

Oxidative stress response in dye degrading bacterium *Lysinibacillus* sp. RGS exposed to Reactive Orange 16, degradation of RO16 and evaluation of toxicity

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Abstract *Lysinibacillus* sp. RGS degrades sulfonated azo dye Reactive Orange 16 (RO16) efficiently. Superoxide dismutase and catalase activity were tested to study the response of *Lysinibacillus* sp. RGS to the oxidative stress generated by RO16. The results demonstrated that oxidative stress enzymes not only protect the cell from oxidative stress but also has a probable role in decolorization along with an involvement of oxidoreductive enzymes. Formation of three different metabolites after degradation of RO16 has been confirmed by GC-MS analysis. FTIR analysis verified the degradation of functional groups of RO16, and HPTLC confirmed the removal of auxochrome group from the RO16 after degradation. Toxicity studies confirmed the genotoxic, cytotoxic, and phytotoxic nature of RO16 and the formation of less toxic products after the treatment of *Lysinibacillus* sp. RGS. Therefore, *Lysinibacillus* sp. RGS has a better perspective of bioremediation for textile wastewater treatment.

Keywords *Lysinibacillus* sp. RGS · Superoxide dismutase · Catalase · Cytotoxicity · Genotoxicity · Decolorization

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Introduction

The recalcitrant nature and inappropriate release of synthetic dyes in the water bodies causes threat to the environment (Vandevivere et al. 1998; Alinsafi et al. 2006). Direct discharge of colored wastewater in the water bodies pollutes the aquatic environment by changing pH, chemical oxygen demand (COD), dissolved oxygen concentration, and biological oxygen demand (BOD) of water. Due to color and chemicals, sunlight fails to penetrate up to bottom of water bodies which affects the solar-driven water ecosystem (Pinheiro et al. 2004). Therefore, the proper treatment of textile wastewater is necessary before its discharge in the water bodies (Supaka et al. 2004). Though physical and chemical methods clean the textile wastewater efficiently, they are unsuccessful to provide a permanent solution due to high treatment cost, secondary pollution problems, and unable to remove recalcitrant azo dyes completely (Chacko and Subramaniam 2011). Therefore, in situ life forms from affected sites have fascinated researchers to utilize them for cleanup of the environment (Hao et al. 2000). These environment cleaners are well-adapted to the affected sites and sometimes use these pollutants as a source of nutrition. Use of microorganisms for the remediation purpose is an economic and ecofriendly way of textile dye degradation. Several bacteria have obtained the capability of mineralizing chemical substances that were not at all present in the environment before the beginning of modern synthetic chemistry (Pérez et al. 2013). Bacteria possess the ability to metabolize synthetic dyes by the action of various oxidative and reductive enzyme systems (McMullan et al 2001).

Xenobiotic compounds are known to be mutagenic in nature, and in some cases, metabolites formed are also toxic in nature (Achary and Panda 2009). For this reason, it is essential to measure the toxicity of contaminant individually or in mixture and their metabolites to evaluate the

practicability of the bioremediation. While considering textile dye toxicity, previous studies showed that textile dyes adversely affects erythrocyte count of freshwater fish *Gambusia affinis* (Sharma et al. 2009) and also they are phytotoxic and cytogenotoxic (Patil and Jadhav 2013) in nature. So, the textile dyes toxicity removal must be taken into consideration during textile dye degradation.

Upon contact with pollutants, antioxidant enzymes of pollutant-degrading bacteria change their activity. Pollutant-degrading bacteria might experience the oxidative stress when exposed to pollutants (Zhang et al. 2012). The oxidative stress produced in bacteria in response to many contaminants has been broadly studied (Geckil et al. 2003; Frederick et al. 2001; Zhang et al. 2012). Research is mainly focused on free radical-mediated damage in biological systems exposed to textile dye wastewater. However, these studies mainly focused on free radical-mediated plant cell damage (Jadhav et al. 2009; Phugare et al. 2011). To our information, no earlier studies have examined the occurrence and action of superoxide dismutase (SOD) and catalase (CAT) in bacteria treated with textile dyes. Bacterial antioxidant response to textile dyes have been focused in current studies which is important for bioremediation. This study was aimed to determine the dye degrading potential of *Lysinibacillus* sp. RGS and to understand the possible role of catalase and superoxide dismutase in protection of bacterial cells against oxidative stress as well as in the enhancement of biodegradation process.

Material and methods

Chemicals, microbiological media, and dyestuff

The textile dyes Reactive Green 19A, Reactive Yellow 84, Reactive Red 120, Reactive Blue 160, Reactive Red 31, Reactive Green 19A, Solvent Red 24, Basic Green 4, Diamond Green 4, Pigment Orange 31, Reactive Red 120, and Reactive Orange 16 (RO16) were collected from Manpasand textile Industry, Ichalkaranji, Maharashtra, India. 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,6-dichlorophenolindophenol (DCIP), and NADH were purchased from Sigma Chemical Company (USA). L-tyrosine, methyl red (MR), and nutrient broth were obtained from Hi-Media Laboratory, India. Ascorbic acid, tartaric acid, *n*-propanol, and other fine chemicals were obtained from SRL Chemicals, India. All chemicals used were of the highest purity.

Organism and culture conditions

Lysinibacillus sp. RGS is a potent dye degrading bacterium previously reported by Saratale et al. (2012). The pure culture

of *Lysinibacillus* sp. RGS was maintained on nutrient agar slants and stored in test tubes at 4 °C and subcultured monthly. *Lysinibacillus* sp. RGS culture (1 mL inoculum; 6.0×10^7 CFU/mL) was inoculated into 3-mL nutrient broth (beef extract, 3 g L⁻¹; peptone, 10 g L⁻¹; NaCl, 5 g L⁻¹; pH 7) and incubated at 30 °C under static condition. The 24-h grown culture (A530 nm=0.3) were again inoculated in semisynthetic media (RO16, 0.050 g L⁻¹; (NH₄)₂SO₄, 0.28 g L⁻¹; NH₄Cl, 0.23 g L⁻¹; KH₂PO₄, 0.067 g L⁻¹; MgSO₄·7H₂O, 0.04 g L⁻¹; CaCl₂·2H₂O, 0.025 g L⁻¹; NaHCO₃, 1.0 g L⁻¹; Peptone, 0.5 g L⁻¹; pH 7).

