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Influence of organic and inorganic compounds on oxidoreductive decolorization of sulfonated azo dye C.I. Reactive Orange 16

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ABSTRACT

An isolated bacterial strain is placed in the branch of the *Bacillus* genus on the basis of 16S rRNA sequence and biochemical characteristics. It decolorized an individual and mixture of dyes, including reactive, disperse and direct. *Bacillus* sp. ADR showed 88% decolorization of sulfonated azo dye C.I. Reactive Orange 16 (100 mg L^{-1}) with 2.62 mg of dye decolorized g^{-1} dry cells h^{-1} as specific decolorization rate along with 50% reduction in COD under static condition. The optimum pH and temperature for the decolorization was 7–8 and 30–40 °C, respectively. It was found to tolerate the sulfonated azo dye concentration up to 1.0 g L^{-1}. Significant induction in the activity of an extracellular phenol oxidase and NADH–DCIP reductase enzymes during decolorization of C.I. Reactive Orange 16 suggest their involvement in the decolorization. The metal salt ($CaCl_2$), stabilizers (3,4-dimethoxy benzyl alcohol and *o*-tolidine) and electron donors (sodium acetate, sodium formate, sodium succinate, sodium citrate and sodium pyruvate) enhanced the C.I. Reactive Orange 16 decolorization rate of *Bacillus* sp. ADR. The 6-nitroso naphthol and dihydroperoxy benzene were final products obtained after decolorization of C.I. Reactive Orange 16 as characterized using FTIR and GC–MS.

1. Introduction

Increasing cost of the water for an industrial sector has made the treatment and reuse of textile effluent increasingly attractive for the industry. The common method for the treatment of wastewater in the textile finishing industry is a physicochemical flocculation in combination with the biological treatment [1]. The conventional treatment of the colored effluents produces a lot of sludge and does not remove all dyes, thus preventing recycling of the treated wastewater. Textile industries have huge interest to develop more effective waste water treatment systems, such as physical, chemical and biological or combination of both.

A number of biotechnological approaches have been suggested, including the use of bacteria or fungi, often in combination with physicochemical processes for the treatment of dyes and effluent [2,3]. Wood-rotting fungi were effectively degrading the variety of azo dyes under aerobic conditions [4]. Fungal treatment of the effluents is usually time consuming [5]. Recently, the pure bacterial cultures have been reported to degrade the higher concentration of dyes. The *Pseudomonas* sp. SUK1 and *Bacillus* sp. UVS completely decolorized the 1 g L^{-1} and 250 mg L^{-1} of textile dyes within 14 and 84 h of incubation under static condition, respectively [6,7]. Aerobic bacteria have been described for an oxidative decolorization of many dyes from several classes including sulfonated. In contrast, under anaerobic conditions, the decolorization of many azo dyes via reduction of an azo bond has been shown by anaerobic as well as facultative anaerobic bacteria [8,9]. There are numbers of hypotheses which suggest the mechanism of azo dye decolorization by bacteria using cytosolic enzymes, non-specific extracellular reduction and extracellular enzymatic mechanism. Sulfonated azo dyes were unable to transport through the cell membrane, hence recalcitrant to the biodegradation. Thus, isolation of bacteria which oxidize the sulfonated azo dyes by an extracellular enzymatic mechanism is of considerable interest for the effective biodegradation of the textile dyes.

Very few bacteria (*Bacillus licheniformis*, *Bacillus natto*, *Bacillus sphaericus*, *Bacillus subtilis* and *Pseudomonas desmolyticum* NCIM 2112) expressed the phenol oxidase activity that catalyzed the oxidation of sulfonated azo dyes [10–12]. Organic and inorganic compounds increase the decolorization efficiency of phenol oxidase producing fungal microorganism [13–16].

In this paper, we are reporting the involvement of extracellular oxidase and intracellular reductase enzymes in the decolorization of sulfonated azo dyes from bacterial isolate *Bacillus* sp. ADR. We have also studied the kinetics of dye decolorization, effect of metal salts, electron donors and stabilizers on sulfonated azo dye decolorization performance of *Bacillus* sp. ADR.

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2. Experimental

2.1. Dyestuff, chemicals and microbiological media

All chemicals were of highest purity and of an analytical grade. Sulfonated azo dye C.I. Reactive Orange 16 (mostly used for dying in the textile industries of Ichalkaranji and solapur, India) and other dyes were obtained from textile industries of Ichalkaranji, India. The 3,4-dimethoxy benzyl alcohol (veratryl alcohol) and other fine chemicals were obtained from SRL Chemicals, India. Peptone, yeast extract and agar powder were obtained from Hi-media Laboratory, India.

2.2. Isolation, screening and identification of microorganism

The bacterium was isolated by procedure reported earlier [7]. The isolated organism was aerobic, Gram positive, rod shaped and motile in nature. It showed positive tests for citrate utilization, urea hydrolysis, catalase, oxidase and arginine hydrolysis.

2.3. 16S rRNA sequencing and analysis

The 16S rRNA sequencing of an isolated organism was done in geneOmbio Technologies Pvt. Ltd., Pune, India. The 16S rRNA sequence was initially analyzed at DDJB server (http://www.ddbj.nig.ac.jp) using BLAST (blastn) tool and corresponding sequences were downloaded. The evolutionary history was inferred using the Neighbor-Joining method [17]. The bootstrap consensus tree inferred from 1000 replicates and was considered to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [18]. The clock calibration is used as 0.01 (time node^{-1} height) to convert distance to time. The phylogenetic tree was linearized assuming equal evolutionary rates in all lineages [19]. The tree is drawn to the scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [20]. Phylogenetic analyses were conducted in MEGA4 package [21].

2.4. Microorganism, media and culture condition

Bacillus sp. ADR was grown at 30 °C under static condition and maintained on nutrient agar at 4 °C. Decolorization experiment was carried out in the nutrient broth (g L^{-1}; NaCl, 5; peptone, 10; and yeast extract, 3).

2.5. Decolorization experiments

A loopful of bacterial culture *Bacillus* sp. ADR was inoculated in the 250 mL Erlenmeyer flask containing 100 mL nutrient broth. It was incubated at static and shaking (120 rpm) condition for the growth. The C.I. Reactive Orange 16 (100 mg L^{-1}) was added in each flask after 24 h of incubation and reincubated at 40 °C. Aliquots (3 mL) of the culture media were drawn after regular intervals. Suspended particles were removed from the culture medium by centrifugation at 5000 rpm for 20 min. Decolorization was monitored by measuring the absorbance of the supernatant at 487 nm using Hitachi U-2800 Spectrophotometer. The cell concentration was determined using OD of the culture at 620 nm (OD_{620}). The relation between bacterial cell concentration and OD_{620} was 1.0 OD_{620} = 1.818 g dry cell weight L^{-1}. The COD was measured by the procedure reported earlier [22]. The ability of *Bacillus* sp. ADR to decolorize the C.I. Reactive Orange 16 was tested at a wide pH (1–14) and temperature range (4–50 °C). The potential of bacterial strain to tolerate the higher concentration of C.I. Reactive Orange 16 (200, 400, 600, 800 and 1000 mg L^{-1}) and repeated addition of C.I. Reactive Orange 16 (100 mg L^{-1}) in a fed batch manner was also checked. Percent decolorization and the dry cell weight at different time intervals were measured. Decolorization kinetics was determined using Michaelis–Menten equation, $v_{dye} = v_{dye,max}$ [Dye]/K_m + [Dye]; where $v_{dye,max}$ denoted maximum decolorization rate, K_m denoted Michaelis–Menten constant and [Dye] represented the concentration of dye. The decolorization of various textile dyes individually and in mixture was checked at static condition. The mixture of dyes was prepared by adding reactive, disperse and direct dyes (30 mg L^{-1} concentration of each dye; C.I. Reactive Yellow 84A, Reactive Blue 28, C.I. Reactive Blue 172, C.I. Disperse red 50, C.I. Disperse Brown 118, Direct Orange RL and Direct Brown MR). Decolorization of mixture of textile dyes was measured at 530 nm (λ_{max} of mixture of dyes). All decolorization experiments were performed in three sets. Abiotic (without microorganism) controls were always included.

