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Decolorization and biodegradation of reactive dyes and dye wastewater by a developed bacterial consortium

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Abstract A bacterial consortium (consortium GR) consisting of *Proteus vulgaris* NCIM-2027 and *Micrococcus glutamicus* NCIM-2168 could rapidly decolorize and degrade commonly-used sulfonated reactive dye Green HE4BD and many other reactive dyes. Consortium GR shows markedly higher decolorization activity than that of the individual strains. The preferable physicochemical parameters were identified to achieve higher dye degradation and decolorization efficiency. The supplementation of cheap co-substrates (e.g., extracts of agricultural wastes) could enhance the decolorization performance of consortium GR. Extent of mineralization was determined with TOC and COD measurements, showing nearly complete mineralization of Green HE4BD by consortium GR (up to 90% TOC and COD reduction) within 24 h. Oxidoreductive enzymes seemed to be involved in fast decolorization/degradation process with the evidence of enzymes induction in the bacterial consortium. Phytotoxicity and microbial toxicity studies confirm that the biodegraded products of Green HE4BD by consortium GR are non-toxic. Consortium GR also shows significant biodegradation and decolorization activities for mixture of reactive dyes as well as the effluent from actual dye manufacturing industry. This confers the possibility of applying consortium GR for the treatment of industrial wastewaters containing dye pollutants.

Keywords Biodegradation · Decolorization · Microbial consortium · Sulfonated reactive dye · Green HE4BD · Phytotoxicity assay · Dye wastewater

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Introduction

A variety of synthetic dyestuffs released by the textile industries pose a threat to the environmental safety. Azo dyes are the largest class of synthetic dyes because of the ease and cost effectiveness of their synthesis and the greatest variety of colors. Azo dyes, containing one or more azo bond (–N=N–), account for 60–70% of all textile dyestuffs used (Khehra et al.

2005). During textile dyeing process, the amount of dye lost in the effluent is dependent up on the class of dye used which varies from 2 to 50% (Sheth and Dave 2009). In case of reactive group of azo dyes, due to low levels of fixation with the textile fiber, up to 50% of the initial dye mass used in the dyeing process remains in the spent dye bath in its hydrolyzed form which no longer has affinity for the fabric and therefore cannot be reused in the dyeing process (Watanapokasin et al. 2008). The presence of reactive dyes in waste streams posing a serious concern in effluent treatment plants due to their color, biorecalcitrance and potential toxicity to pristine ecosystems (Jadhav et al. 2010). Very small quantity of reactive group of azo dyes shows their visual effect with adverse impact in terms of total organic carbon (TOC) and chemical oxygen demand (COD) and thus causing severe environmental problems worldwide (Jirasripongpun et al. 2007). Removal of reactive group of azo dyes from textile effluents is difficult by using typical wastewater treatment processes due to their stability and resistance towards light or oxidizing agents. Typical reactive dyeing process requires higher pH and salt concentrations which further complicate the management of used reactive dye baths (Rys and Zollinger 1989).

Colored textile industrial effluents are one of the most obvious indicators of water pollution. These colored industrial effluents are often contaminated with harmful or poisonous chemical pollutants when withdrawn on the land affects the germination rate of several plants thereby decreasing the soil fertility (Pourbabaee et al. 2006). In addition due to the presence of sediments, suspended and dissolved solids which are repositories for toxic dyes, causing rapid depletion of transparency of water, water quality and dissolved oxygen in the receiving water (Asgher et al. 2007). Some investigators report that azo dyes and their metabolites are toxic, carcinogenic and mutagenic in nature which leads to the formation of tumors, cancers and allergies besides growth inhibition of bacteria, protozoan, algae, plants and different animals (Chen 2002; Gottlieb et al. 2003). Existing physical/chemical methods are not suitable because they usually involve the complicated procedures, are expensive, unable to remove the recalcitrant azo dyes and their organic metabolites completely and generate a significant amount of sludge (Pearce et al. 2003). Due to these bottlenecks of the conventional treatment methods, there is an urgent need to develop more effective and ecofriendly treatment methods for such type of pollutants.

In the current scenario, microbial or enzymatic treatment offers an indispensable, ecofriendly and cost-effective solution towards restoring azo dye polluted ecosystems and could help to reduce the enormous water consumption compared to physicochemical methods. Now a days recent research is focused on the treatment of this pollution source efficiently by applying different biotechnological approaches (Rai et al. 2005).Varieties of microorganisms including bacteria, fungi, yeasts, actinomycetes and algae are capable of degrading azo dyes, among which bacterial cells represent an inexpensive and promising tool for the removal of various azo dyes from textile dye effluents (Dafale et al. 2008). Bacteria capable of dye decolorization, either in pure cultures or in consortia, have been reported (Telke et al. 2008; Kalyani et al. 2009; Chen and Chang 2007; Moosvi et al. 2005, 2007). It has been demonstrated that synergistic metabolic activities of mixed microbial consortium can lead to complete mineralization of azo dyes (Tony et al. 2009). The mechanism of microbial degradation of azo dyes involves the reductive cleavage of azo bonds (–N=N–) with the help of azoreductase under anaerobic conditions resulted into the formation of colorless solutions (Chang et al. 2001). For the reduction of azo dyes, reduction to the anion radical occurs by a fast one-electron transfer reaction, followed by a second, slower electron transfer event to produce the stable dianion (Zimmermann et al. 1982). Thus the functional group of azo dye with higher electronic density might be unfavorable to this second electron transfer to form the dianion, leading to low or no capability for decolorization (Pearce et al. 2003). Due to this reason sulfonated reactive group of azo dyes are normally considered to be more recalcitrant than carboxylated azo dyes. In addition, the rate limiting step during bacterial decolorization of sulfonated azo dyes is the permeation through the bacterial cell membrane (Lourenco et al. 2000).

In the present study, we attempted to use a developed bacterial consortium GR for faster decolorization of various sulfonated reactive azo dyes commonly used in various textiles, paint, garments and allied industries in India. In particular, the potential of consortium GR in removing a toxic, sulfonated reactive dye Green

HE4BD was investigated. To elucidate the decolorization mechanism of Green HE4BD in between individual strains and the microbial consortium by studying the difference in oxidoreductive enzymatic status has been studied. Phytotoxicity and microbial toxicity study was performed to examine whether the degradation products of Green HE4BD by consortium GR have toxic effects. The extent of mineralization of the Green HE4BD as well as mixture of reactive dyes during microbial decolorization was also determined by using TOC and COD measurement. To make the study practically more applicable, finally we investigated the potential of consortium GR for the decolorization and degradation of actual textile effluents.

Materials and methods

Dyes and chemicals

Green HE4BD, Golden Yellow HE4R, Orange 3R were obtained from Manpasand textile industry, Ichalkaranji and Violet 5R, Red ME4BL and Red M2BN were obtained from Shriram Textile Industries, Solapur, India. ABTS [(2,2-azinobis (3-ethylbenzothiazolin-6-sulfonic acid)], NADH were purchased from Sigma Chemical Company (USA). Yeast extract and glucose were obtained from Showa Chemicals. Catechol, *n*-propanol, tartaric acid, and other fine chemicals were obtained from SRL Chemicals, India. The agricultural raw materials such as rice husk and rice straw were collected from local farmers whereas sugarcane bagasse and wood shavings were obtained from local industries of Taiwan. The raw materials were air dried, milled and sieved through a 0.2 mm screen before storing at room temperature prior to use. The textile effluent was obtained from local industry.