Preparation of cell-free extract

Lysinibacillus sp. RGS culture was inoculated into 3-mL nutrient broth and under static condition incubated at 30 °C. The 24-h grown culture (A530 nm=0.3) was again inoculated in semisynthetic media (pH 7) incubated at 30 °C for 24 h and centrifuged at 3,000×g for 15 min to harvest cells. For sonication (Sonics-vibracell ultrasonic processor), cells (8.2 g L⁻¹) were suspended in potassium phosphate buffer (50 mM, pH 7.4) and giving 8 strokes each of 15 s with a 2-min interval at 4 °C, keeping sonifier output at 40 A. Supernatant obtained after centrifugation of this crude extract at 5,000×g for 15 min was used as a source of enzyme. Enzyme source from the cells obtained after complete decolorization was obtained by similar procedure (2 h for RO16, 0.050 g L⁻¹).

Establishment of optimum conditions for decolorization

To study the effect of different environmental situations on the decolorization performance of RO16 by *Lysinibacillus* sp. RGS, the decolorization experiments have been carried out (24-h-grown cells; dye concentration 50 mg L⁻¹); at different temperatures (20–65 °C), pH values (2–12), and initial dye concentrations (50–500 mg L⁻¹) under static condition. By adjusting the pH of semisynthetic media (with 0.1 M HCl and 0.1 M NaOH), effect of different pH was studied prior to cells and dye addition. Similarly, before addition of dye, flasks containing cells suspended in semisynthetic media were kept at respective temperature for 15 min to reach the specific temperature to study effect of temperature. The incubation was continued after dye addition at respective temperature. The decolorization was monitored after 1 h of incubation. In order to examine the effect of initial dye concentrations on the decolorization performance, different concentrations of dye ranging from 50 to 500 mg L⁻¹ were added into the decolorization flask containing bacterial cells (3 mL inoculum of 24 h grown bacteria) in semisynthetic media. The dye removal was measured and used to determine the proportion of dye removed. The percent decolorization was measured at different time intervals.

Decolorization tests of RO16 and dye mixture

Screening of different individual dyes (each 50 mg L⁻¹; Reactive Green 19A, Reactive Red 120, Reactive Red 31, Reactive Yellow 84, Solvent Red 24, Basic Green 4, Diamond Green 4, Reactive Blue 160, Pigment Orange 31, and RO16) has been done to examine decolorization performance of *Lysinibacillus* sp. RGS. The flasks containing 100 mL semi-synthetic media and dye (each 50 mg L⁻¹) were inoculated with 3 mL culture of *Lysinibacillus* sp. RGS for bacterial decolorization study. Aliquots (2 mL) of the culture broth were subsampled after regular time intervals for analysis of percent decolorization. For separation of cell mass, the aliquot

was centrifuged at 3,000×g for 15 min. Decolorization was measured by calculating change in the absorbance of supernatant of the respective dyes at the maximum absorption wavelength (λ_{max}).

For mixture of dyes, i.e., Reactive Yellow 84, Reactive Green 19A, Reactive Red 120, Reactive Blue 160, and RO16 (concentration 50 mg L⁻¹ of each dye), the decolorization performance was studied using same method. All decolorization experiments were carried out in triplicates. All the time, abiotic controls (semisynthetic media + dye) were included. The decolorization of mixture of dyes was determined by measuring removal of ADMI from the aqueous solutions. ADMI removal percent (%) was calculated as follows:

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

Outcome of repeated addition of RO16

Outcome of repeated addition of RO16 on the decolorization performance of *Lysinibacillus* sp. RGS was studied. Cells grown for 24 h (in 3-mL nutrient media) of *Lysinibacillus* sp. RGS were inoculated in RO16 containing semisynthetic media. RO16 (50 mg L⁻¹) was repeatedly added into a batch culture (100 mL) after decolorization of dye. The percent decolorization and time was monitored after each cycle. Decolorization was supervised up to the maximum, until no further decolorization was observed.

Enzyme induction during decolorization of RO16, repeated batch cycles, and dye mixture

The enzyme activities of different oxidoreductive enzymes were monitored at different time intervals (i.e., after 50 % of decolorization, after complete decolorization, and second and third cycle of repeated batch).

The oxidative enzymes were examined in the cell-free extract using spectrophotometer. For laccase activity, a reaction mixture of 2.5 mL containing 10 % ABTS as a substrate in 20 mM potassium phosphate buffer (pH 4.0) was used and absorbance was measured at 420 nm (Hatvani and Mecs 2001). For tyrosinase activity, reaction mixture used was 2.5 mL containing sodium acetate buffer (20 mM, pH 4.0) and 100 μ M of L-tyrosine as a substrate. To start the reaction, 0.2 mL of enzyme solution was added, and increase in absorbance was measured at 280 nm (Kadam et al. 2011). Lignin peroxidase activity was determined by using a 2.5-mL reaction mixture (100 mM *n*-propanol, 250 mM tartaric acid, 10 mM H₂O₂), and the formation of propanaldehyde was monitored at 300 nm (Hatvani and Mecs 2001). Reaction

mixture for veratryl alcohol activity contains 1 mM veratryl alcohol in 0.1 M citrate phosphate buffer (pH 3.0) and 0.2 mL enzyme. Formation of veratraldehyde was monitored at 310 nm (Jadhav et al. 2009). Molar extinction coefficient of respective substrate of enzyme was used to calculate enzyme activity of each enzyme. All enzyme assays were performed in triplicate, and specific enzyme activity were represented by taking average of three experiments.

