of DR5B by individual plant and bacteria and also by the consortium-PP. Dye solution showed 43, 56 and 60 % germination inhibition in case of *S. vulgare* and 58, 62 and 67 % in case of *P. mungo* as compared to metabolites formed by *P. putida*, *P. grandiflora* and consortium-PP, respectively. Consortium-PP metabolites solution showed

Table 2 Phytotoxicity studies of Direct Red 5B and its degradation products

Parameters	*Sorghum vulgare*					*Phaseolus mungo*				
	(A)	(B)	(C)	(D)	(D)	(A)	(B)	(C)	(D)	(D)
Germination %	100	40	70	90	100	90	30	70	80	90
Plumule (cm)	4.82 ± 0.04	2.26 ± 0.07*	3.48 ± 0.05$	4.23 ± 0.14$	4.63 ± 0.07$	7.57 ± 0.15	3.53 ± 0.09*	6.03 ± 0.18$	6.63 ± 0.18$	7.57 ± 0.12$
Radicle (cm)	3.10 ± 0.09	1.27 ± 0.09*	2.47 ± 0.19$	2.80 ± 0.01$	3.03 ± 0.01$	2.82 ± 0.04	0.75 ± 0.03*	1.43 ± 0.09$	2.27 ± 0.07$	2.67 ± 0.03$

(A) water (B) Direct Red 5B (C) extracted metabolites of dye degraded by *P. putida* strain PgH (D) extracted metabolites of dye degraded by *P. grandiflora* Hook. (E) extracted metabolites of dye degraded by Consortium-PP

Values are a mean of three experiments ± SEM. Root and shoot lengths of plants grown in Direct Red 5B are significantly different from that of plants grown in distilled water by * $P < 0.001$. Root and shoot lengths of plants grown in the extracted metabolites are also significantly different from that of plants grown in Direct Red 5B by $ $P < 0.001$

more germination when compared to metabolites given by individual plant or bacteria showing the formation of harmless metabolites by consortium-PP. Low percentages of germination of *P. mungo* and *S. vulgare* with reduced lengths of the plumule and radicle in dye solution of 2,000 ppm concentration, indicate the toxicity of this dye to these plants, while the metabolites formed after the degradation of the dye by *P. putida*, *P. grandiflora* and consortium-PP were found to be nontoxic and therefore showed more germination percentage and proper development of shoots and roots when compared to toxic dye solution.

Conclusion

Phytoremediation as rightly defined; is the use of plants and their rhizosphere microflora for the degradation and detoxification of environmental pollutants. The strategy of this study was to achieve an enhanced degradation of DR5B by using a naturally occurring consortium which involved *P. grandiflora* and its root-associated bacteria, *P. putida*. The model dye DR5B was decolorized completely and more efficiently by consortium-PP than individual plants and bacteria. There was a notable percentage increase in dye decolorization by consortium-PP. The metabolites formed after degradation of the dye were found to be nontoxic when applied on seeds of *S. vulgare* and *P. mungo* as healthy and proper germination was observed. Thus, it can be concluded that such naturally found synergisms of plants and their root-associated microflora, if used for degradation of textile dyes could lead to an eco-friendly approach for textile wastewater cleanup. The potential of such a naturally existing synergism of plants and microorganisms for practical phytoremediation is yet to be scrutinized. Basic processes of phytoremediation are still largely not clear and hence require further basic and applied research to optimize its field performance. Studies on consortium-PP for the degradation of textile wastewater in real-life conditions are underway.

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