Microorganisms and culture conditions

The pure strain of *Proteus vulgaris* (NCIM-2027) and *Micrococcus glutamicus* (NCIM-2168) were obtained from National Chemical Laboratory (NCL, Pune, India). Pure culture was maintained on the nutrient agar slants and stored in test tubes at 4°C and subcultured monthly. The pure cultures of *P. vulgaris* and *M. glutamicus* were grown in 250 ml Erlenmeyer flask, containing 100 ml nutrient broth containing (g l^{-1}): beef extract, 3; peptone, 10; NaCl, 5, pH 6.6 at 37 and 30°C for 24 h, respectively under static condition. The bacterial consortium GR was developed by aseptically transferring the 0.5 ml suspension of 24 h grown culture of each of the individual strains; *P. vulgaris* (PV) and *M. glutamicus* (MG) in 250 ml Erlenmeyer flasks containing 100 ml nutrient broth, pH 6.6 and incubated at 37°C for 24 h under static condition. Individual strains used in this study *P. vulgaris* and *M. glutamicus* were inoculated as 1 ml suspension of 24 h grown culture, respectively, to maintain the same cell count in the pure culture and in the consortium. To study the effect of carbon and nitrogen sources on the decolorization of Green HE4BD, semi-synthetic medium with different carbon and nitrogen sources (1% each) such as glucose, sucrose, peptone, urea, beef extract, lactose, casein and yeast extract were used (Saratale et al. 2009). In addition, to make the process economically feasible, 1 g of rice husk, rice straw, bagasse powder and wood shavings were mixed with 100 ml distilled water individually and autoclaved at 121°C for 20 min. Thereafter, 10 ml extract of each agricultural waste was added into semi-synthetic medium to examine for its effects on the decolorization performance of Green HE4BD by consortium GR.

Decolorization studies

The 24 h grown consortium GR and individual strains (*P. vulgaris* and *M. glutamicus*) cells were incubated with different reactive dyes such as, Green HE4BD, Golden Yellow HE4R, Orange 3R, Violet 5R, Red ME4BL and Red M2BN (Table 1) at concentration, 50 mg l^{-1}, individually and incubated at 37°C under static condition (no aeration and agitation). An aliquot (3 ml) of the culture media was withdrawn at the different time intervals. Aliquot was centrifuged at 10,000 rpm for 15 min to separate cell mass. Supernatant was used to determine the decolorization by measuring the change in absorbance of culture supernatants at the maximum absorption wavelength (λ_{max}) of the respective dyes. Decolorization performance of mixture of different combination of reactive dyes with concentration (50 mg l^{-1} each) by developed consortium GR was studied in 250 ml Erlenmeyer flask containing 100 ml nutrient broth at 37°C under static condition. In addition dye wastewater decolorization study was carried out in the synthetic media (containing 0.5% peptone) with (1:1) proportion.

Establishment of optimum operation conditions

To evaluate the effects of operational and environmental conditions on the decolorization performance by individual (PV and MG) strains and the developed consortium GR, the batch decolorization experiments were carried out (24 h grown cells; dye concentration 50 mg l^{-1}); at different agitation speeds (0 and 150 rpm), temperatures (30–50°C), pH values (5–12), and initial dye concentrations (50–250 mg l^{-1}) at 37°C under static condition. Usually the enzymatic decolorization activity of azo dye decolorizers are strongly inhibited by the presence of oxygen (Chang et al. 2001), the above experiments were performed under the static-incubation conditions (i.e., neither aeration nor agitation), except for the experiments investigating the effect of the agitation on the decolorization. Further, the decolorization of continuous addition of dye aliquots (50 mg l^{-1}) to culture media was also studied by consortium GR in the nutrient broth, under static condition without supplement of additional nutrients. Studies on the effect of various carbon and nitrogen sources were carried out in the synthetic medium (dye concentration, 50 mg l^{-1}) at 37°C, under static condition, by using developed consortium GR. The synthetic medium consisted of (g l^{-1}): Green HE4BD, 0.050; $(NH_4)_2SO_4$, 0.28; NH_4Cl, 0.23; KH_2PO_4, 0.067; $MgSO_4{\cdot}7H_2O$, 0.04; $CaCl_2{\cdot}2H_2O$, 0.022; $FeCl_3{\cdot}6H_2O$, 0.005; yeast extract, 0.2; NaCl, 0.15; $NaHCO_3$, 1.0 and 1 ml per liter of a trace element solution containing (g l^{-1}) $ZnSO_4{\cdot}7H_2O$, 0.01; $MnCl_2{\cdot}4H_2O$, 0.1; $CuSO_4{\cdot}5H_2O$, 0.392; $CoCl_2{\cdot}6H_2O$, 0.248; $NaB_4O_7{\cdot}10H_2O$, 0.177 and $NiCl_2{\cdot}6H_2O$, 0.02. For preculture, 10% inoculum (with an optical density of 1.0 at 620 nm) was applied, and the culture was grown in the nutrient broth for 24 h before it was used for inoculation into the synthetic medium. All decolorization experiments were performed in triplicates. Abiotic controls (without microorganism) were always included.

Preparation of cell free extract

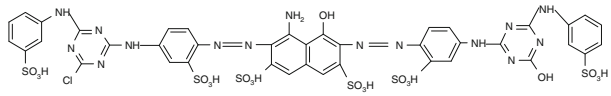
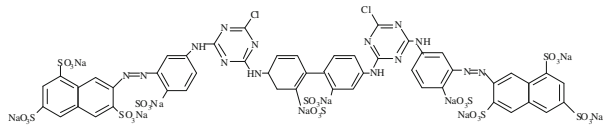
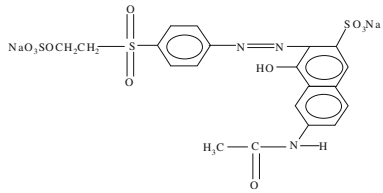
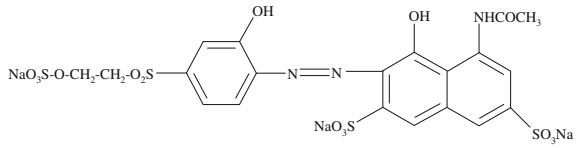
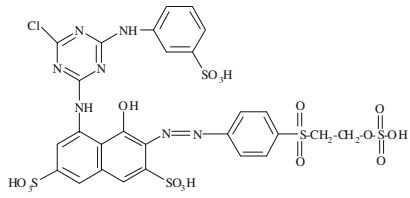
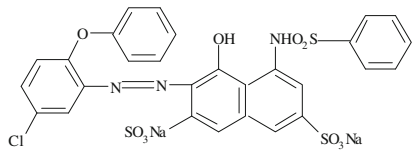
The developed consortium GR and individual organisms (*P. vulgaris* and *M. glutamicus*) cells were grown in 250 ml Erlenmeyer flasks containing 100 ml nutrient broth, pH 6.6, incubated at 30°C (for *M. glutamicus*) and 37°C (for GR and *P. vulgaris*) for 24 h under static condition and harvested by centrifugation at 10,000×*g* for 15 min. The culture supernatant obtained after centrifugation during the harvesting of cell biomass was directly used as a source of extracellular enzymes in order to determine enzymatic status directly contact with target. The harvested cells were suspended in potassium phosphate buffer (50 mM, pH 7.4) for sonication (Sonics-vibracell ultrasonic processor), keeping sonifier output at 40 amp and giving eight strokes each of 40 s with a 2 min interval at 4°C. These extracts were used as an intracellular enzyme source without centrifugation. Similar procedures were followed to the cells obtained after 24 h incubation with consortium GR (complete decolorization) and individual strains. Protein content was estimated by the Biuret method. Protein concentration of cell free extract was kept constant (2.0 mg ml^{-1}) for the enzymatic studies.