For azoreductase activity, decrease in the methyl red concentration was monitored at 430 nm in a reaction mixture of 2.5 mL (4.45 μ M methyl red, 50 mM sodium phosphate buffer (pH 5.5), and 20 μ M NADH). NADH-DCIP reductase activity was measured as reported earlier (Saratale et al. 2007). All enzyme assays were performed thrice, and enzyme activity was measured using molar extinction coefficient of substrate used.

Oxidative stress enzymes during decolorization

CAT and SOD activity were determined spectrophotometrically at 240 and 550 nm, respectively. CAT activity was determined in a reaction mixture of 3 mL containing 10 mM H₂O₂ in 50 mM sodium phosphate buffer (pH 7.0), and absorbance was measured at 240 nm. One unit of SOD was measured in a reaction mixture of 2 mL, the amount of enzyme required for 50 % inhibition of 75 mM NBT at 25 °C.

Analytical methods

COD, BOD, alkalinity, and hardness were checked using (APHA, American Public Health Association 1998) protocols, and the total organic carbon (TOC) of the medium were calculated as described previously (Saratale et al. 2009).

UV-visible spectral analysis of cell-free sample, before and after dye decolorization, was carried by using Shimadzu UV spectrophotometer (UV 1800), and changes in its absorption scale in the visible range (400–800 nm) were documented (Waghmode et al. 2011). HPTLC analysis was performed using precoated TLC silica gel (60 F 254) plates supplied by Merck. Using a micro syringe (HPTLC Camag, Switzerland) 10 μ L of the samples (RO16, metabolites of RO16) were spotted on TLC plates. The solvent system used was methanol:toluene:acetic acid:ethyl acetate (3:1:0.5:0.5 v/v). The RO16 chromatogram was scanned at 260 nm (Joshi et al. 2010). Stereoscopically pure KBr (95:5) was mixed with residues obtained after evaporation of solvent extracts for FTIR analysis. Shimadzu 8400S spectrophotometer in the mid-infrared region of 400–4,000 cm^{-1} with 16-scan speed was used for studies. Identification of metabolites obtained after degradation of RO16 was carried out using QP2010 GC-MS (Shimadzu) (ionization voltage 70 eV, carrier gas was helium gas, flow rate 1 mL min^{-1} , 33 min run time) (Lade et al. 2012). Gas chromatography was conducted with a Resteck column (0.25 \times 30 mm; XTI-5) in temperature programming mode. Column oven temperature was 280 $^{\circ}\text{C}$, and injection temperature was 200 $^{\circ}\text{C}$. Temperature was hold at 50 $^{\circ}\text{C}$ for 1 min then rose to 280 $^{\circ}\text{C}$ at 10 $^{\circ}\text{C}$ rise per min.

Toxicity study RO16 and dye mixture

Before use, *Allium cepa* bulbs (weight, 4–10 g; diameter 12–20 mm) were washed under running tap water and loose outer scales and dry bases were removed. Root primordials were exposed to RO16 diluted to 400, 500, and 600 ppm concentrations and dye mixture (500 ppm), treated samples of both, and distilled water. The experiments were conducted using a procedure described earlier (Patil and Jadhav 2013). After measuring root length and mitotic index, their mean was calculated and scored to evaluate nuclear aberrations, fragments and loss of chromosomes, multinucleated cells at each point, and micronuclei formation (Carita and Marin-Morales 2008). Mitosis index was calculated by number of mitotic cells observed divided by total number of cells observed.

The comet assay was carried out as described by (Chakraborty et al. 2009). A fluorescence microscope was used to analyze 25 randomly chosen nuclei from each slide. % DNA damage (% T), tail moment, and tail length (μm) were measured using a computerized image analysis system (Comet version 1.5).

The phytotoxicity study was performed by exposing seeds of *Sorghum vulgare* and *Phaseolus mungo* (10 seeds of each) separately to 10-mL sample of untreated and treated RO16 and dye mixture along with distilled water as a control sample. The control set was carried out using distilled water at the

same time. Germination (%) and length of plumule (cm) and radical (cm) were measured after 7 days. Experiment has been repeated two times at room temperature.

Statistical analysis

One-way analysis of variance (ANOVA) with the Tukey–Kramer multiple comparisons test was used for data analysis.

Result and discussion

Decolorization experiments

The decolorization potential of microorganism was assessed by examining its ability to degrade various textile dyes. All 10 dyes monitored showed decolorization as follows: Reactive Yellow 84 (complete decolorization in 5 h), Reactive Green 19A (95 % in 24 h), Reactive Red 120 (complete decolorization in 6 h), Reactive Blue 160 (complete decolorization in 3 h), Reactive Red 31 (complete decolorization in 6 h), Solvent Red 24 (complete decolorization in 8 h), Basic Green 4 (80 % in 24 h), Diamond Green 4 (complete decolorization in 24 h), Pigment Orange 31 (complete decolorization in 3 h), and RO16 (complete decolorization in 2 h), while in sterile, cell-free medium, decolorization did not occur up to 48 h of incubation suggesting the absence of abiotic decolorization. As RO16 showed complete decolorization in 2 h, this industrially important toxic azo dye was taken for further studies. *Lysinibacillus* sp. RGS could decolorize RO16 rapidly and thus was found to be better than the bacterial consortium DAS reported earlier (Jadhav et al. 2008). To confirm the decolorization, UV-vis spectroscopic analysis was carried out. The absorption spectra of RO16 before and after treatment by *Lysinibacillus* sp. RGS in visible range were taken. Peak responsible for absorption maxima of parent dye (495 nm) were completely disappeared in the sample obtained after decolorization (Fig. S1) confirming the complete removal.

