Scientific paper

Kinetics and Mechanism of Reactive Red 141 Degradation by a Bacterial Isolate *Rhizobium radiobacter* MTCC 8161

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Abstract

A bacterium identified as *Rhizobium radiobacter* MTCC 8161 was isolated from effluent treatment plant of textile and dying industry of Ichalkaranji, India. The bacterial isolate *Rhizobium radiobacter* MTCC 8161 was capable of decolorizing various azo, triphenylmethane (TPM), disperse and reactive textile dyes with decolorizing efficiency varying from 80–95%. This strain decolorized (90%) a deep red sulfonated diazo dye Reactive Red 141 (50 mg/L) with 0.807 mg of dye reduced/g of dry cells/h of specific decolorization rate in static anoxic condition at optimum pH 7.0 and temperature 30 °C with 83.33% reduction in COD. The degradation efficiency of this strain using urea and yeast extract showed fast decolorization among different carbon, nitrogen source. The induction of various oxidative and reductive enzymes indicates involvement of these enzymes in color removal. Phytotoxicity studies revealed less toxic nature of decolorized products (1000 mg /L) as compared to original dye. FTIR spectroscopy and GC–MS analysis indicated naphthalene diazonium, p-dinitrobenzene and 2- nitroso naphthol as the final products of Reactive Red 141.

Keywords: Isolation, Sulfonated diazo dye, Biodegradation, *Rhizobium radiobacter*, Phytotoxicity and GC-MS.

1. Introduction

More than 50% azo dyes are used annually, due to simple diazotization reaction mechanism for the production. Around 2000 of them are used in the textile, leather, plastics, paper, cosmetics and foods industries.[1–2] Azo dyes are characterized by the presence of one or more azo groups substituted with aromatic amines. A substituent often found in azo dyes is the sulfonic acid group ($-SO_3H$). The azo dyes containing this substituent are called as sulfonated azo dyes. Sulfonated azo dyes are widely used in the different industries. The fixation rate of these reactive dyes (including azo dyes) in dying is as low as 50%,[3] which results the release of 10–15% water soluble azo dyes into the environment through wastewater discharge.[4] Sulfonated and unsulfonated azo dyes have a negative aesthetic effect on the wastewater and some of these compounds and biodegraded products are toxic, carcinogenic and mutagenic.[5] Azo dyes have large number of structural diversity and they are designed to resist the physical, chemical and microbial attack.[6] Currently various chemical, physical and biological treatment methods are used to remove the color. Because of the high cost and disposal problems, most of the chemical and physical methods for treating dye wastewater are not widely applied in the textile industries.[7] The ecofriendly microbial decolorization and detoxification is a cost competitive alternative to the physical and chemical methods.[8] Early work on biodegradation was more focused on an anaerobic conditions;[9] hence the sequential anaerobic and aerobic bacterial degradation system is efficient in degradation of the sulfonated azo dyes.[10] Recently, degradation of benzidine based azo dye by *Pseudomonas desmolyticum* NCIM 2112[11] and unidentified bacterial consortium[12] had been reported at static anoxic condition. The literature information about the kinetics of decolorization and the environmental factors affecting the decolorization rates is relatively scarce. Monoazo dye decolorization has been reported to follow first-order kinetics with respect to dye concentration by several authors,[13–14] whereas other reports mention zero-order[15–16] or even half-order kinetics.[17] Biodegradation of azo dyes is mainly biological process, either enzymatically catalyzed

reaction involving various oxidative enzymes[18–19] (lignin peroxidase, laccase, and other oxidases) and reductive enzymes[20] (azo reductase and nonspecific reductases) or the reaction with enzymatically reduced electron carriers.[21] The involvement of nonspecific reductases (MG reductase and DCIP reductase) in decolorization of texile dyes was reported earlier.[11]

In this piece of work, we report the isolation and characterization of bacterial isolate capable of decolorizing Reactive Red 141 azo dye in static anoxic condition and also the enzymes (lignin peroxidase, azoreductase, dichlorophenol endophenol reductase, MG reductase and aminopyrine *N*-demethylase) responsible for biodegradation of Reactive Red 141. The objective of this work is to investigate the potential of *R. radiobacter*, decolorization kinetics, degradation metabolites of dyes and fate of toxicity of dye and its degraded metabolites.