2.6. Effect of metal salts, stabilizers and electron donors on the decolorization of C.I. Reactive Orange 16

The effect of electron donors on the azo dye decolorization was studied by the addition of sodium acetate, sodium formate, sodium pyruvate, sodium succinate and sodium citrate at 30 mM concentration in 100 mL nutrient broth [23]. Effect of various metals salts, viz. $MgCl_2$, $MnCl_2$, $CaCl_2$, $CuSO_4$, $FeCl_3$, and $ZnSO_4$ on the decolorization was studied by supplementing them in nutrient broth at a final concentration of 0.5 mM. In order to elucidate the role of enzymes in the decolorization, various stabilizers, viz. 50 μM 3,4-dimethoxy benzyl alcohol and 50 μM *o*-tolidine were directly added into the nutrient broth.

To quantify the influence of electron donors, metal salts and stabilizers on decolorization of C.I. Reactive Orange 16, we have calculated the specific decolorization rate of *Bacillus* sp. ADR in the presence of them. The specific decolorization rate was expressed as mg of dye decolorized g^{-1} of dry cells h^{-1}.

2.7. Enzyme assays

2.7.1. Preparation of cell free extract

The 24 h grown cells of *Bacillus* sp. ADR were harvested by the centrifugation at 7000 rpm for 20 min. The cell pellets were suspended in 50 mM potassium phosphate buffer (pH 7.4) and sonicated (30 s, 50 amplitude, 7 strokes) at 4 °C. The sonicated cells were centrifuged in cold condition (4 °C; 7000 rpm; 20 min) and used as the source of intracellular enzymes. Culture media free of cells was used as a source of extracellular enzyme.

2.7.2. Enzyme activities

All enzyme activities were assayed in the cell free extract as well as culture media at room temperature (25 °C). Phenol oxidase activity was determined using the procedure reported earlier [24]. The assay mixture containing 1 mM 3,4-dimethoxy benzyl alcohol and 20 mM sodium citrate buffer (pH 3.0). The reaction was initiated by the addition of an enzyme. The formation of oxidized product was monitored at 310 nm (ε_{310} 9300 M^{-1} cm^{-1}). Lignin peroxidase activity was determined by monitoring the conversion of 3,4-dimethoxy benzyl alcohol to 3,4-dimethoxybenzaldehyde in the presence of hydrogen peroxide at 310 nm as described earlier [13]. One unit of the enzyme activity was defined as the amount of enzyme required to produce 1 μmol of oxidized product min^{-1} under the assay conditions. Phenol oxidase activity was determined using various phenolic and nonphenolic substrates such as *o*-tolidine, hydroquinone, guaiacol, catechol and tyrosine by the

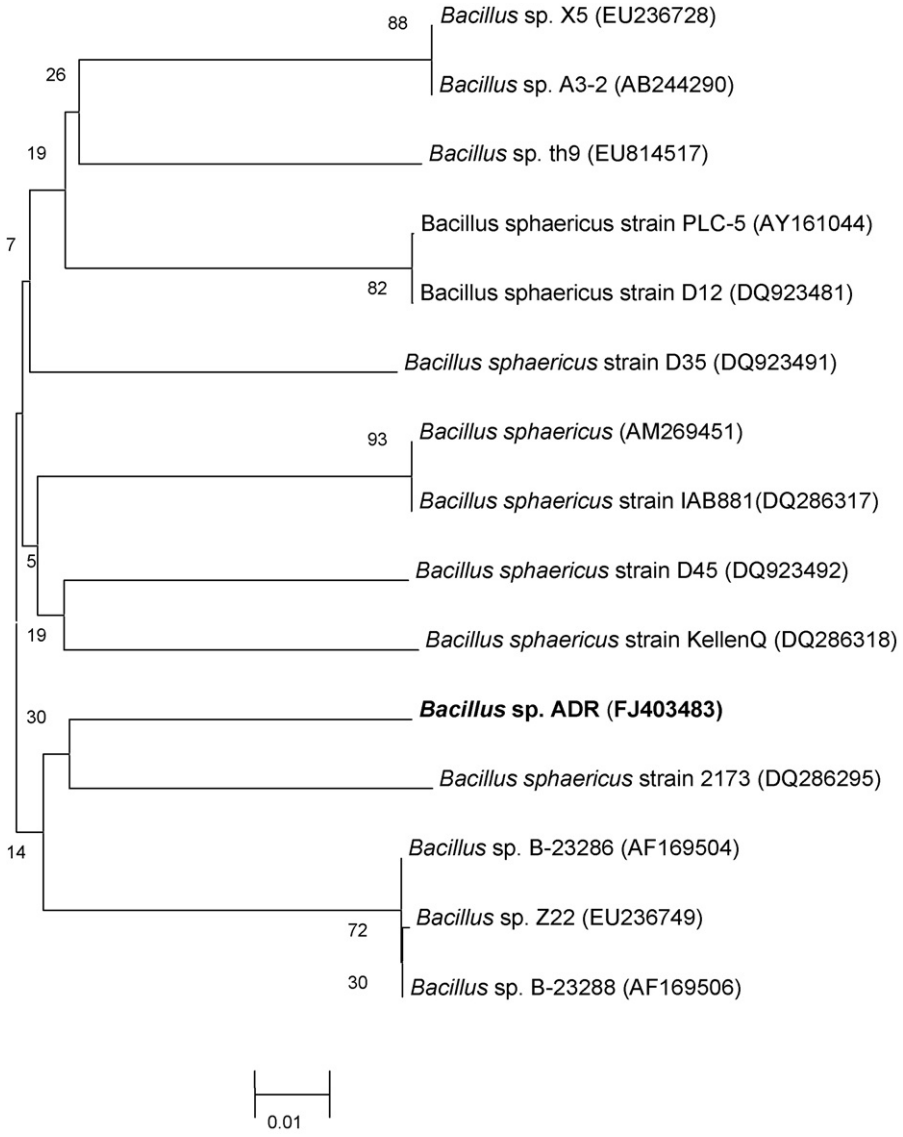


Fig. 1. Phylogenetic analysis of 16S rRNA sequence of *Bacillus* sp. ADR.

procedure reported earlier [25]. Veratryl alcohol oxidase assay was determined in the reaction mixture containing 1 mM 3,4-dimethoxy benzyl alcohol and 20 mM sodium citrate buffer (pH 3.0). The enzyme activity was defined as amount of H_2O_2 formed mg^{-1} of enzyme min^{-1}. H_2O_2 estimation was done by procedure reported earlier [26]. The NADH–DCIP reductase, malachite green reductase and azoreductase assays were carried out using the procedures reported earlier [27–29]. One unit of enzyme activity was defined as amount of enzyme required to reduce 1 μM of substrate min^{-1}. All the enzyme assays were run in triplicate.