Establishment of optimum conditions for the decolorization

Lysinibacillus sp. RGS showed 85 % decolorization at broad pH (6–8) and temperature (20–37 $^{\circ}\text{C}$) ranges, while the maximum decolorization was observed at pH 7 and temperature 30 $^{\circ}\text{C}$ (Fig. S2 and S3). As dye wastewater generally have higher pH and varying temperature, the broad pH and temperature stability of this strain favors the field applicability. The complete decolorization required 2, 2.3, 3, 5, and 9 h for 50, 100, 150, 200, and 250 mg L^{-1} concentration, respectively (Fig. S4). Similarly, time required for decolorization was increased with subsequent increase in dye concentration. In presence of the higher dye concentrations (300 and

500 mg L⁻¹), *Lysinibacillus* sp. RGS showed low decolorization performances of 92 and 72 % after 48 h of incubation, respectively. The higher dye concentrations imparted a toxic effect on microbial degradation potential. The other reasons could be inadequate biomass or improper cell-to-dye ratio for the uptake of higher concentrations of dye (Jadhav et al. 2008). Growth of microorganisms was greatly inhibited in case of azo dyes having sulfonic acid (SO₃H) groups on aromatic rings (Kalyani et al. 2009).

Outcome of repeated addition of RO16

Decolorization capability of *Lysinibacillus* sp. RGS was evaluated up to 25 cycles by repeated additions of RO16 (50 mg L⁻¹) under static condition (Fig. 1). Complete decolorization of the dye was observed up to the 21st cycle, further, the decolorization up to 94 % within 200 min at 22nd cycle and 90, 84, and 78 % was found within 240 min at 23, 24 and 25th cycle, respectively. In case of the repeated batch cycle, the growth medium was added only at initial stage, cells might have entered in stationary or death phase due to lack of nutrients resulting in decrease in the decolorization.

While considering the results of increasing dye concentration and repeated batch addition, direct initial exposure 300 mg L⁻¹ dye concentration was inhibitory for *Lysinibacillus* sp. RGS and therefore it was unable to decolorize but after repeated addition of a total of 1,050 mg L⁻¹, dye concentration was found to be decolorized. Similar results were obtained during the decolorization and detoxification studies of Remazol Red and textile effluent (Saratale et al. 2012). Based on these observations, oxidative stress enzymes were studied after first, second, and third repeated batch cycle to check oxidative stress produced on bacteria and to their find possible role in decolorization.

Enzyme induction during decolorization of RO16 and dye mixture

The enzyme activities of laccase, tyrosinase, veratryl alcohol oxidase, lignin peroxidase, NADH-DCIP reductase, and azoreductase were monitored at different time intervals (Table 1) (i.e., after 50 % of decolorization, after complete decolorization, and second and third cycle of repeated batch) to understand the enzyme induction mechanism more precisely related to the oxidative stress enzymes. The induction of enzymes pattern after the 50 % of decolorization showed that almost all oxidative as well as reductive enzymes were induced (Table 1). Among them, azoreductase showed more induction as compared to other enzymes. Azoreductase also showed its role in proposed biodegradation pathway (Fig. 4). It was found that the activities of oxidative as well as reductive enzymes were significantly reduced after complete decolorization (2 h) as compared to activities obtained after 50 % decolorization (45 min). During 50 % of decolorization as RO16 was available, presence of target molecule, i.e., RO16, might have promoted the enzyme activities. Induction in activities of oxidative as well as reductive enzymes during 50 % decolorization might be due to presence of target molecule, i.e., RO16, while decreased enzyme activities after complete decolorization were due to absence of RO16. In the repeated dye decolorization study, it was observed that time required for decolorization was reduced considerably (15 min) but at the same time all oxidative enzymes (laccase, lignin peroxidase, tyrosinase, and veratryl alcohol oxidase) were induced in minute quantity but all reductive enzymes (azoreductase and DCIP reductase) increased tremendously. Oxidoreductive enzymes have been found to be involved in the biological degradation of dyes (Karigar and Rao 2011). In this study, oxidative stress enzymes were considerably induced at each repeated batch cycle (Table 1). Induction of

Fig. 1 Effect of repeated addition of RO16 on decolorization performance of *Lysinibacillus* sp. RGS

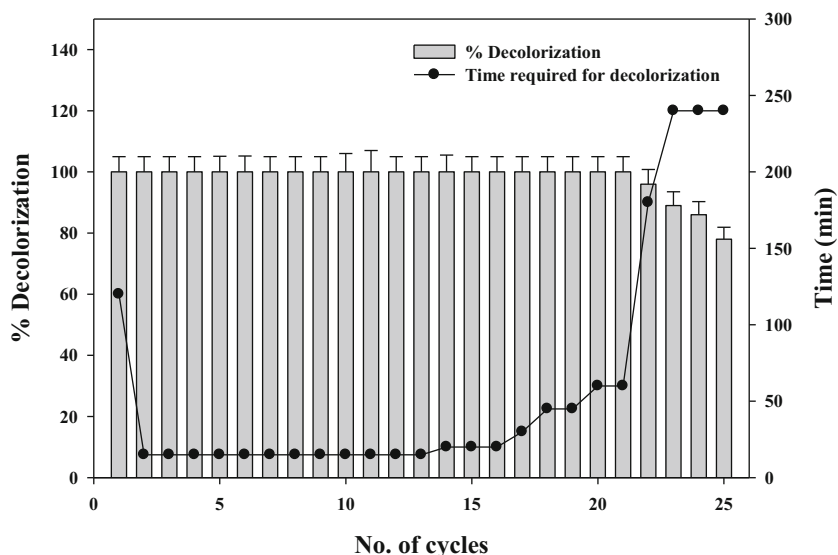


Table 1 Dye decolorizing and oxidative stress enzymes study for decolorization of RO 16 and dye mixture by *Lysinibacillus* sp. RGS