Enzyme assays

The intracellular and extracellular activities of lignin peroxidase and laccase were assayed spectrophotometrically. Lignin peroxidase activity was determined by monitoring the formed 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 (Telke et al. 2008). Laccase activity was determined in a reaction mixture of 2 ml containing ABTS (10%) in 0.1 M acetate buffer (pH 4.9) and measured as an increase in the optical density at 420 nm (Hatvani and Mecs 2001). These enzyme assays were carried out at 37°C, where reference blanks containing all components except the assayed enzyme. One unit of enzyme activity was defined as a change in absorbance unit per minute per mg protein of the enzyme.

The azoreductase activity was assayed by the modified version of the earlier method (Kalyani et al. 2009), in which the decrease in the Methyl Red concentration (measured at 440 nm) was monitored in a 2.2 ml reaction mixture containing 152 μM Methyl Red, 50 mM sodium phosphate buffer (pH 5.5) and 20 μM NADH. Enzyme activity was calculated by using molar extinction coefficient of 42.8 mM cm^{-1} of Methyl Red. Riboflavin reductase NAD(P)H: Flavin oxidoreductase was measured by the method reported by Fontecave et al. (1987) with some modification. In this aerobic assay, the flavin reductase catalyzes the reduction of riboflavin, and the reduced riboflavin is immediately reoxidized by oxygen. Cell extract was added to a solution (final volume, 1 ml) containing

Table 1 Chemical structures of the industrial reactive dyes used in this study

Name of reactive dye	Chemical structure
Green HE4BD (GHE) (C.I. Reactive Green 19A) CAS No: 61931-49-5	
Golden Yellow HE4R (GYHE4R) (C.I. Reactive Golden Yellow 84) CAS No: 61951-85-7	
Orange 3R (O3R) (C.I. Reactive Orange16) CAS No: 12225-88-6	
Violet 5R (V5R) (C.I. Reactive Violet 5) CAS No: 12226-38-9	
Red ME4BL (RME4BL) (C.I. Reactive Red 195) CAS No: 93050-79-4	
Red M2BN (RM2BN) (C.I. Acid Red 249) CAS No: 6416-66-6	

100 μM of Tris–HCl (pH 7.5), 25 μmol of NADPH and 0.003 unit of riboflavin. The decrease in absorbance at 340 nm was measured spectrophotometrically. Reaction rates were calculated by using a molar extinction coefficient of 6.3 mM cm^{-1}. One unit of enzyme activity was defined as a microgram of riboflavin reduced per minute per mg of protein. NADH–DCIP (dichlorophenol indophenol) reductase activity was assayed by following the procedures reported by Bhosale et al. (2006). The assay mixture contained 50 μM DCIP, 50 μM NADH in 50 mM potassium phosphate buffer (pH 7.4) and 0.1 ml of enzyme solution in a total volume of 5.0 ml. The DCIP reduction was monitored at 595 nm. One unit of enzyme activity was defined as a microgram of DCIP reduced per min per mg of protein. All enzyme assays were conducted in triplicate and the average rates were calculated to represent the enzyme activity.

Analytical methods

Decolorization was quantitatively analyzed using UV–Visible spectrophotometer (Hitachi U-2800) whereas biodegradation was monitored by thin layer

chromatography (TLC) and high performance liquid chromatography (HPLC). During UV–Visible spectral analysis, changes in absorption spectrum in the decolorized medium (400–800 nm) were recorded in comparison with the results from the control runs. The percentage of decolorization was calculated as follows (Saratale et al. 2006),

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

The average decolorization rate (μg h^{-1}) was calculated as follows,

$$\text{Average decolorization rate} = \frac{C \times \%D \times 1000}{100 \times t}$$

where C is the initial concentration of dye (mg l^{-1}) and %D is the dye decolorization (%) after time t (h).

After complete decolorization (pH 6.6) at 37°C by consortium GR (after 24 h for individual organisms), culture broth was centrifuged at 10,000×*g* for 15 min and equal volume of ethyl acetate was used to extract the metabolites from clear supernatant. The extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness in a rotary evaporator. The crystals obtained were dissolved in small volume of HPLC grade methanol and used for analysis. TLC analysis was carried out on silica gel sheet (Sigma Aldrich) using mobile phase composed of methanol, ethyl acetate, *n*-propanol, water, and acetic acid (1:2:3:1:0.2 v/v). The samples of 0 h (control) and after 24 h incubation with consortium GR (complete decolorization) and individual strains were analyzed and the spots were developed using an iodine chamber. HPLC analysis (Waters model no. 2690) was carried out on C_{18} column (symmetry, 4.6 × 250 mm) with methanol as mobile phase at flow rate of 1.0 ml min^{-1} for 10 min at 470 nm. The analysis results were compared with that of the control dye.

The degree of biodegradation (mineralization) of Green HE4BD reduction in total organic carbon (TOC) of the culture before and after decolorization was measured by using Liqui TOC II apparatus (Elementar Analysensysteme GmbH Company, Germany) equipped with an IR detector. The detailed procedure for TOC analyses was described in our recent work (Saratale et al. 2009). In addition reduction in the chemical oxygen demand (COD) after 24 h incubation with consortium GR (complete decolorization) and individual strains was determined (APHA–AWWA–WEF 1998). The nutrient medium was used as blank and similar condition was used for test. Similar procedure was employed for the mixture of reactive dyes and dye wastewater study. The COD was calculated as follows,

$$\text{COD}\ \left(\text{mg}\ l^{-1}\right) = \frac{(A - B) \times N \times 1000 \times 8}{\text{Volume of sample (ml)}}$$

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

For the mixture of dyes and dye wastewater, the true color level independent of hue was measured using the American Dye Manufacturers Institute (ADMI 3WL) tristimulus filter method (APHA–AWWA–WEF 1998 Chen et al. 2003). ADMI removal percent (%) is the ratio between the removal of ADMI value at any contact time and the ADMI value at initial concentration and calculated as follows,

$$\text{ADMI removal ratio}\ (\%) = \frac{\text{Initial ADMI}_{(0h)} - \text{Observed ADMI}_{(t)}}{\text{Initial ADMI}_{(0h)}} \times 100\%$$

where $ADMI_{(0h)}$ and $ADMI_{(t)}$ are the initial ADMI value (at 0 h) and the ADMI value after a particular reaction time (t), respectively.

Growth of microorganisms in dye containing medium was measured by the gravimetric method after drying at 80°C until constant weight. Standard methods (APHA–AWWA–WEF 1998) were used to determine the biomass concentration.

Total nitrogen and total phosphorous measurement

Total nitrogen (T-N), total phosphorous (T-P) of the dye wastewater before and after treatment with consortium-GR were measured using the C-mac test kit (USEPA approved method), following the instructions of the manufacturer. Total nitrogen and total phosphorous were calculated in terms of mg l^{-1} T-N and mg l^{-1} T-P, respectively.

Microbial and phytotoxicity studies

The microbial toxicity of untreated and treated dye (ethyl acetate extracted and dried) in relation to soil

microbial flora; *Rhizobium radiobacter*, *Acinetobacter* sp. *Pseudomonas desmolyticum* (NCIM-2112), *Cellulomonas biazotea* (NCIM-2550), isolated *Escherichia coli* DH5α, *Micrococcus glutamicus* (NCIM-2168) and *Proteus vulgaris* (NCIM-2027) were studied. All microorganisms were inoculated on the nutrient agar. Four wells of 8.0 mm diameter were made on the nutrient media containing plates. Two wells were filled with 0.1 ml of untreated dye and other two with 0.1 ml of ethyl acetate extracted and dried dye metabolites at concentration of 100 and 300 ppm, respectively. The plates were incubated at 30 ± 2°C for 48 h. Zone size of inhibition (diameter in cm) surrounding the well represented the index of toxicity.