Protein concentration was determined by the Lowry method [30] with bovine serum albumin as the standard.

2.8. Extraction and analysis of the metabolites formed after biodegradation

The culture medium was centrifuged at 5000 rpm for 20 min after 12 and 24 h of incubations. Culture supernatant containing the metabolites formed after decolorization was extracted using equal volume of ethyl acetate; dried over anhydrous Na_2SO_4 and concentrated in a rotary vacuum evaporator. Thin layer chromatogram (TLC) was obtained by spotting metabolites obtained after decolorization on the silica gel coated glass plate. The composition of mobile phase was *n*-propanol:water:ethanol:ethyl acetate:glacial acetic acid (3:1:2:2:0.5; v/v). The control dye and metabolites obtained after its decolorization were visualized in an iodine chamber. High performance liquid chromatography (HPLC) analysis was carried out (Waters model no. 2690) on C18 column (symmetry, 4.6 mm × 250 mm) by isocratic method with 10 min run time. The mobile phase was methanol with flow rate of 0.75 mL min^{-1} and UV detector at 487 and 370 nm. The 10 μL of sample was manually injected into the injector port. Fourier transform infrared spectroscopy (FTIR) analysis was done in the mid IR region of 400–4000 cm^{-1} with 16 scan speed. The pellets prepared using spectroscopic pure KBr (5:95) and fixed in the sample holder for the analyses. Rotary vacuum concentrated sample was dissolved in methanol and used for gas chromatography mass spectroscopy (GC–MS) analysis using a QP 5000 mass spectrophotometer (Shimadzu). The ionization voltage was 70 eV. Gas chromatography was conducted in temperature programming mode with a Resteck column (0.25 mm × 30 mm; XTI-5). The initial column temperature was 40 °C for 4 min, then increased linearly at 10 °C min^{-1} to 270 °C and held at 4 min. The temperature of injection port was 275 °C and GC–MS interface was maintained at 300 °C. The helium was

used as carrier gas with a flow rate 1 mL min^{-1} and 30 min run time. The compounds were identified on the basis of mass spectra and using the NIST library stored in the computer software of the GC–MS (version 1.10 beta Shimadzu) [31].

2.9. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparison test. Readings were considered significant when the *P*-value was $\leq$0.05.

3. Results

3.1. Isolation, screening and identification of microorganism

Bacterial isolate which efficiently decolorized various textile sulfonated azo dyes (100 mg L^{-1}; C.I. Reactive Orange 16, C.I. Reactive Red 2, C.I. Reactive Yellow 84A, C.I. Reactive Blue 59 and C.I. Reactive Blue 172) in the nutrient broth was selected. Selected strain was identified as *Bacillus* sp. ADR on the basis of morphology and 16S rRNA sequence. The phylogenetic position of the strain *Bacillus* sp. ADR was determined on the basis of the 16S rRNA sequence (785, FJ403483). Fig. 1 showed the phylogenetic relationship between the strain ADR and other related microorganisms. The homology assay result indicates the strain ADR was in the phylogenetic branch of the *Bacillus* genus and showed 99% sequence homology with other related *Bacillus* sp.

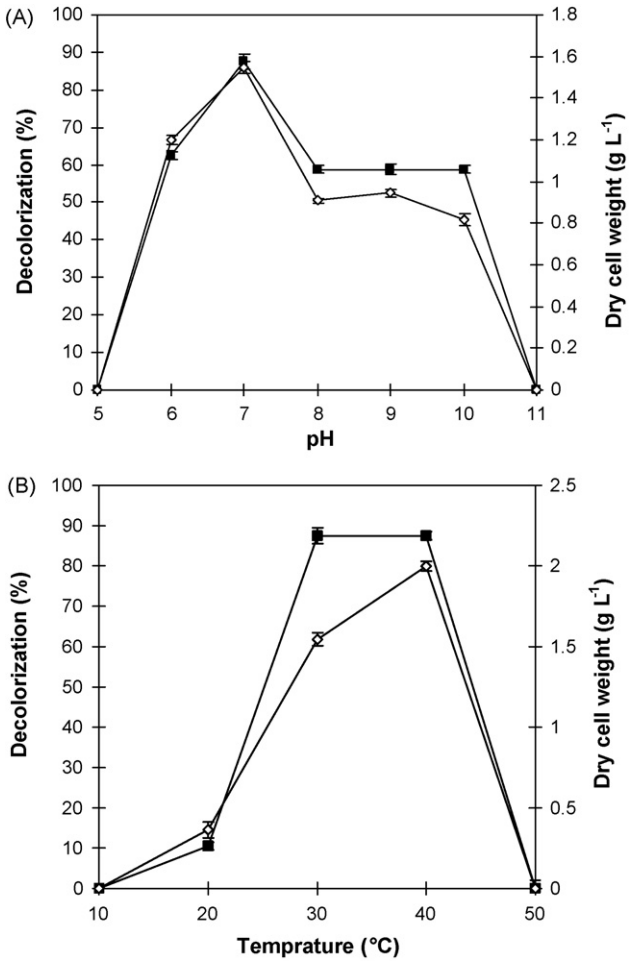


Fig. 2. Effect of pH (A) and temperature (B) on decolorization of C.I. Reactive Orange 16 by *Bacillus* sp. ADR. %decolorization (■) and dry cell weight; g L^{-1} (◊).

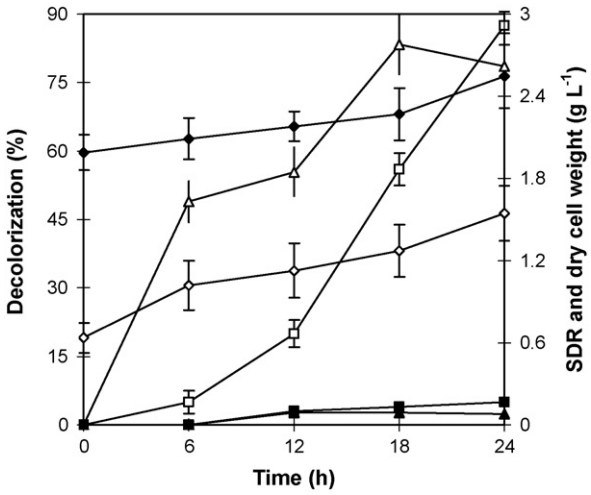


Fig. 3. Comparison of static culture with shake culture. Specific decolorization rate (SDR) expressed as mg of dye decolorized g^{-1} of dry cells h^{-1}; static culture (△), shaking culture (▲). Biomass g L^{-1}; static culture (◊), shaking culture (♦). %decolorization; static condition (□), shaking condition (■).

3.2. Growth and the decolorization of dyes

The *Bacillus* sp. ADR grew well in the pH range 6.0–10.0. The optimum pH and temperature for C.I. Reactive Orange 16 decolorization was 7.0 and 30–40 °C (Fig. 2A and B). It showed decolorization of C.I. Reactive Orange 16 in the nutrient broth and nutrient agar slant. It showed higher specific decolorization rate (2.62 mg of dye decolorized g^{-1} dry cells h^{-1}) and percent decolorization at static condition as compared to shaking (Fig. 3). It showed 50% reduction in COD after 24 h of incubation under static condition. It also showed excellent decolorization of various individual and mixtures of textile dyes (Table 1).