	Control	After 50 % decolorization (45 min)	After complete decolorization (1st cycle)	After complete decolorization (2nd cycle)	After complete decolorization (3rd cycle)	Dye mixture
Laccase ^a	0.3±0.02	1.4±0.12***	0.7±0.09**	0.6±0.04*	0.5±0.04*	0.4±0.03
Tyrosinase ^b	1.1±0.06	2.2±0.16**	1.8±0.07*	1.8±0.03*	1.4±0.01	2.1±0.13**
Lignin peroxidase ^c	0.3±0.04	0.4±0.05*	0.4±0.02*	0.3±0.01	0.2±0.01	0.4±0.02*
Veratryl alcohol oxidase ^d	1.2±0.04	2.6±0.14***	2.4±0.05***	2.3±0.07***	1.7±0.22	2.1±0.06**
Azoreductase ^e	0.3±0.02	5.7±0.37***	2.8±0.17***	4.9±0.12***	7.2±0.05***	3.1±0.06***
DCIP reductase ^f	0.5±0.02	3.9±0.04***	3.8±0.07***	4.9±0.05***	5.1±0.02***	3.2±0.04***
Oxidative stress enzymes						
Catalase ^g	0.01±0.05	1.5±0.02***	2.6±0.01***	4.6±0.02***	6.9±0.02***	
Superoxide dismutase ^h		15.1±0.2	30.9±0.02	33.03±0.07	35.7±0.01	

Values are mean of three experiments ± SEM

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Significantly different from control (0 h) by one-way ANOVA with Tukey–Kramer comparison test)

^a μg of ABTS oxidized mL⁻¹ enzyme min⁻¹ mg enzyme⁻¹

^b μg of L-tyrosine oxidized mL⁻¹ enzyme min⁻¹ mg enzyme⁻¹

^c μg of *n*-propanol oxidized mL⁻¹ enzyme min⁻¹ mg enzyme⁻¹

^d μg of veratryl alcohol oxidized mL⁻¹ enzyme min⁻¹ mg enzyme⁻¹

^e μg of methyl red reduced mL⁻¹ enzyme min⁻¹ mg enzyme⁻¹

^f μg of DCIP reduced mL⁻¹ enzyme min⁻¹ mg enzyme⁻¹

^g μg of H₂O₂ utilized mL⁻¹ enzyme min⁻¹ mg enzyme⁻¹

^h Inhibition of NBT reduction (by 50 %) mL⁻¹ enzyme min⁻¹

catalase during biodegradation of sulfonated azo dyes has been previously reported in fungi (Laxminarayana et al. 2010) and bacteria (Joshi et al. 2013). Repeated additions of RO16 lead to oxidative stress in *Lysinibacillus* sp. RGS showing CAT and SOD activities by bacterial cells. The dye molecules might have transported to intracellular region causing induction in reductive enzymes such as azoreductase.

In case of dye mixture, laccase (939 %), tyrosinase (173 %), lignin peroxidase (357.2 %), veratryl alcohol oxidase (36.87 %), NADH-DCIP reductase (100 %), and azoreductase (100 %) are induced notably showing their mutual role in the degradation process.

Analytical methods

To evaluate pollution level of the RO16 by *Lysinibacillus* sp. RGS, environmental parameters were determined by measuring the percent reduction in initial and final organic content. *Lysinibacillus* sp. RGS displayed complete decolorization of RO16 sequentially with a significant TOC reduction (72 %) within 2 h. *Lysinibacillus* sp. RGS remove TOC potentially as compared to Fenton/UV-C process for Reactive Black 5 (Lucas and Peres 2007), showing a TOC removal 46 %. The *Lysinibacillus* sp. RGS showed complete decolorization of RO16 with 75 % reduction in COD within 2 h at static conditions. The other physicochemical parameters like hardness, alkalinity, BOD were reduced by 24, 53, and 69 %,

respectively. In case of dye mixture, TOC, COD, alkalinity, hardness, BOD, and ADMI were reduced up to 39, 41, 22, 39, 59, and 78 %, respectively. There was no change in TOC, COD, and BOD in control set containing the semisynthetic medium with RO16 after 2-h incubation.

Lysinibacillus sp. RGS made use of these dyes and their reaction intermediates as the carbon source achieving mineralization of the dye compound. *Lysinibacillus* sp. RGS did not only decolorize the dye but also reduced hardness, alkalinity, BOD, TOC, and COD after treatment. Thus, from the environmental as well as economical point of view, *Lysinibacillus* sp. RGS could be a good alternative to physicochemical methods for dye treatment.

The spectrophotometric analysis of RO16 decolorization by *Lysinibacillus* sp. RGS at 400 to 800 nm showed significant reduction in absorbance as compared to control RO16 (Fig. S1). The HPTLC analysis of degraded metabolites showed different degradation pattern with different R_f values in decolorized dye samples as compared to control RO16. The R_f values of control dye were 1.08 and 0.47 whereas the dye and its products gave the R_f values of 1.07, 1.04, 0.77, and 0.02 (Fig. 2) indicating the biodegradation of RO16. The FTIR spectrum of control dye RO16 compared with extracted metabolites obtained from 2-h culture is shown in Fig. 3. The spectrum of control dye displayed a peak at 3,329.4 cm⁻¹ for N–H stretching, a peak at 2,964 cm⁻¹ for asymmetric O–H stretch and band for C–H stretching at 2,829.1 cm⁻¹ was