Phytotoxicity tests were performed in order to assess the toxicity of the untreated and treated dye (by consortium GR). The ethyl acetate extracted products of Green HE4BD degradation were dried and dissolved in sterile distilled water to make a final concentration of 300 ppm for phytotoxicity studies. The phytotoxicity study was carried out (at room temperature) on *Phaseolus mungo* important in the Indian agriculture (Pourbabaee et al. 2006; Kalyani et al. 2009). Ten seeds of *Phaseolus mungo* under study were regularly supplied with distilled water as a control. Green HE4BD (300 ppm) and their degradation products (300 ppm) for 7 days. Volumes for supplement of water, dye and degraded dye metabolites were kept same, i.e. 10 ml per day for seeds taken in watch glasses. Toxicity effect was measured in terms of percent germination, lengths of plumule and radical of the plant after 7 days.

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

Screening of various reactive dyes for decolorization

The effectiveness of microbial decolorization depends on the survival, adaptability and the activity of enzymes produced by microorganisms present in the consortium. The decolorization capacity of a microorganism can be tested by examining its potential to degrade various dyes (Senan and Abraham 2004). Thus, the ability of the pure culture as well as the developed consortium were tested to decolorize various industrial reactive dyes, such as Green HE4BD, Golden Yellow HE4R, Orange 3R, Violet 5R, Red ME4BL and Red M2BN (at a concentration of 50 mg l^{-1} each) (Table 1). The decolorization was conducted with the dye-supplemented nutrient broth at 37°C under static condition. As shown in Table 2, the average decolorization rate of the consortium GR was significantly higher than that observed for individual strains. This might be attributed to the synergistic reaction of single strains in the consortium GR (Chen and Chang 2007). Similar findings were reported by Khehra et al. (2005) who observed a higher decolorization rate of AR-88 dye by consortium HM-4 (combinations of four selected bacterial isolates) when compared with its constituent pure strains. The highly toxic and recalcitrant sulfonated reactive azo dye (Green HE4BD) could be decolorized within 24 h by consortium GR under static condition at pH 8, 37°C and dye concentration of 50 mg l^{-1}. The maximum average decolorization rate was 2083 μg h^{-1} (Table 2). In contrast, under the identical conditions, *P. vulgaris* and *M. glutamicus* required more time (70 and 42 h, respectively) to achieve complete decolorization and exhibit lower decolorization rates of 694 and 1190 μg h^{-1}, respectively (Table 2). The slower decolorization with individual strains may be due to structural differences, higher molecular weight and presence of inhibitory groups likes $–SO_3H$ and $–SO_3Na$ in the dyes (Moosvi et al. 2005; Junnarkar et al. 2006). The advantage of mixed culture is apparent as some strains can collectively carry out such biodegradation tasks that no individual pure strain can achieve due to synergistic metabolic activities of microbial community. In microbial consortium the individual strains may attack the dye molecule at different positions or may utilize metabolites produced by the co-existing strains for further decomposition (Tony et al. 2009; Moosvi et al. 2007; Asgher et al. 2007; Jadhav et al. 2008). Apart from decolorization by live cultures, low or negligible decolorization (about 3 to 5%) of Green HE4BD were observed by autoclaved cells of an individual as well as consortium GR indicating that microbial decolorization was primarily

Table 2 Decolorization of various reactive groups of azo dyes (50 mg l^{-1} each) by individual strains and consortium GR at 37°C under static condition

Name of reactive dyes	Chemical class	λ_{max} (nm)	Average decolorization rate (μg h^{-1})		
			PV	MG	GR
Green HE4BD	Reactive diazo	540	694	1190	2083
Golden Yellow HE4R	Reactive diazo	530	943	856	1889
Orange 3R	Reactive monoazo	416	932	1068	1832
Violet 5R	Reactive monoazo	530	863	1158	1864
Red ME4BL	Reactive monoazo	540	912	1024	1466
Red M2BN	Reactive monoazo	550	872	986	1287

PV Proteus vulgaris NCIM-2027, *MG Micrococcus glutamicus* NCIM-2168, *GR* developed consortium

due to biological degradation rather than adsorption. In addition, the medium pH before (initial pH; 6.6) and after decolorization (6.6–6.9) was quite similar, confirming that the decolorization was indeed due to bacterial action rather than pH variation in the medium.

Optimization of physicochemical conditions

The effects of various physicochemical conditions (agitation, temperature, pH, and dye concentration) on the decolorization performance of Green HE4BD by using the consortium GR were studied in detail. Better growth of consortium GR and individual strains were observed under static condition when compared with under shaking condition (data not shown). A complete decolorization of Green HE4BD was obtained under static condition after certain incubation period with an individual as well as the consortium GR, but only 30–35% performance was achieved under shaking condition (data not shown). These results suggest that facultative anaerobic condition was favorable for the decolorization, which could be due to involvement of azoreductase for the decolorization of azo dyes. It was reported that reductive cleavage of azo bonds (–N=N–) by azoreductase is normally inhibited by the presence of oxygen primarily due to competition in the oxidation of reduced electron carriers like NADH with either oxygen or azo groups as electron receptor (Chang et al. 2001; Sheth and Dave 2009). Hence, in this study static conditions were adopted to investigate the bacterial decolorization.

When the decolorization of Green HE4BD (50 mg l^{-1}) was investigated at various temperatures (30–50°C), complete decolorization was observed at (37°C) within 24 h incubation by consortium GR. Further increase in the temperature resulted in a marginal reduction in the decolorization activity (Table 3). Over the pH range of 5–8, the consortium GR achieved the highest decolorization activity for Green HE4BD at pH 8, while the decolorization rate decreased at lower pH (5–7) and higher pH (10–12) (Table 3). It is thought that the pH effect may be more likely related to the transport of dye molecules across the cell membrane, which was considered as the rate-limiting step for the decolorization (Lourenco et al. 2000). Similar results were observed in the decolorization of Reactive Violet 5R by developed consortium JW2, which is a combination of *Paenibacillus polymyxa*, *Micrococcus luteus* and *Micrococcus* sp. (Moosvi et al. 2007).

The decolorization performance of Green HE4BD by consortium GR was also studied at various increasing dye concentration (50, 100, 150, 200 and 250 mg l^{-1}). The consortium GR has the ability to degrade higher dye concentration (200 and 250 mg l^{-1}) of Green HE4BD with moderate decolorization rates (Table 3). In contrast, at higher dye concentration 200 and 250 mg l^{-1}, the pure cultures show poor decolorization performance (PV; 20, 15% and MG 22, 18%) after 4 days, respectively (data not shown). Beyond 250 mg l^{-1} dye concentration, gradual decrease in the decolorization rate by consortium GR was observed probably due to the toxic effect of dyes to the individual bacteria and/or inadequate biomass concentration for the uptake of higher concentrations of dye, as well as blockage of active sites of azoreductase by dye molecule with different structure (Tony et al. 2009).