2. Experimental

2. 1. Dyestuff, Chemicals and Microbiological Media

All the chemicals were of highest purity and of analytical grade. The textile dye Reactive Red 141[22] (Procion Red HE7B) obtained from local industry of Ichalkaranji, India. ABTS (2, 2' – Azinobis, 3-ethylbenzothiazoline-6-sulfonic acid) and tartaric acid were obtained from Sigma Chemicals Company (St. Louis, MO, USA) and BDH Chemicals (Mumbai, India) n-propanol, catechol and other fine chemicals were from SRL Chemicals, India.

2. 2. Isolation, Screening and Identification of Microorganism

The microorganisms present in the soil samples from the effluent disposal site of a textile-dyeing industry located in Ichalkaranji, India were enriched in the nutrient broth containing various textile dyes (50 mg of the dye/L) in static anoxic condition at 30 °C. After 48 h of incubation the 1 mL of cell suspension was transferred into fresh dye containing broth to screen the strain having color removing ability. The screening procedure in liquid medium was continued until decolorization of the broth. A small amount of decolorized broth was transferred into nutrient agar plates containing various textile dyes (50 mg/L). Colonies surrounded by decolorized zones were selected and isolated by streak plate method. Isolates were then screened for their color removal ability in liquid medium and the best isolate was selected. The bacterial isolate was first examined by gram staining and further identification was done by Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India.

2. 3. Strain, Media and Culture Condition

The pure culture of bacterial isolate was grown at 30 °C in static anoxic condition and maintained on nutrient agar slant at 4 °C. Decolorization experiment was carried out in nutrient broth (0.5% NaCl, 1% peptone, 0.2% yeast extract, 0.1% beef extract). Bushnell and Hass medium (g/L: $MgSO_4$, 0.2; K_2HPO_4,1.0; $CaCl_2$, 0.02; $FeCl_3$, 0.05; NH_4NO_3, 1.0; supplemented with yeast extract, 0.05 and Reactive Red 141 (50 mg/L) was used to study the effect of carbon and nitrogen source on decolorization of Reactive Red 141. The different carbon and nitrogen sources (0.5%) such as glucose, mannitol, sucrose, starch, sodium citrate, peptone, yeast extract, urea, ammonium chloride, and ammonium acetate were used in the present study.

2. 4 Decolorization Experiments

2. 4. 1. Decolorization at Static and Shaking Conditions

Decolorization ability of bacterial isolate was tested in shaking and static culture at optimum pH (7.0) and temperature (30 °C). A loopful of bacterial culture was inoculated in 250 mL Erlenmeyer flask containing 100 mL nutrient broth and grown in static anoxic or shaking (120 rpm) conditions at 30 °C temperature. After 24 h, 50 mg/L of dye was added in each flask and incubated in static as well as shaking condition. Aliquots (3 mL) of the culture media were drawn at a regular time intervals and the suspended particles were removed by centrifugation at 5000 rpm for 15 minutes. The supernatant was used for the analysis of COD and decolorization. Decolorization was monitored by measuring the absorbance at 530 nm using Hitachi U-2800 Spectrophotometer and chemical oxygen demand (COD) was determined according to standard method.[23] The cell concentration was determined by attenuance (OD) of 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. Bushnell and Hass medium was used to study the effect of various carbon and nitrogen sources on the decolorization of Reactive Red 141 in static anoxic condition at 30 °C.

2. 4. 2. Decolorization With Increased Dye and Inoculum Concentration

The various concentrations of dye (100, 200, 300, 400, and 500 mg/L) and inoculum (5, 10, 15, 20 and 25%) were added into the nutrient medium in order to examine the effect of initial dye and inoculum concentration on the decolorization in static anoxic condition. Percent decolorization and dry cell weight at different time intervals were measured. The correlation between the specific decolorization rate and dye concentration can be described by Michaelis Menten kinetics ($v_{dye} = v_{dye,max}$ [Dye] / K + [Dye]); where $v_{dye\ max}$ and K_m denoted maximum decolorization rate and Michaelis Menten constant and [Dye] represents the concentration of Reactive Red 141(mg/L).[24]