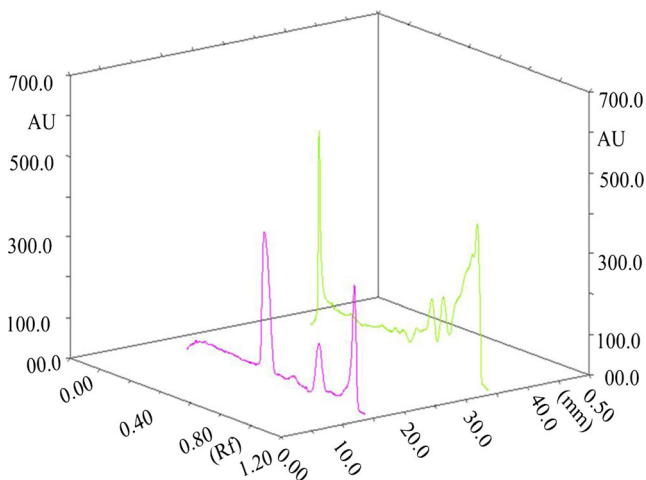


Fig. 2 HPTLC 3-D graph of control dye RO16 (A) and its metabolites (B) obtained after 2 h of degradation by *Lysinibacillus* sp. RGS

observed. Whereas, a peak at $2,359.2\text{ cm}^{-1}$ represented C–H stretching of azo group. Peak at $1,624.2\text{ cm}^{-1}$ showed N = N stretching vibration. In addition, a peak at $1,433.8\text{ cm}^{-1}$ represented C–H vibration. Thus, a change in the spectra in the degraded metabolite sample suggests the degradation of

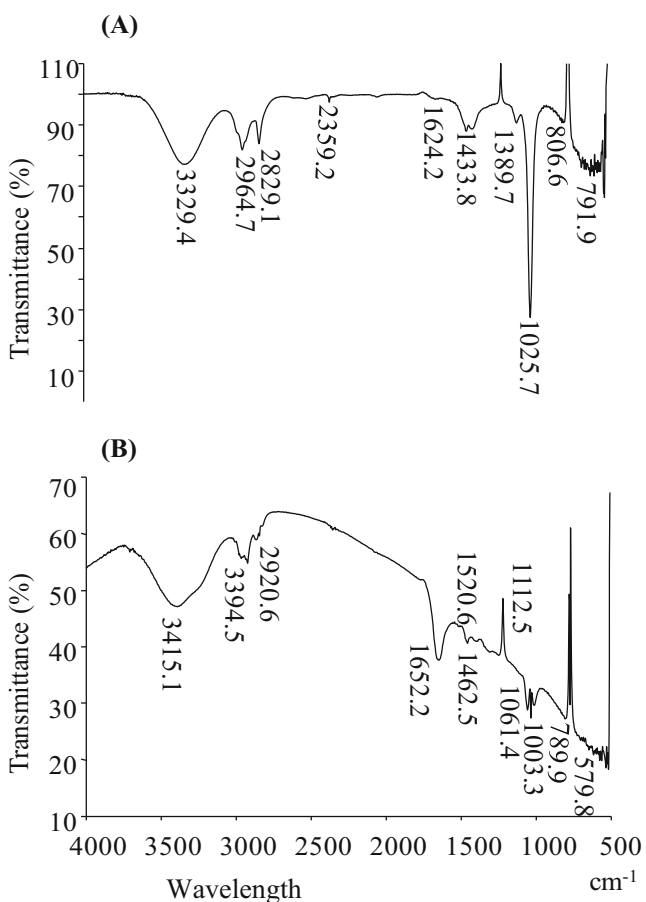


Fig. 3 FTIR analysis of products (extracted with ethyl acetate) formed by degradation of RO16 (a) at 0 h (control), (b) metabolites formed by *Lysinibacillus* sp. RGS strain RGS after complete decolorization (2 h)

RO16 by breakage of various bonds due to the action of microbial enzyme system.

The degradation products formed during RO16 decolorization by *Lysinibacillus* sp. RGS was verified by GC-MS analysis, which shows several peaks in GC-MS total ion chromatogram. The degradation of RO16 by *Lysinibacillus* sp. RGS produced the low molecular weight aromatic compounds. The degradation pathway of RO16 was proposed (Fig. 4) showing probable metabolites produced during biodegradation. Initially, primary reductive cleavage in azo bond of RO16 resulted in the formation of intermediate [A] and [B]. The intermediate [A] after desulfonation gave 2-[(3-aminophenyl) sulfonyl] ethanol which on further deamination and asymmetric cleavage formed (ethylsulfonyl) benzene. Intermediate [B] upon deamination gave sodium 4-hydroxy-5,8-dihydronaphthalene-2-sulfonate; the formation of these two end products confirmed degradation of RO16. Similarly, biodegradation of Remazol Red by catalytic action of *Brevibacillus laterosporus* formed (ethylsulfonyl) benzene as one of the final products as reported earlier (Kurade et al. 2012).

Toxicity studies

Roots of *A. cepa* plants were used to assess cytogenotoxicity (Chakraborty et al. 2009). Various parameters have been studied to assess the toxicity such as root length, mitotic index (MI), micronuclei formation, total number of aberrations, total number of mitotic cells, etc. MI less than negative control indicates affected growth and development by test compound, while MI above negative control indicates uncontrolled cell proliferation (Carita and Marin-Morales 2008). Strong genotoxic effect of RO16 and dye mixture in the mitotic cells of *A. cepa* roots were seen as compared to that of negative control, i.e., distilled water. The mitotic index of RO16 exposed cells was 13.3, while that of the dye mixture was 14.9 which are increased as compared to distilled water (11.2) in both the cases. The MI of metabolites formed after the degradation of RO16 (11.5) and distilled water (11.6) is relatively similar to MI of distilled water. The increased MI in both RO16 and dye mixture might be connected with uncontrolled cell division and tumor formation which have ultimately applied harmful effects on the cells (Carita and Marin-Morales 2008). Along with mitotic index, root length, number of aberrations, and total number of mitotic cells were also affected in the cells of roots exposed to RO16 and dye mixture, which clearly indicates the cytotoxic nature of the test samples while the degraded metabolites showed similar root length, number of aberrations, and total number of mitotic cells as that of distilled water (Table 2), which clearly pointed out the less toxicity of the metabolites.