Table 3 Decolorization performance and incubation time of Green HE4BD by using developed consortium GR at different temperatures, pH and dye concentrations

Parameters	Operational conditions						
	pH[a]						
	5	6	7	8	9	10	12
Decolorization (%)	54	76	CD	CD	60	30	ND
ADR (mg h^{-1})	0.56	1.27	1.79	2.1	0.63	0.25	–
Time (h)	48	30	28	24	48	60	72
	Temperature (°C)[b]						
	30	37	40	45	50		
Decolorization (%)	85	CD	96	85	60		
ADR (mg h^{-1})	1.6	2.1	1.67	1.19	0.63		
Time (h)	36	24	30	36	48		
	Dye concentration (mg l^{-1})[c]						
	50	100	150	200	250		
Decolorization (%)	CD	CD	94	92	CD		
ADR (mg h^{-1})	1.56	1.25	0.79	0.64	1.56		
Time (h)	32	40	60	72	32		

ADR Average decolorization rate (mg h^{-1}), *CD* complete decolorization, *ND* no decolorization

[a] Effect of initial pH (at 37°C, static condition, dye concentration 50 mg l^{-1})

[b] Effect of temperature (at pH 8.0, static condition, dye concentration 50 mg l^{-1})

[c] Effect of initial dye concentration (at 37°C, pH 8.0, static condition)

Effect of co-substrates supplementation on the decolorization

Since the dyes are deficient in carbon source, it seems necessary to supplement additional carbon or nitrogen source to assist biodegradation of dyes by the bacterial consortium (Senan and Abraham 2004). Therefore, in this study, the decolorization performance of Green HE4BD by consortium GR was examined in the presence of an additional carbon and nitrogen sources (1%) and 10 ml extract of agricultural by-products (1%) to obtain efficient and faster decolorization and biodegradation. Table 4 shows that percent decolorization was maximum with purified substrate peptone (100%) and beef extract (100%) within 24 h, whereas in the presence of 10 ml extract of agricultural waste

Table 4 Effect of supplementation of different carbon and nitrogen sources on the decolorization of Green HE4BD by consortium GR

Medium	Decolorization (%)	Incubation time (h)
SM	ND	48
SM + Sucrose	48	48
SM + Glucose	56	48
SM + Peptone	100	24
SM + Urea	90	48
SM + Beef extract	100	24
SM + Casein	ND	48
SM + Lactose	86	48
SM + Yeast extract	68	48
SM + Rice husk[a]	100	30
SM + Rice straw[a]	100	36
SM + Bagasse[a]	30	48
SM + Wood shavings[a]	25	48

SM synthetic medium, *ND* no decolorization

[a] 10 ml extract of each agricultural residue (1%). The extract was prepared by mixing 1 g of rice husk, rice straw, bagasse powder and wood shavings with 100 ml distilled water individually and the mixtures were autoclaved at 121°C for 20 min

rice husk and rice straw, complete decolorization was achieved within 30 and 36 h, respectively. In contrast, lower decolorization efficiency was observed with other supplements of carbon and nitrogen sources within 48 h (Table 4). Jadhav et al. (2010) pointed out that the presence of various carbon and nitrogen sources in medium might have stimulatory or inhibitory effect on enzyme systems involved in the decolorization. Similarly in the presence of carbon and nitrogen sources variation in time required for the decolorization of Green HE4BD by consortium GR was observed. It is thought that in case of complex substrates such as extract of rice husk and rice straw, consortium GR could convert and degrade them, producing some volatile organic acids or alcohols (such as acetic acid and ethanol), which acts as electron donors and apparently induces the reductive cleavage of azo bonds (Yoo et al. 2000; Watanapokasin et al. 2008). The use of agricultural by-products (rice husk and rice straw) instead of pure substrates (peptone and beef extract) for the enhancement of the decolorization of Green HE4BD becomes an ecofriendly and economically feasible process. Utilizing agricultural

lignocellulosic waste (about 1.3×10^{10} metric tons per year) as a supplement to assist or stimulate the degradation of industrial effluents could act as a promising technology and may resolve the problem of the disposal of agro-residues (Saratale et al. 2010).

Enzyme analysis

Bioremediation is the microbial clean-up approach. Microbes can acclimatize themselves to toxic wastes and new resistant strains develop naturally, which can transform various toxic chemicals to less harmful forms. A major mechanism behind biodegradation of different recalcitrant compounds in microbial system is driven by the biotransformation enzymes (Saratale et al. 2007; Bhosale et al. 2006). As a result, those enzymes could be indicators reflecting the performance of biotransformation/biodegradation of the target pollutants. The mixed cultures only provide an average macroscopic view of what is happening in the system and results are not easily reproduced, making thorough and effective interpretation difficult. To get additional insight into the decolorization mechanism, oxidative (lignin peroxidase, laccase) and reductive; (NADH–DCIP reductase, riboflavin reductase, azoreductase) enzyme activities were monitored after 24 h incubation in consortium GR (complete decolorization), *P. vulgaris* and *M. glutamicus* at intracellular and extracellular locations. All enzyme activities were observed at intracellular location instead of lignin peroxidase in consortium GR and individual strains. The results showed significant induction in the extracellular lignin peroxidase (219%), riboflavin reductase (465%), azoreductase (439%) and intracellular lignin peroxidase (487%) by consortium GR relative to that obtained from pure culture of *P. vulgaris* and *M. glutamicus*. Likewise, there was moderate induction in oxidative laccase (139 and 177%) and reductive NADH–DCIP reductase (156 and 151%) by consortium GR compared to individual strains (Table 5). In contrast, slight induction in intracellular lignin peroxidase (139%) and moderate induction in riboflavin reductase (198%), and azoreductase (181%) enzyme activity by consortium GR relative to *M. glutamicus* was observed (Table 5). The role of oxidoreductive enzymes in the decolorization of sulfonated reactive azo dyes have been characterized in *Rhizobium radiobacter* MTCC 8161 (on Reactive Red 141), and *Pseudomonas* sp. SUK1 (on Reactive Red 2) (Kalyani et al. 2009; Telke et al. 2008). The higher induction of oxidoreductive enzymes in consortium might be due to synergistic effect of both microorganisms. This enzymatic mechanism leads to the decrease in time required for the decolorization of Green HE4BD by the consortium GR as compare to that of an individual organism (Table 5).

Evaluation of decolorization and biodegradation of Green HE4BD

To disclose the possible mechanism of the dye decolorization, we analyzed the products of biotransformation of Green HE4BD by UV–Visible spectroscopy,

Table 5 Oxidoreductive enzyme activity profiles after 24 h incubation in Green HE4BD-containing medium with consortium GR (complete decolorization), *Proteus vulgaris* NCIM-2027 and *Micrococcus glutamicus* NCIM-2168

Enzymes	*Proteus vulgaris*	*Micrococcus glutamicus*	Consortium GR
Lignin peroxidase (intracellular)[a]	0.056 ± 0.009	0.016* ± 0.004	0.078 ± 0.005
Lignin peroxidase (extracellular)[a]	0.081 ± 0.01	0.141 ± 0.04	0.178 ± 0.04
Laccase[a]	0.046 ± 0.008	0.036 ± 0.004	0.064 ± 0.01
Riboflavin reductase[b]	6.7 ± 1.23	15.72 ± 1.24	31.16* ± 4.95
NADH-DCIP reductase[c]	19.01 ± 0.51	19.63 ± 0.71	29.66** ± 2.12
Azoreductase[d]	2.76 ± 0.78	6.67* ± 0.20	12.12*** ± 0.95

Values are mean of three experiments, SEM (±), significantly different from the *Proteus vulgaris* at * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ and by one-way ANOVA with Tukey–Kramer Multiple Comparisons Test