3.3. Effect of increasing concentration and fed batch addition of C.I. Reactive Orange 16

The time required for the decolorization of C.I. Reactive Orange 16 was increased with an increasing concentration of C.I. Reactive Orange 16 (Fig. 4). The decolorization rate increased 1.25 times

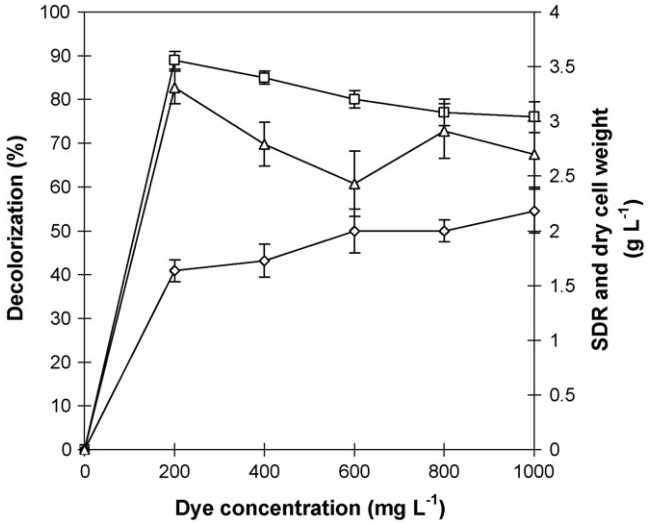
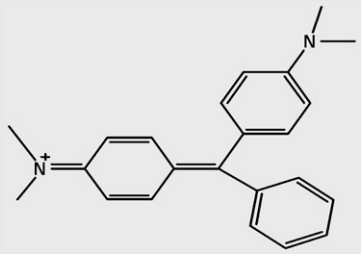
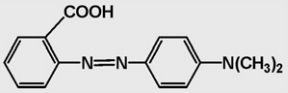
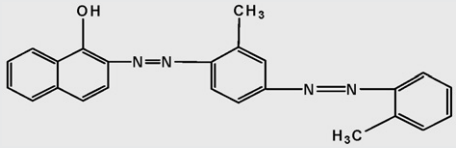
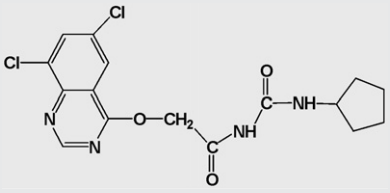
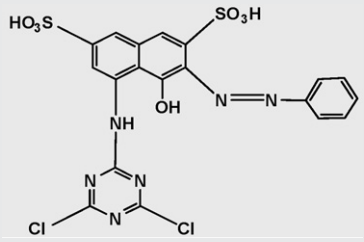
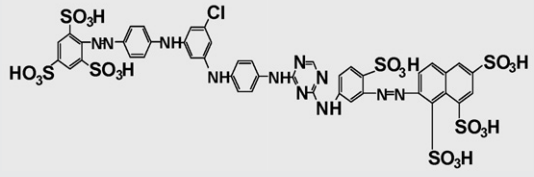
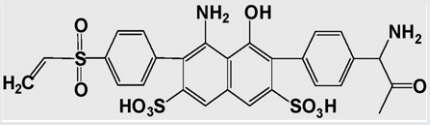


Fig. 4. Influence of increasing dye concentration on biomass and specific decolorization rate of *Bacillus* sp. ADR. Biomass; g L^{-1} (◊), specific decolorization rate (SDR) expressed as mg of dye decolorized g^{-1} of dry cells h^{-1} (△) and %decolorization (□).

Table 1
Decolorization of textile dyes by *Bacillus* sp. ADR.

Dyes	Chemical structure	λ_{max} (nm)	Decolorization (%)	Time (h)
C.I. Basic green 4		620	94	60
C.I. Acid Red 2		430	86	12
C.I. Solvent Red 24		530	90	24
C.I. Direct Red 111	NA	520	90	24
C.I. Disperse Brown 118		440	90	07
C.I. Reactive Red 2		540	90	24
C.I. Reactive Yellow 84A		530	90	24
C.I. Reactive Blue 59	NA	560	92	24
C.I. Reactive Blue 172		610	92	24
Mixture of textile dyes		570	90	36

NA = not available.

at 200 mg L^{-1} concentration of C.I. Reactive Orange 16 as compared to decolorization rate at 100 mg L^{-1} concentration. Further increase in the dye concentration decreased the decolorization rate and percent decolorization, but it was above 2.4 mg of dye decolorized g^{-1} of dry cells h^{-1} and 75%, respectively. The kinetic constants estimated from the experimental data were 3.31 mg of dye decolorized g^{-1} of dry cells h^{-1} for $v_{dye,max}$ and 100 mg L^{-1} for K_m. *Bacillus* sp. ADR decolorized repeated addition of dye (100 mg L^{-1}) for seven cycles without any extra additional supplements (Fig. 5).

3.4. Effect of metal salts, stabilizers and electron donors on the decolorization of C.I. Reactive Orange 16

The specific decolorization rate of *Bacillus* sp. ADR was considerably increased in the presence of sodium acetate (4.53), sodium formate (3.49), sodium citrate (2.97) and sodium pyruvate (4.68) over control (2.62). $CaCl_2$ (4.01) increased the specific decolorization rate of *Bacillus* sp. ADR by 1.5 times and all other metal salts decreased the specific decolorization rate ($MgCl_2$, 2.13; $MnCl_2$, 2.12; $CuSO_4$, 1.87; $FeCl_3$, 1.66 and $ZnSO_4$, 0.65), but none of the metal salts

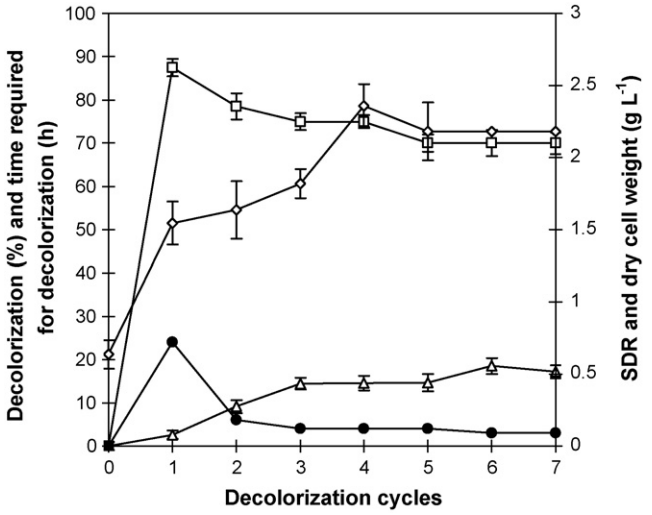


Fig. 5. The fed batch decolorization ability of *Bacillus* sp. ADR. Specific decolorization rate (SDR) expressed as mg of dye decolorized g^{-1} of dry cells h^{-1} (△), %decolorization (□), time required for decolorization in h (●), and dry cell weight in g L^{-1} (◊).

Table 2
Enzymatic status during decolorization of C.I. Reactive Orange 16 after different time interval (0, 12 and 24 h).