The fed batch decolorization of Reactive Red 141 dye was also studied, in this study 50 mg/L dye was added into the 24 h grown culture (100 mL nutrient broth) of bacterial isolate, after decolorization 50 mg/L dye added into the decolorized broth without supplement of additional nutrient. Dye was added continuously until culture does not lose decolorization ability. All decolorization experiments were performed in three sets. Abiotic (without microorganism) controls were always included. The dye concentration was determined by monitoring the absorbance of dye at 530 nm. The percent decolorization was determined by procedure reported earlier.[25]

2. 5. Enzyme Assays

2. 5. 1. Preparation of Cell Free Extract

The 24 h grown cells of bacterial isolate were harvested by centrifugation (7000 rpm, 20 minutes) and suspended in 50 mM potassium phosphate buffer (pH 7.4) and sonicated (5.0 second, 50 amplitude, 7 strokes) at 4 °C. The sonicated cells were centrifuged and the supernatant was used as source of enzyme.

2. 5. 2. Enzyme Assay

All the enzymatic activities were assayed in cell free extract as well as cultural supernatant. Lignin peroxidase (LiP) assay was performed by procedure reported earlier.[26] One unit of enzyme activity corresponds to change in 0.1 U of absorbance /minute. Enzyme activity was expressed as units of enzyme/minute/mg of protein. Aminopyrine *N*-demethylase and NADH–DCIP reductase activity was determined using a procedure reported earlier.[27] The malachite green (MG) reductase assay was carried out using procedure reported earlier.[28] Azoreductase assay mixture contained the 4.45 μM of methyl red (MR) and 100 μM NADH in 50 mM phosphate buffer (pH 7.4) and 0.1 mL of enzyme solution. This reaction mixture was pre-incubated for 4 minutes followed by the addition of NADH and monitored for the decrease in color absorbance (430 nm) at room temperature. Azoreductase activity was expressed in terms of MR reduction which was calculated by using molar absorption coefficient of 0.023 $\mu M^{-1} cm^{-1}$ at 430 nm.[29] All enzyme assays were run in triplicate and average rates were calculated.

2. 6. Extraction and Analysis of Biodegraded Product

After 48 h of decolorization, the entire decolorized medium was centrifuged at 5000 rpm for 20 minutes. Supernatant of dye decolorized components were extracted using equal volume of ethyl acetate, dried over anhydrous Na_2SO_4 and concentrated in a rotary vacuum evaporator. The decolorization was monitored by Uv-vis spectrophotometer (Hitachi U-2800). The biodegradation was analyzed by TLC, HPLC and FTIR spectroscopy. HPLC analysis was carried out (Waters model no. 2690) on C_{18} column (symmetry, 4.6 × 250 mm) by isocratic method with 10 minutes run time.[11] The mobile phase was methanol with flow rate of 0.75 mL/minute and Uv detector at 370 nm. Biodegradation of Reactive Red 141 was characterized by Fourier Transform Infrared Spectroscopy (Perkin Elmer, Spectrum one) and compared with control dye. FTIR analysis was done in the mid IR region of 400–4000 per cm with 16 scan speed. The pellets prepared using spectroscopic pure KBr (5 : 95), were fixed in sample holder and analyses were carried out. Rotary vacuum concentrated sample was dissolved in methanol and GC-MS analysis of metabolites was carried out 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 minutes, then increased linearly at 10 °C per minute to 270 °C and held at 4 minutes. The temperature of injection port was 275 °C and GC/MS interface was maintained at 300 °C. The helium was carrier gas; flow rate was 1 mL per minute and 30 minutes run time. The compounds were identified on the basis of mass spectra and using the NIST library stored in the computer software (version 1.10 beta Shimadzu) of the GC-MS.

2. 7. Toxicity Study

Phytotoxicity test were performed in order to assess the toxicity of Reactive Red 141 and its degraded metabolites. Tests were carried out according to the procedure reported earlier[30] on two kinds of seeds which are commonly used in Indian agriculture: *Triticum aestivum* and *Oryza sativa*. The Reactive Red 141 and ethyl acetate extracted product (dry) were dissolved separately in distilled water and made the final concentration of 1000 mg/L. Toxicity study was done by growing the seeds (10) of each plant species separately into control, dye and extracted product sample. Germination (%), length of plumule and radical was recorded after 13 days.