[a] U min^{-1} mg $protein^{-1}$

[b] μg of riboflavin reduced mg^{-1} min^{-1}

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

[d] μg Methyl Red reduced min^{-1} mg $protein^{-1}$

TLC, and HPLC. UV–Visible spectral analysis (400–800 nm) of the Green HE4BD showed maximum absorbance (λ_{max}) at 640 nm and decrease in the absorbance of samples withdrawn after decolorization by individual bacterial strains and consortium GR (Fig. 1). Evidence for the removal of the dye can be observed with absorbance at λ_{max} being virtually zero after 24 h and an increase in absorbance towards UV region. These results indicate that the color removal by individual strains and its consortium GR may be largely attributed to the biodegradation. TLC analysis of the extracted metabolites after 24 h of incubation in the consortium GR (complete decolorization), *P. vulgaris* and *M. glutamicus* displayed the appearance of two spots in the pure cultures, whereas three spots in consortium GR with the R_f values of PV (0.78, 0.45), MG (0.89, 0.55) and consortium GR (0.90, 0.77, 0.60) (data not shown). TLC analysis indicates formation of compounds other than the original dye, thereby suggesting the biodegradation of Green HE4BD. The TLC result also indicates the formation of different metabolites in consortium GR when compared to the pure cultures. HPLC elution profile shows the presence of new peak with marked decrease in intensity at a different retention time when compared to control (1.735 min) (Fig. 2a) and consortium GR (major peaks at 2.876 and 2.279 min and minor peaks at 2.657, 3.727 and 3.989 min) (Fig. 2b). The role of oxidoreductive enzymes in the biodegradation of sulfonated diazo reactive dyes; Navy Blue HE2R (Reactive Blue 172) by an isolated *Exiguobacterium* sp. RD3, Reactive Red 141

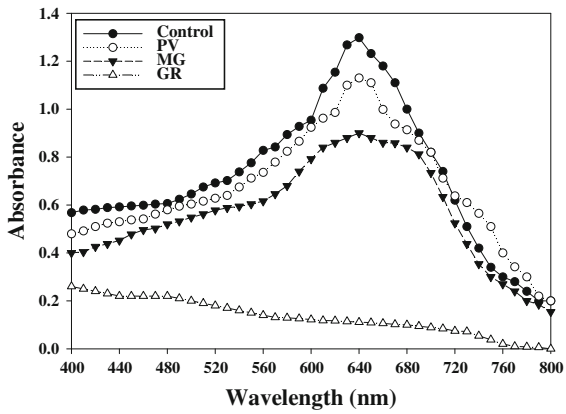


Fig. 1 UV–Visible spectral scans of Green HE4BD (50 mg l^{-1}) after 24 h decolorization by consortium GR (complete decolorization), *Proteus vulgaris* NCIM-2027 and *Micrococcus glutamicus* NCIM-2168

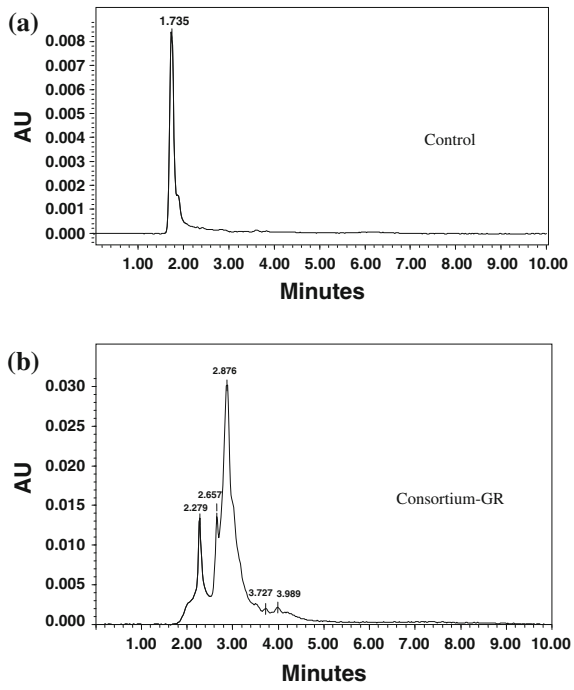


Fig. 2 HPLC chromatogram of **a** control Green HE4BD, **b** metabolites formed by consortium GR after complete decolorization (24 h incubation)

by isolated *Rhizobium radiobacter* MTCC 8161 and Reactive Red 2 by isolated *Pesudomonas* sp. SUK1 was reported (Dhanve et al. 2008; Telke et al. 2008; Kalyani et al. 2009). In those studies, the formation of naphthalene moiety as end products were observed which are nontoxic in nature. Similarly, in this study analytical studies confirmed the biodegradation of Green HE4BD and we supposed that the formation of smaller molecular weight intermediates (naphthalene moieties) are formed by the consecutive action of oxidoreductive enzymes present in consortium GR which may be present in a small amount in the decolorized solution.

To evaluate the level of decolorization and biodegradation of Green HE4BD by individual strain as well as by consortium GR, we have determined the percentage of mineralization (represented by TOC and COD removal ratio) by measuring the initial and final organic content. The consortium GR shows nearly complete decolorization of Green HE4BD accompanied with significant TOC reduction (84%) within 24 h. In contrast, using pure cultures resulted in a lower TOC removal ratio of the Green HE4BD as only 38 and 52% was achieved by PV and MG, respectively (Fig. 3). The significant removal of COD

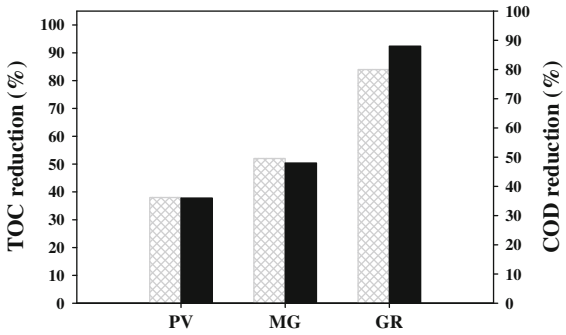


Fig. 3 The percentage of TOC and COD reduction of Green HE4BD by individual strains (PV, *Proteus vulgaris* NCIM-2027; MG, *Micrococcus glutamicus* NCIM-2168) and developed consortium GR after 24 h incubation

content was observed in consortium GR (88% COD removal), much higher than that of the pure culture (PV; 36% and MG; 48%) after 24 h incubation (Fig. 3). Our TOC results showed a good potential compared to the combination of ozonation and microbial treatment using isolated *Pandoraea* sp. EBR-01 for various reactive dyes (Kurosumi et al. 2008), which obtained a TOC removal of up to 70–80%. Moreover, the COD removal efficiency is better than that reported earlier (Dhanve et al. 2008; Kalyani et al. 2009), as a COD reduction of 55.55 and 52% was observed for Reactive Blue 172 by *Exiguobacterium* sp. RD3 and Reactive Red 2 by *Pseudomonas* sp. SUK1, respectively.