Enzymes	0 h	12 h	24 h
Extracellular phenol oxidase[a]	633 ± 02	930 ± 07***	619 ± 03
Intracellular phenol oxidase[b]	26 ± 3.0	12 ± 2.0***	02 ± 0.3***
Azo reductase[b]	1.04 ± 0.52	0.51 ± 0.07***	0.51 ± 0.07***
DCIP reductase[b]	340 ± 01	398 ± 01	828 ± 02***
MG reductase[b]	82 ± 05	71 ± 02	39 ± 03***

All enzyme activities checked after different time interval (0, 12 and 24 h). Values are mean of three experiments ± SEM.
*** Significantly different from control cells at $P < 0.001$ by one-way analysis of variance (ANOVA) with Tukey–Kramer comparison test.
[a] Enzyme activity, U L^{-1} of culture min^{-1}.
[b] Enzyme activity, U mg^{-1} of protein min^{-1}.

inhibited complete decolorization. The 3,4-dimethoxy benzyl alcohol (3.97) and *o*-tolidine (3.41) increased the decolorization rate of *Bacillus* sp. ADR by 1.51 and 1.30 times, respectively, as compared to control (2.62).

3.5. Enzymatic analysis

Extracellular phenol oxidase activity was significantly (259%, $P < 0.001$) increased after 12 h of incubation. Activity of an intracellular phenol oxidase, azoreductase and MG reductase significantly decreased after 12 and 24 h of incubations as compared to control (0 h) (Table 2). NADH–DCIP reductase activity was significantly increased (245%, $P < 0.001$) after 24 h of incubation as compared to control (Table 2). Enzyme assay of the cells grown in absence of the stabilizers showed decrease in extracellular phenol oxidase activity after 24 h of incubation (Table 2). However, in the presence of stabilizer the extracellular phenol oxidase activity was not decreased after 24 h of incubation (Table 3). The culture supernatant did not show the activities of lignin peroxidase, polyphenol oxidase, veratryl alcohol oxidase, azo reductase, NADH–DCIP reductase and MG reductase. Oxidation of various phenolic and nonphenolic substrates by crude extracellular oxidase enzyme was observed (Table 4).

Table 4
Oxidation of phenolic and nonphenolic substrates by crude extracellular phenol oxidase.

Substrates	λ_{max} (nm)	Specific activity[a]
3,4-Dimethoxy benzyl alcohol	310	0.150
o-Tolidine	366	0.125
Hydroquinone	248	0.005
L-DOPA	470	0.075
Guaiacol	420	0.017
p-Cresol	400	ND
Tyrosine	278	ND

One unit of enzyme activity was defined as the amount of enzyme required to increase 1.0 ABS unit min^{-1}.
[a] U mg^{-1} of protein min^{-1}.

3.6. Analysis of the metabolites formed after biodegradation of C.I. Reactive Orange 16

The difference in Rf value of dye (0.92) and formed metabolite (0.80) indicate the biodegradation of C.I. Reactive Orange 16. Decolorized sample did not show absorption peak at 487 and 530 nm (data not shown). Considerable difference in the FTIR spectrum of C.I. Reactive Orange 16 and metabolites obtained after its decolorization indicate biodegradation (Fig. 6). The FTIR spectrum of C.I. Reactive Orange 16 showed the specific peaks in fingerprint region for unsubstituted or multisubstituted benzene and naphthalene ring, supported by the peaks at 627 cm^{-1} for C–C bending vibrations, 664 cm^{-1} for –C–N– bending vibrations, 833 cm^{-1} for S=O stretching vibrations, 1005 cm^{-1} for C–CH_3 bending vibrations, 1054 cm^{-1} for C=O stretching vibrations. The group frequency region showed specific peaks for the functional groups, the peaks at 1409 cm^{-1} for C=C stretching vibrations of benzene ring, 1671 cm^{-1} for N=N stretching vibrations, 1853 cm^{-1}, 2924 cm^{-1} for C–H stretching vibrations in –CH_3 and –CH_2 group, 3427 cm^{-1}

Table 3
Enzymatic status during decolorization of C.I. Reactive Orange 16 in presence of 3,4-dimethoxy benzyl alcohol and *o*-tolidine.

Enzymes	Stabilizers					
	3,4-Dimethoxy benzyl alcohol			*o*-Tolidine		
	0 h	12 h	24 h	0 h	12 h	24 h
Extracellular phenol oxidase[a]	633 ± 04	933 ± 05**	933 ± 05**	633 ± 03	932 ± 04***	932 ± 03***
Intracellular phenol oxidase[b]	26 ± 2.0	11 ± 1.0	02 ± 0.3***	26 ± 4.0	11 ± 3.0***	02 ± 0.3***
Azo reductase[b]	1.04 ± 0.14	0.83 ± 0.07***	0.77 ± 0.07***	1.04 ± 0.06	0.79 ± 0.08***	0.70 ± 0.08**
DCIP reductase[b]	340 ± 05	460 ± 09	398 ± 04	340 ± 05	460 ± 07	368 ± 03
MG reductase[b]	82 ± 02	70 ± 02**	30 ± 01**	82 ± 02	69 ± 06	32 ± 02*

All enzyme activities checked after different time interval (0, 12 and 24 h). NA, no activity. Values are mean of three experiments ± SEM.
* Significantly different from control cells at $P < 0.05$ by one-way analysis of variance (ANOVA) with Tukey–Kramer comparison test.
** Significantly different from control cells at $P < 0.01$ by one-way analysis of variance (ANOVA) with Tukey–Kramer comparison test.
*** Significantly different from control cells at $P < 0.001$ by one-way analysis of variance (ANOVA) with Tukey–Kramer comparison test.
[a] Enzyme activity, U L^{-1} of culture min^{-1}.
[b] Enzyme activity, U mg^{-1} of protein min^{-1}.

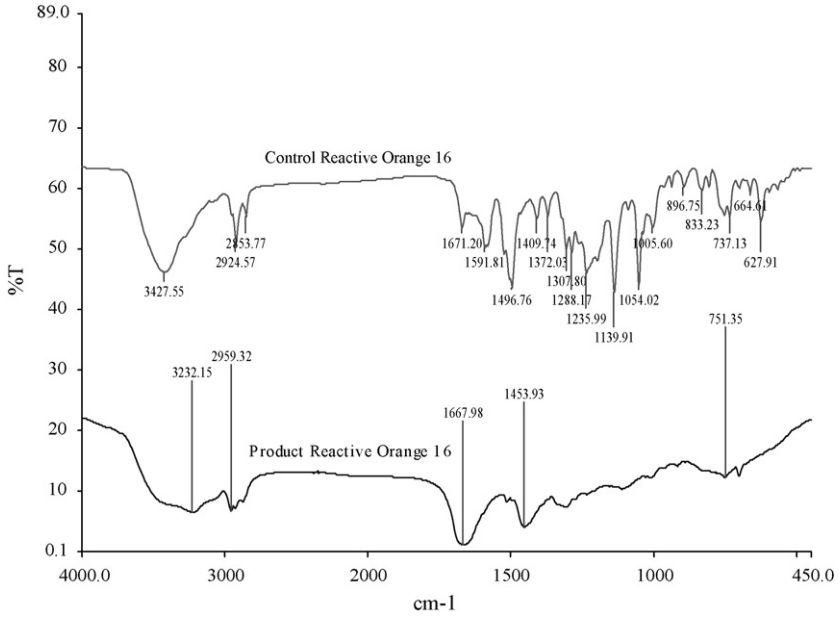


Fig. 6. FTIR spectra of C.I. Reactive Orange 16 and its degradation metabolites.

for N–H stretching vibrations. FTIR spectrum of the metabolites obtained after decolorization showed peaks at 751.35 cm^{-1} for C–N stretching vibrations, 1103.96 cm^{-1} for C–H bending vibrations, 1302.68 cm^{-1} for C–OH stretching vibrations, 1453.93 cm^{-1} for N=O stretching vibrations, 1660 cm^{-1} for C–H bending vibrations and 2959.32 cm^{-1} for C–H stretching vibrations supports the formation of 6-nitroso naphthol and dihydroperoxy benzene. HPLC analysis of C.I. Reactive Orange 16 showed a single peak at 1.94 min (Fig. 7A). The metabolites obtained after decolorization showed the peaks at 2.10, 2.42, 2.76 and 3.17 min (Fig. 7B). Proposed biodegradation pathway of sulfonated azo dye C.I. Reactive Orange 16 was shown in Fig. 8 on the basis of GC–MS analysis of the metabolites obtained after decolorization of C.I. Reactive Orange 16 (Table 5).