Commet assay is a microelectrophoretic technique for the DNA damage visualization in individual cell. It is a sensitive, rapid, and relatively simple method for detecting DNA

Fig. 4 Proposed pathway for biodegradation of RO16 by *Lysinibacillus* sp. RGS

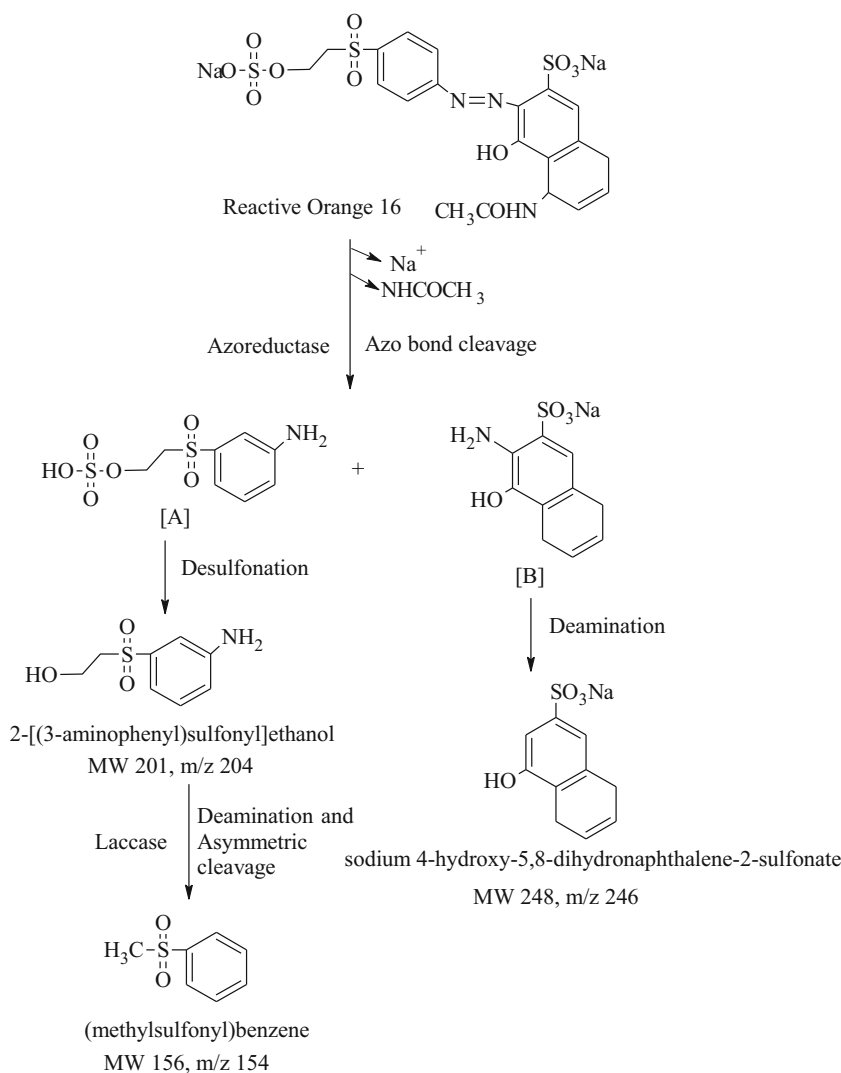


Table 2 Effect of untreated and treated RO16 and dye mixture on chromosomal aberrations in the *Allium cepa* tests

Parameters	RO 16	Extracted Metabolites	Distilled water	Dye mixture	Extracted Metabolites
RL	3.2± 0.07***	4.4±0.08*	4.6± 0.01	3.4± 0.1***	4.1± 0.1***
MI	13.3	11.5	11.2	14.9	11.6
MN	4	1	1	4	1
CB	4	1	1	6	2
TA	9	2	1	8	1
TMO	247	115	141	283	169

Values are mean of three experiments ± SEM

RL Root length, MI Mitotic index, MN Micronuclei formation, CB Chromosomal break, TA Total number of aberrations, TMO Total number of mitotic cells observed

* $P < 0.05$; *** $P < 0.001$ (significantly different from control (0 h) by one-way ANOVA with Tukey–Kramer comparison test)

damage in individual cells (Achary and Panda 2009). In this study, the *A. cepa* bulbs were exposed to 600 and 500 ppm concentrations of treated and untreated RO16 and dye mixture, respectively. Isolated nuclei from *A. cepa* meristems are highly sensitive to the electric field due to naked condition (Fig. 5). The parameters measured were tail length (μm), percentage of tail DNA (% of DNA in comet tail), and tail moment. DNA damage observed for RO16 sample and dye mixture showed significant increase. In control sample of RO16, the DNA was intact and in electric field, it does not form tailing move uniformly. DNA causes double-stranded breaks that lead to formation of tailing in an electric field in presence of dye. Similar results were observed in coal fly ash exposed *A. cepa* bulbs (Chakraborty et al. 2009).