Decolorization of mixture of various reactive dyes and dye wastewater

The economic removal of the polluting dyes is an important issue on industrial effluent discharge. In India, an average mill discharges about 1.5 million litres of dye-contaminated effluent per day, which leads to chronic and acute toxicity (Sandhya et al. 2005). Since many textile plants have a rural location and municipal treatment-cost payments are high, it may be economical to build a waste treatment plant on site. Dyes of different structures are often used in the textile processing industry and therefore the effluents from the industry are markedly variable in composition. The composition of textile effluent consists of a mixture of many synthetic dyes and the effluent characteristics (such as pH, dissolved oxygen, organic, and inorganic chemical content etc.) depend greatly upon the textile processing (Chen et al. 2003). Thus, the microbial population used in the treatment process for removing color from those effluents must have the capability of decolorizing a mixture of different dyes. By considering this perspective, the decolorization performance of the developed consortium GR on mixture of reactive dyes was examined. The dye mixtures tested were: Ist set (Green HE4BD, Golden Yellow HE4R, Orange 3R); IInd set (Violet 5R, Red ME4BL, Red M2BN), and IIIrd set containing mixture of all six reactive dyes each at a concentration of 50 mg l^{-1} in batch culture at 37°C under static condition. The results show that consortium GR could achieve high color removal performance for Ist, IInd and IIIrd set, giving a 86, 92 and 74% decolorization in terms of ADMI removal ratio within 72 h (Fig. 4; Table 6). In addition to color removal, consortium GR shows substantial reduction in TOC and COD for Ist set (72 and 69%), IInd set (78 and 74%), and for IIIrd set (68 and 62%), indicating mineralization of dye mixture (Fig. 5a, b). Literature shows that developed consortium SKB-II displayed up to 57% decolorization of a mixture of four dyes (Congo Red, Bordeaux, Ranocid Fast Blue and Blue BCC) at a concentration of 2.5 mg l^{-1} for each dye after 120 h incubation (Tony et al. 2009).

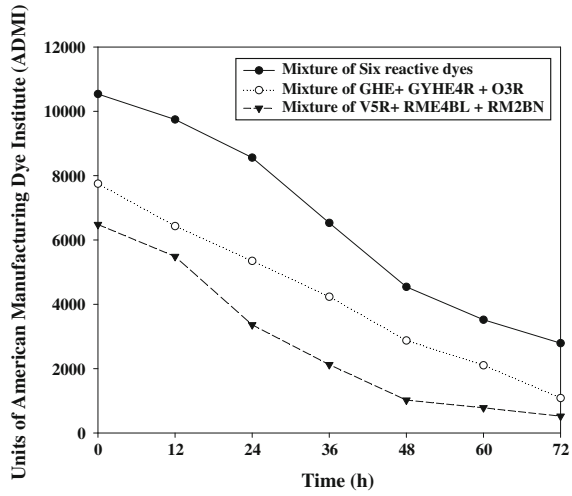


Fig. 4 Time-course profiles of color removal (in terms of ADMI removal ratio) of two different mixtures of three reactive dyes (namely, Green HE4BD/Golden Yellow HE4R/Orange 3R and Violet 5R/Red ME4BL/Red M2BN; 50 mg l^{-1} each), as well as a mixture of all six reactive dyes (50 mg l^{-1} each) by consortium GR

Table 6 Decolorization performance of mixture of various reactive groups of azo dyes (50 mg l^{-1} each) by consortium GR at 37°C under static condition

Name of reactive dyes	Initial ADMI value	Final ADMI value	Decolorization (%)	Time (h)
Green HE4BD + Golden Yellow HE4R + Orange 3R	7750	1085	86	72
Violet 5R + Red ME4BL + Red M2BN	6480	519	92	72
Mixture of all above six dyes[a]	10,540	2792	74	72

ADMI American dye manufacturing dye institute

[a] Concentration of dye 50 mg l^{-1} each

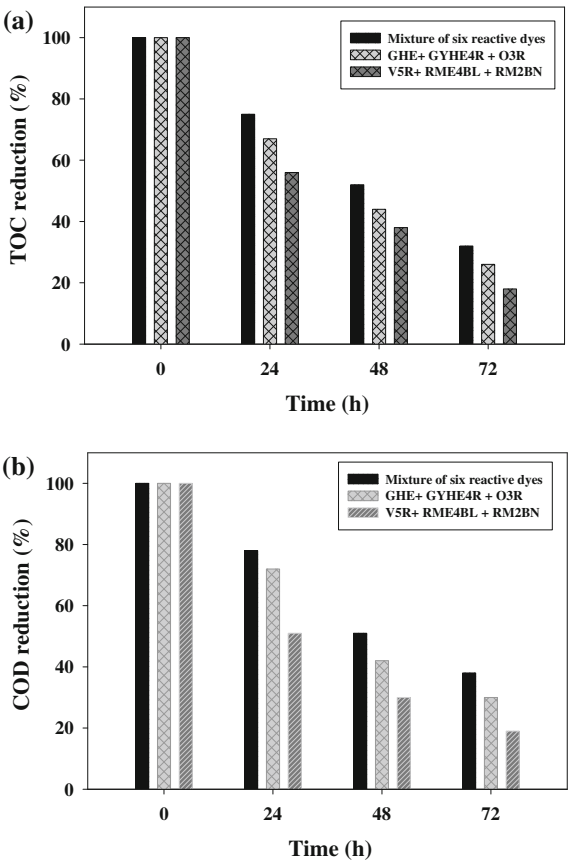


Fig. 5 Percentage of **a** TOC reduction and **b** COD reduction of two different mixtures of three reactive dyes (namely, Green HE4BD/Golden Yellow HE4R/Orange 3R and Violet 5R/Red ME4BL/Red M2BN; 50 mg l^{-1} each), as well as a mixture of all six reactive dyes (50 mg l^{-1} each) by consortium GR at different incubation time

Several research groups have recently demonstrated the possibility of utilizing microbial communities for the biotreatment of dye-containing wastewater from textile and dyestuff industries (Moosvi et al. 2007; Sandhya et al. 2005; Jiranuntipon et al. 2008; Sheth and Dave 2009). Consortium GR was also applied for the actual dye wastewater, showing significant decolorization in terms of ADMI value (75%), TOC (64%) and COD (60%) removal after 96 h incubation. This indicates partial mineralization of textile effluents (Table 7). It was reported that higher nitrogen and phosphorous content in the wastewater shows toxic effects (Ong et al. 2009). In this perspective, removal efficiency of total phosphorous and total nitrogen by consortium GR was investigated. After 96 h incubation, significant reduction in T-N (69%) and T-P (63%) was observed (Table 7). The foregoing results suggest that decolorization by consortium GR might be largely attributed by biodegradation instead of biosorption for mixture of dyes and wastewater. Furthermore, Table 8 shows that the efficiency of our microbial consortium GR is better or comparable to that reported in the relevant studies. In addition, the results also indicate that the individual strains among the microbial consortium might have synergistic effect, resulting in a significant improvement in TOC and COD removal ratio. Thus, potential of consortium GR in concern with diversity of dyes and their nonspecificity could be useful for the treatment of the textile effluents by using appropriate bioreactor.

Phytotoxicity and microbial toxicity studies

For sulfonated azo dyes, both aromatic sulfonic and azo groups and their metabolic intermediates (sulfonated and unsulfonated aromatic amines) represent important groups of environmental pollutants having toxic nature (Gottlieb et al. 2003; Junnarkar et al. 2006; Chen 2002). Improper disposal of dyeing effluents containing reactive azo dyes causes serious environmental and health hazards, they are being disposed off in water bodies and this water is being used for an agriculture purpose. Use of untreated and

Table 7 Summary of the textile wastewater study after treatment by consortium GR at different time intervals

Parameters	Treatment by consortium GR			
	Initial value (0 h)	After 24 h	After 48 h	After 96 h
pH	8.4	7.9	7.8	7.8
ADMI	13,690	9865	5567	3456
COD (mg l^{-1})	18,600	14,568	11,256	7440
TOC (mg l^{-1})	19,870	15,469	12,586	7550
T-N (mg l^{-1})	400	300	225	125
T-P (mg l^{-1})	140	112	89	53

Table 8 Comparison of the decolorization performance of different reactive dyes by using microbial consortium