4. Discussion

Sulfonated azo dyes are recalcitrant to the biodegradation and widely used in the textile industries of Ichalkaranji and Solapur region, India. Thus, we have selected the C.I. Reactive Orange 16 as a model sulfonated azo dye for the optimization of decolorization process. *Bacillus* sp. ADR efficiently decolorized the individual and the mixture of textile dyes (reactive, disperse and direct dyes). The *Bacillus* sp. ADR showed 88% decolorization of the C.I. Reactive Orange 16 (100 mg L^{-1}) under static condition along with 50% reduction in COD. This observation suggests the ability of *Bacillus* sp. ADR to mineralize the sulfonated azo dyes. Similarly, complete mineralization of Methyl Orange (after 7 days incubation at static condition) by an isolated *Bacillus* sp. was reported previously [32]. The optimum growth conditions (at pH 7.0, 30–40 °C) for *Bacillus* sp. ADR and the ability of decolorization at alkaline pH (8.0–10.0) indicate the use of this microorganism for the decolorization of textile effluent that mostly present in alkaline range. Most of the lignolytic fungi required longer time for the growth and dye decolorization [33,34]. White rot fungus *Dichomitus squalens* poorly decolorized triphenylmethane dyes [34] while, the *Bacillus* sp. ADR efficiently decolorized malachite green, a triphenylmethane dye. *Bacillus* sp. ADR has capability of repeated decolorization of sulfonated azo dye up to seven cycles indicate its industrial applicability. The ability of *Bacillus* sp. ADR to decolorize the mixture of azo dyes and higher concentration (1 g L^{-1}) of an individual azo dye suggests its potential for the treatment of textile effluent.

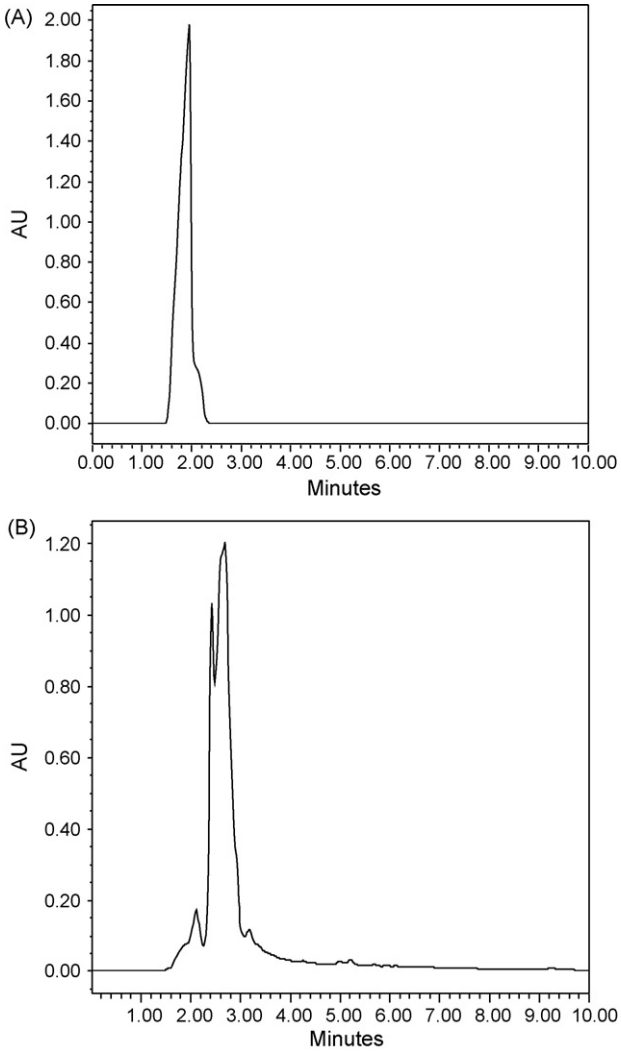


Fig. 7. HPLC elution profile of C.I. Reactive Orange 16 (A) and its degradation metabolites (B).

Table 5
GC–mass spectral data of metabolites formed after C.I. Reactive Orange 16 degradation.