To study the effect of RO16 and dye mixture on seed germination, root length, and plant growth, phytotoxicity tests have been carried out (Table 3). The germination rate, root, and shoot length of *P. mungo* and *S. vulgare* were drastically affected when treated with RO16 and dye mixture. In contrast,

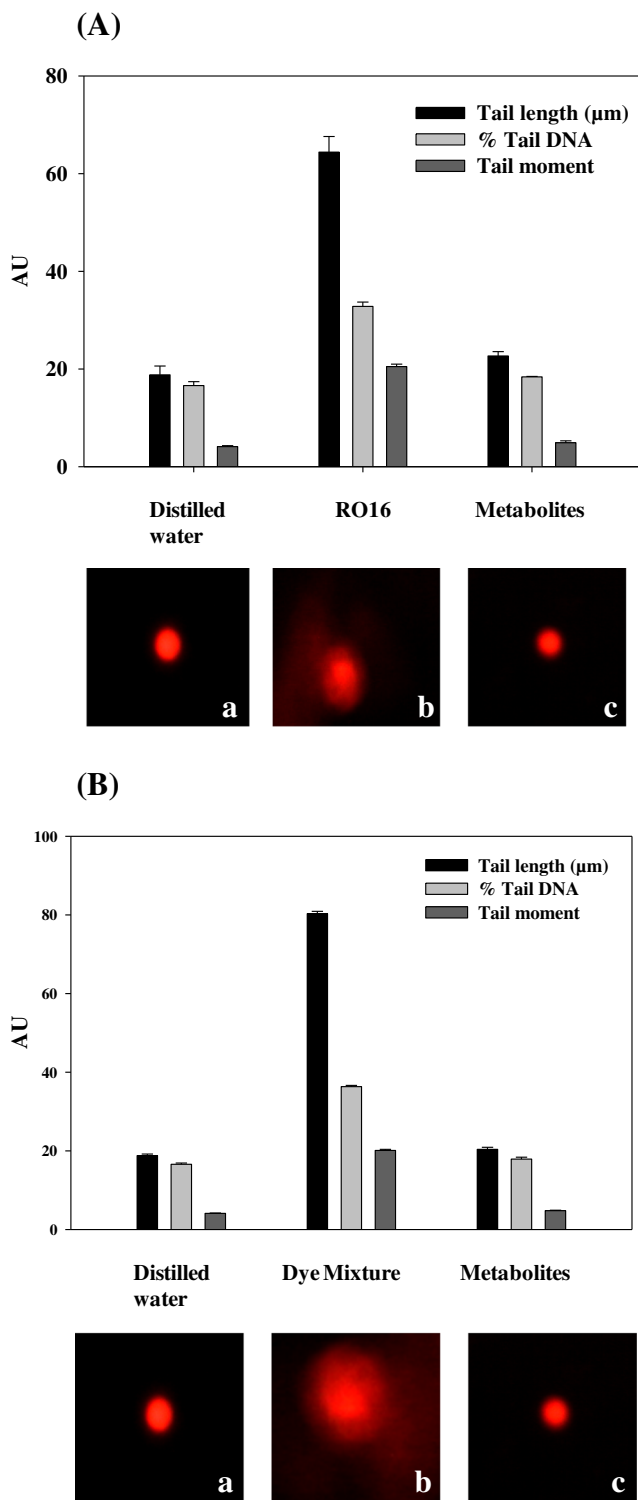


Fig. 5 Toxicity analysis using root cells of *Allium cepa* exposed to RO16, dye mixture and their metabolites. *A* Comets observed in distilled water (a), RO16 (b), and its degraded metabolite (c) along with detection of DNA damage. *B* comets observed in distilled water (a), dye mixture (b), and its degraded metabolite (c) along with detection of DNA damage

germination rate, root, and shoot length of *P. mungo* and *S. vulgare* grown in degradation products of RO16 and dye

Table 3 Phytotoxicity analysis of metabolites obtained after degradation of RO16 and dye mixture by *Lysinibacillus* sp. RGS

Parameters studied	<i>Phaseolus mungo</i>				<i>Sorghum vulgare</i>				
	Water	RO16	Extracted Metabolites	RO16	Water	RO16	Extracted Metabolites	Dye mixture	Extracted Metabolites
Germination (%)	100	40	100	30	100	30	80	20	90
Shoot length (cm)	25.6±0.01	7.2±0.10***	25.6±0.08	4.8±0.01***	17.8±0.03	4.8±0.01***	10.2±0.40*	8.0±0.01***	14.7±0.30*
Root length (cm)	9.9±0.4	1.4±0.62***	7.9±0.40*	2.9±0.23***	8.9±0.10	2.9±0.23***	8.2±0.21	0.5±0.01***	7.8±0.30

Values are mean of three experiments ± SEM

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (significantly different from control (0 h) by one-way ANOVA with Tukey–Kramer comparison test)

mixture were found equivalent to distilled water-treated *P. mungo* and *S. vulgare* seeds. This proved the lower toxicity of the metabolites formed after decolorization of RO16 and textile dye mixture. Phytotoxicity test concerning *P. mungo* and *S. vulgare* confirmed that the biodegradation products of RO16 and dye mixture did not interfere with the germination and growth of plants.

Hence, it is possible to use *Lysinibacillus* sp. RGS-treated effluent sample for irrigation purpose as it degrades various textile azo dyes and produces the metabolites which shows relatively similar seed germination, root length, and shoot length as in the distilled water. Similar results of phytotoxicity were observed during low-dose irradiation pretreatment of microbial decolorization and degradation of Reactive Red 120 (Paul et al. 2013).

Conclusion

This report suggests effect of oxidative stress enzyme in bacteria during textile dye degradation. Significant enhancement of oxidative stress enzymes such as CAT and SOD during the decolorization of RO16 shows its probable role in decolorization of RO16. *Lysinibacillus* sp. RGS was able to rapidly degrade sulfonated azo dye RO16 and dye mixture; therefore, it could prove to be useful for the treatment of textile dyestuffs and textile industry effluent by developing appropriate bioreactor. Analytical studies (HPTLC, GC-MS, and FTIR) confirmed the degradation of RO16 to different metabolites. Cytogenotoxicity and phytotoxicity studies proved the harmless nature of the metabolites when compared to the parent dye. The studies on reactor development using *Lysinibacillus* sp. RGS for treatment of real textile effluents are underway.

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