Name of strain	Name of azo dyes; concentration	Decolorization (%)	Time (h)	Average decolorization rate (μg h^{-1})	References
Consortium SKB-II (*Bacillus vallismortis* and *Bacillus megaterium*)	Congo red; 10 mg l^{-1}	96	120	80	Tony et al. (2009)
Bacterial consortium RVM11.1	Reactive Violet 5; 200 mg l^{-1}	94	37	5081	Moosvi et al. (2005)
Acclimatized microbial consortium (*Pseudomonas aeruginosa* and *Bacillus circulans*, and unidentified NAD1 and NAD6)	Reactive Black 5; 100 mg l^{-1}	70–90	48	1875	Dafale et al. (2008)
Four bacterial isolates consortium [*Bacillus cereus* (BN-7), *Pseudomonas putida* (BN-4), *Pseudomonas fluorescence* (BN-5) and *Stenotrophomonas acidaminiphila* (BN-3)]	Acid Red 88; 60 mg l^{-1}	78	24	1950	Khehra et al. (2005)
	Acid Red 119; 60 mg l^{-1}	99	24	2475	
	Acid Red 97; 60 mg l^{-1}	94	24	2350	
	Acid Blue 113; 60 mg l^{-1}	99	24	2475	
	Reactive Red 120; 60 mg l^{-1}	82	24	2050	
Fungus bacterial coculture (*Penicillium* sp. QQ and *Sphingomonas xenophaga* QYY)	Reactive Brilliant red X-3B; 50 mg l^{-1}	87.8	72	604	Gou et al. (2009)
Bacterial consortium of (*Enterobacter* sp., *Serratia* sp., *Yersinia* sp. and *Erwinia* sp.)	Reactive Red 195; 30 mg l^{-1}	90	48	563	Jirasripongpun et al. (2007)
Five-member bacterial consortium; (*Alcaligenes faecalis*, *Sphingomonas* sp. EBD, *Bacillus subtilis*, *Bacillus thuringiensis* and *Enterobacter cancerogenus*)	Direct Blue-15; 50 mg l^{-1}	92.14	24	1920	Kumar et al. (2007)
Mixed bacterial consortium JW-2 (*Paenibacillus polymyxa*, *Micrococcus luteus* and *Micrococcus* sp.)	Reactive Violet 5R; 100 mg l^{-1}	100	36	2778	Moosvi et al. (2007)
Microbial consortium DAS (three different isolated *Pseudomonas* sp.)	Reactive Orange 16; 100 mg l^{-1}	100	48	2083	Jadhav et al. (2010)
Consortium GR (*Proteus vulgaris* NCIM2027 and *Micrococcus glutamicus* NCIM2168)	Reactive Green 19 A; 50 mg l^{-1}	100	24	2083	This study

treated dyeing effluents containing water for the agriculture purpose has direct impact on the fertility of soil (Kalyani et al. 2009). Therefore, it is of concern to assess the phytotoxicity and microbial toxicity of the Green HE4BD before and after degradation by consortium GR.

Seed germination and plant growth bioassays are the most common techniques used to evaluate the phytotoxicity. The relative sensitivity towards the dye Green HE4BD and its degradation products by consortium GR in relation to *Phaseolus mungo* was studied. The mean of plumule length and radical length of *Phaseolus mungo* were 14.63 ± 1.17 and 6.03 ± 0.74 cm, respectively, of 10 seeds in distilled water as a control with 100% germination. When treated with 300 mg l^{-1} concentration of Green HE4BD, the germination rate of *Phaseolus mungo* seeds (60%) was inhibited and the plumule and radical length were drastically affected. In contrast, treating with 300 mg l^{-1} of degradation products had minor effect on the plant, as the germination ratio was 100% and the plumule length and radical length of *Phaseolus mungo* were 10.32 ± 1.37 and 4.83 ± 0.84 cm, respectively (Table 9). These results suggest that the degradation products of Green HE4BD were non-toxic to the common crop *P. mungo*.

In microbial toxicity, the control with a dye concentration of 100 and 300 mg l^{-1} shows zone of inhibition (Table 10). In contrast, the extracted metabolites of dye (100 mg l^{-1}) by consortium GR did not show any growth inhibition in all soil microorganisms. However, when the concentration was increased to 300 mg l^{-1}, slight inhibition for some microorganisms was observed (Table 10). Similarly, no zone of inhibition for soil microorganisms was observed in the degraded metabolites of Reactive Red by *Pseudomonas aeruginosa* NGKCTS (Sheth and Dave 2009). Thus, both phytotoxicity and microbial toxicity studies show good germination as well as significant growth in the plumule and radical for *Phaseolus mungo* and growth of soil microorganisms on dye metabolites, indicating that the metabolites generated after biodegradation of Green HE4BD is less toxic when compared

Table 9 Phytotoxicity on *Phaseolus mungo* using original dye (Green HE4BD) and metabolites formed after biodegradation with consortium GR

	Germination (%)	Plumule (cm)	Radical (cm)
Distilled water	100	14.63 ± 1.17	6.03 ± 0.74
Green HE4BD[a]	40	1.28** ± 0.88	0.88** ± 0.56
Extracted metabolites[a]	100	10.32** ± 1.37	4.83* ± 0.84

[a] Concentration = 300 ppm

Values are mean of three experiments, SEM (±), significantly different from the control (seeds germinated in distilled water) at * $P < 0.05$, ** $P < 0.01$, by one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparisons test

Table 10 Microbial toxicity studies of Green HE4BD and its metabolites formed after biodegradation by consortium GR

Name of microbial strain	Zone of inhibition (cm)			
	Green HE4BD[a]	Extracted metabolites[a]	Green HE4BD[b]	Extracted metabolites[b]
Rhizobium radiobacter	0.30	NI	0.55	0.1
Acinetobacter sp.	0.4	NI	0.75	0.1
Pseudomonas desmolyticum NCIM-2112	0.25	NI	0.50	0.15
Cellulomonas biazotea NCIM-2550	0.19	NI	0.50	0.1
Micrococcus glutamicus NCIM-2168	0.12	NI	0.40	0.1
Proteus vulgaris NCIM-2027	0.30	NI	0.95	NI
Escherichia coli DH5α	0.25	NI	0.60	NI

NI no inhibition

[a] Concentration = 100 ppm

[b] Concentration = 300 ppm

to the original dye. The feature of detoxification reveals additional advantage of using consortium GR for the decolorization and biodegradation of the target dye.

Conclusions

Bacterial consortium comprising of *Proteus vulgaris* NCIM-2027 and *Micrococcus glutamicus* NCIM-2168 was developed in this study. The bacterial consortium was able to degrade sulfonated toxic Green HE4BD with significant decolorization rate relative to individual strains. Significant induction in oxidoreductive enzymes at intracellular location in consortium GR compared to pure cultures shows difference in the metabolic actions during assimilation of Green HE4BD. The TOC and COD analysis shows that the consortium GR could nearly completely mineralize this dye with nontoxic residual metabolites evaluated by phytotoxicity and microbial toxicity tests. This enhanced effect of consortium GR was due to the coordinated metabolic interactions of individual strains. The Consortium GR was tested to decolorize and mineralize mixture of reactive dyes and actual dye wastewater shows significant efficiency in the color removal (in terms of reduction in ADMI value) as well as the reduction of TOC and COD. The ability of consortium to utilize cheap co-substrate such as rice husk and rice straw for dye decolorization represents an advantage for treatment of textile industry wastewaters. However, potential of consortium GR needs to be demonstrated for its application in treatment of real dye bearing wastewaters using appropriate bioreactor systems.

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