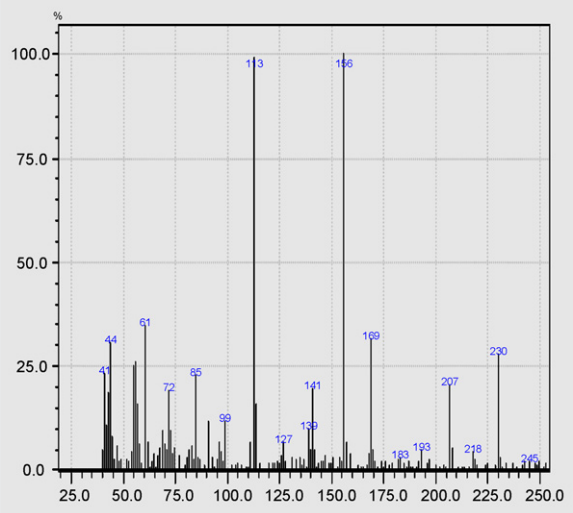
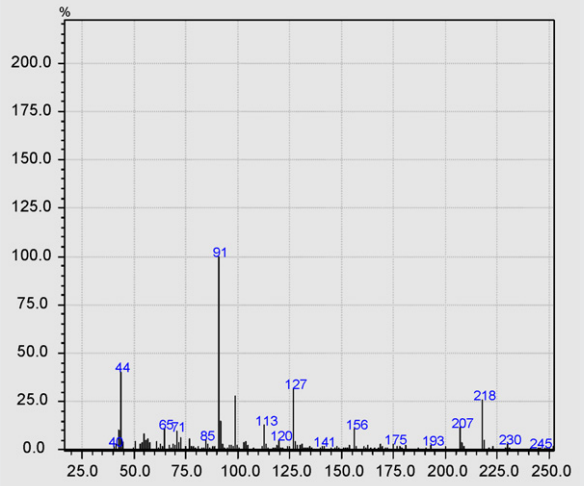
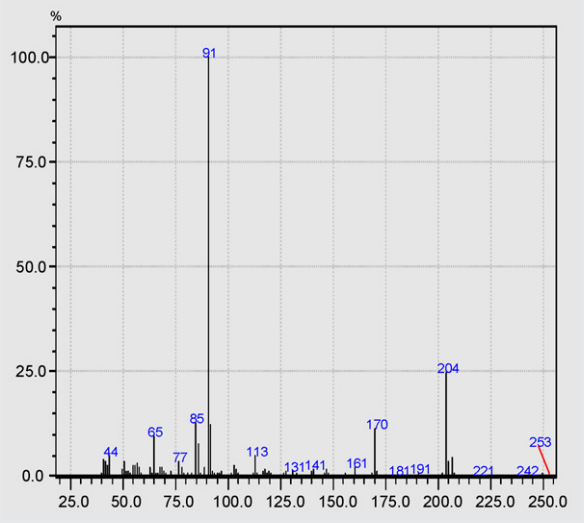
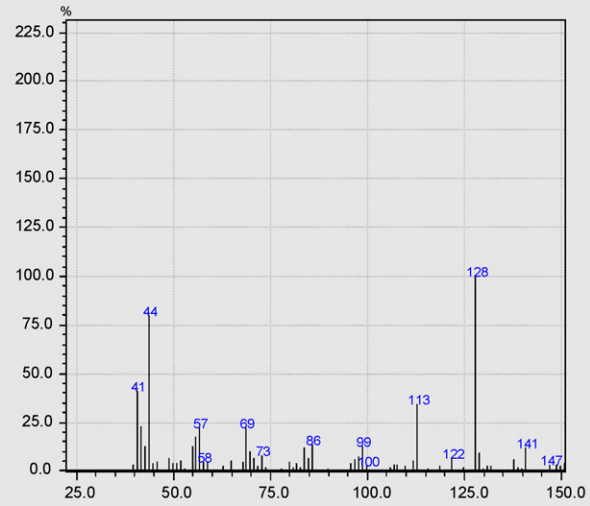
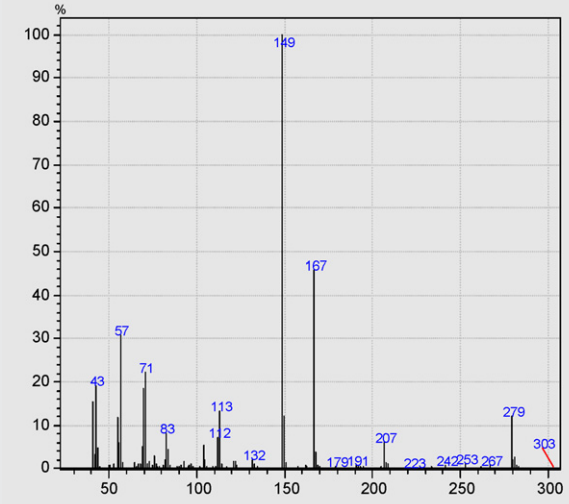
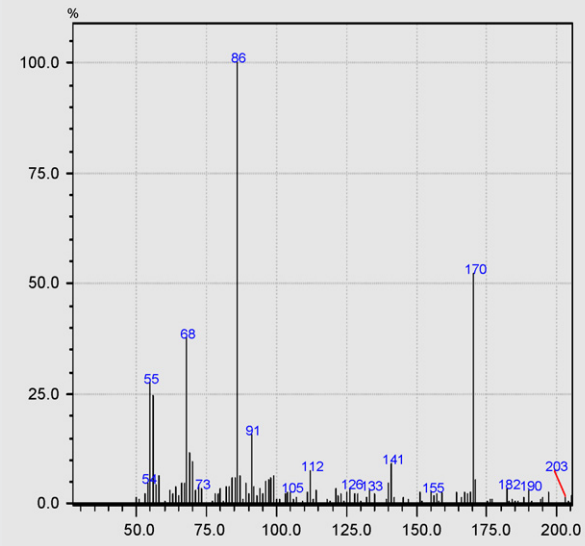
Sr. no.	Rt. (min)	Mw (*m*/*z*)	Area (%)	Name of metabolites	Mass peaks
[I]	ND	295	ND	Phenyl sulfone ethoxy sulfate dizonium	ND
[II][a]	25.87	230	2.64	Hydroperoxy ethyl sulfone phenyl dizonium	
[III][a]	25.74	218	1.56	Dihydroperoxy methyl sulfone benzene	
[IV][a]	26.60	204	4.92	Dihydroperoxy phenyl sulfone	

Table 5 (*Continued*)

Sr. no.	Rt. (min)	Mw (*m*/*z*)	Area (%)	Name of metabolites	Mass peaks
[V][a]	8.85	141	01	Dihydroperoxy benzene	
[VI][a]	26.70	279	17.60	6-Acteamido naphthol 2-sulfonic acid	
[VII][b]	19.65	170	6.90	6-Nitroso naphthol	

ND, not detected.

[a] Metabolites formed after 12 h of decolorization of C.I. Reactive Orange 16.

[b] Metabolites formed after 24 h of decolorization of C.I. Reactive Orange 16.

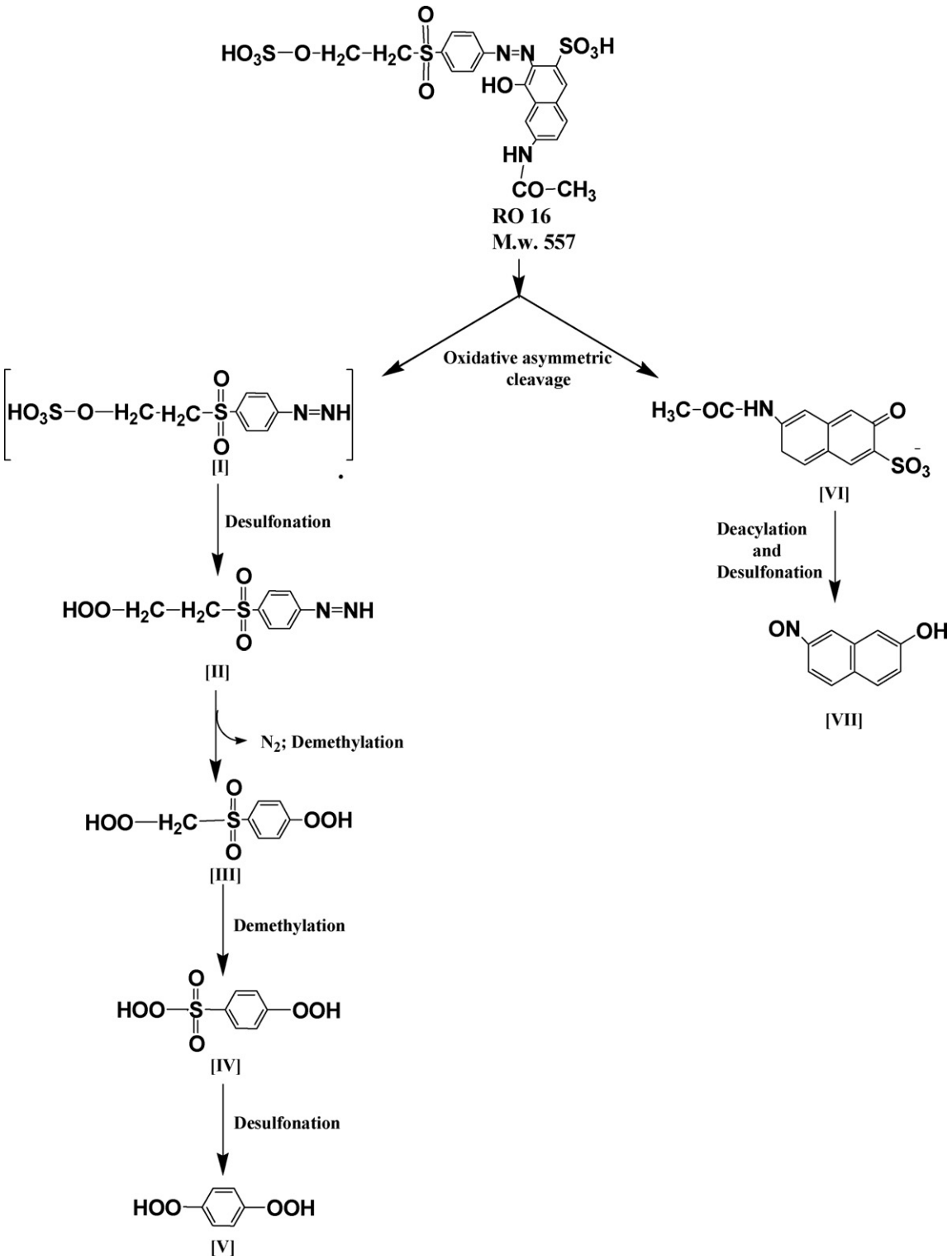


Fig. 8. Proposed pathway of C.I. Reactive Orange 16 degradation by *Bacillus* sp. ADR.

Extracellular oxidase secreted by *Bacillus* sp. ADR oxidized the phenolic (guaiacol, L-dihydroxy phenyl alanine and hydroquinone) and nonphenolic (*o*-tolidine and 3,4-dimethoxy benzyl alcohol) substrates similar to the eukaryotic laccases. *Bacillus* sp. ADR oxidase neither required H_2O_2 for the oxidation of phenolic and nonphenolic substrates nor produced H_2O_2 during oxidation indicate absence of peroxidase and veratryl alcohol oxidase activities in the culture supernatant. *Bacillus* sp. ADR oxidase was unable to oxidize tyrosine suggests absence of polyphenol oxidase activity [35]. Hence, the enzyme is named as "laccase like *Bacillus* sp. ADR phenol oxidase." The ability of *Bacillus* sp. ADR phenol oxidase to react with nonphenolic substrates explained the existence of bacterial phenolic and nonphenolic oxidation systems without lignin peroxidase. Previous reports showed an involvement of polyphenol oxidase from *B. licheniformis*, *B. natto* and *B. sphaericus* in the decolorization of textile dyes [10]. The high stable laccase from *Bacillus*

subtilis SF spores was used for the decolorization of dyes. Significant induction in the phenol oxidase activity during decolorization of sulfonated azo dye C.I. Reactive Orange 16 and decolorization in agar medium suggests its involvement in the decolorization. Induction of NADH–DCIP reductase suggests its involvement in decolorization of C.I. Reactive Orange 16. The exact role of NADH–DCIP reductase is still unknown. It catalyzes the reduction reaction using NADH as electron donor. There is no clear evidence of anaerobic and aerobic azo reductases or non-specific enzymes that catalyze the reduction of a wide range of electron-withdrawing contaminants, including azo dyes [36]. The combined action of oxidoreductases (phenol oxidase and NADH–DCIP reductase) from *Bacillus* sp. ADR might be responsible for the decolorization of sulfonated azo dye C.I. Reactive Orange 16. Lower decolorization rate in shaking cultures might be due to the repressed extracellular phenol oxidase synthesis [37].

Various electron donors and electron acceptors are always present in the environment [38,39]; thus it is important to study the effects of different electron donors and acceptors on bacterial azoreduction. The electron donors, viz. sodium acetate, sodium formate, sodium succinate, sodium citrate and sodium pyruvate enhanced the decolorization rate of *Bacillus* sp. ADR. Similar results were reported earlier [23]. NADH–DCIP reductase requires NADH as an electron donor; thus, sodium acetate, sodium formate, sodium citrate, and sodium pyruvate might act as artificial electron donors for NADH–DCIP reductase. Increased decolorization rate of *Bacillus* sp. ADR in presence of $CaCl_2$ was due to the activation of enzymes involved in decolorization of C.I. Reactive Orange 16. $CuSO_4$, $FeCl_3$ and $ZnSO_4$ decreased the decolorization rate of *Bacillus* sp. ADR, but unable to inhibit complete decolorization at 0.5 mM concentration of metal salts. However, azo dye decolorization by basidiomycete fungus was completely inhibited at 0.1 mM concentration of metal ions [40]. The earlier reports showed an increase in the fungal decolorization rate by the stabilizers of oxidative enzymes [13–16]. The activity of phenol oxidase enzyme was increased after 12 h of incubation and further decreased after 24 h of incubation in absence of stabilizers. In presence of 3,4-dimethoxy benzyl alcohol and *o*-tolidine, the phenol oxidase activity was not decreased after 24 h of incubation. This indicates C.I. Reactive Orange 16 decolorization rate of *Bacillus* sp. ADR was enhanced by stabilizing the extracellular phenol oxidase using 3,4-dimethoxy benzyl alcohol and *o*-tolidine.

HPLC analysis of metabolites formed after biodegradation of C.I. Reactive Orange 16 showed the peaks with different retention times than original dye which indicates the biodegradation of C.I. Reactive Orange 16 into different metabolites. The absence of peak at 1671 cm^{-1} (–N=N– stretching vibrations) in FTIR spectrum of C.I. Reactive Orange 16 degradation products suggested the breakdown of azo bond. We have proposed the biodegradation mechanism on the basis of metabolites identified using GC–MS and enzyme action. Azo dyes can be cleaved symmetrically or asymmetrically, with an active site available for an enzyme to excite the molecule. The induced status of phenol oxidase after 12 h of decolorization and formation of 6-acetamido naphthol 2-sulfonic acid and hydroperoxy ethyl sulfone phenyl dizonium intermediates suggested asymmetric cleavage of C.I. Reactive Orange 16 by phenol oxidase enzyme. Significant induction of NADH–DCIP reductase after 24 h of decolorization and formation of dihydroperoxy benzene and 6-nitroso naphthol intermediates suggested reduction of intermediate metabolites.

Bacillus sp. ADR phenol oxidase is capable of oxidizing several substrates of laccase. This indicates the enzyme may be subspecies of the laccase family. Currently, we have no additional information to determine whether *Bacillus* sp. ADR phenol oxidase has a different reaction spectrum from that of eukaryotic laccase. Regardless of the poor understanding of prokaryotic laccases to date, the oxidase activities detected in environmental *Bacillus* strains suggest the similar enzymes are widespread among this group of bacteria. The previous studies have shown the involvement of phenol oxidases in sporulation, pigmentation of spores, Mn^{2+} oxidation, UV and H_2O_2 resistance in *Bacillus* sp. [41–43]. As suggested in fungi, these enzymes could also play a crucial role in degrading phenolic and nonphenolic compounds to benefit the saprophytic life cycle of soil bacteria in the natural environment. *Bacillus* and related bacteria could be proven as abundant source of phenol oxidase and similar useful enzymes.

5. Conclusion

This study demonstrates the decolorization of sulfonated azo dye C.I. Reactive Orange 16 by combined action of phenol oxidase and NADH–DCIP reductase enzymes of *Bacillus* sp. ADR. *Bacillus* sp. ADR has potential to detoxify sulfonated azo dyes because this reaction releases azo linkages as molecular nitrogen, which prohibits aromatic amine formation. The decolorization efficiency of *Bacillus* sp. ADR could be enhanced by using the metal salt ($CaCl_2$), stabilizers (3,4-dimethoxy benzyl alcohol and *o*-tolidine) and electron donors (sodium acetate, sodium formate, sodium succinate, sodium citrate and sodium pyruvate). Thus, *Bacillus* sp. ADR could be a good biocatalyst for the treatment of textile dyes and industrial effluents.

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