2. 8. Statistical Analysis

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

3. Results and Discussion

3. 1. Isolation, Screening and Identification of Microorganism

The bacterial isolate which efficiently decolorized the various textile dyes in submerged culture was selected.

Table 1. Morphological, cultural and biochemical test for identification of strain.

Test	*R. radiobacter*
Colony morphology	
Configuration	Circular
Margin	Entire
Elevation	Slightly raised
Surface	Smooth
Opacity	Translucent
Gram's reaction	Negative
Cell shape	Very small rod
Size (μm)	Length: 1 – 2 μ, Width : < 1μ
Motility	+
Biochemical Tests	
Growth on MacConkey agar	+ (NLF)
Citrate utilization	+
H2S production	+
Urea hydrolysis	+
Catalase test	+
Oxidase test	+
Nitrate reduction	+
Argenine hydrolysis	+
Phosphatase	W

NLF = Non-Lactose Fermenter, w = weak.

The isolated strain was identified and deposited as *Rhizobium radiobacter* MTCC 8161 based on morphological, cultural and biochemical tests (Table 1) into the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India. The sulfonated azo dyes are recalcitrant in nature, thus the isolation of bacteria capable of decolorization and mineralization of sulfonated azo dyes has proven difficult.[31] Our isolated bacterial strain *R. radiobacter* efficiently decolorized and degraded the sulfonated diazo dye Reactive Red 141 at static anoxic condition.

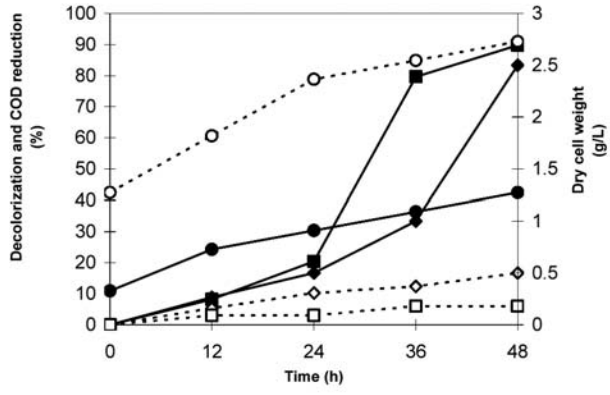

Figure 1. Comparison of shake culture with static culture with respect to effect on cell growth, dye decolorization and COD reduction in nutrient broth. Biomass: shake culture (○), static culture (●). Dye decolorization: shake culture (□), static culture (■), and COD reduction: shake culture (◇), static culture (◆).

3. 2. Effect of Static and Shaking Condition

The cell growth in shaking condition was much higher than static condition but there was little decolorization (6%) and COD reduction (16.66%) in shaking condition, while 90% decolorization with 83.33% decrease in COD was observed at static anoxic condition within 48 h (Figure 1). The *pseudomonas* sp. decolorized the Direct Black 38 within 5 days under anaerobic condition[32] and *Stenotrophomonas acidaminiphila* (BN-3) decolorized the C. I. Acid Red 88 within 60 h.[33] The pH of the culture medium remains constant (pH 7.0) during the entire decolorization process.

3. 3. Effect of Initial Dye and Inoculum Concentration

R. radibacter was efficiently decolorizes the increasing concentration of dyes (100, 200, 300, 400 and 500 mg/L) with a decolorizing efficiency varying from 60–90%, where the diazo dye Reactive Black 5 decolorization by *Candida oleophila* was significantly inhibited

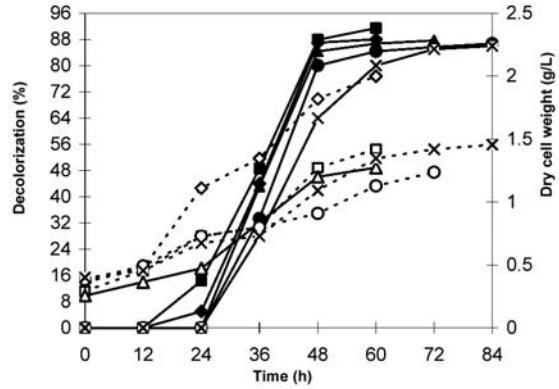

Figure 2. Effect of increasing dye concentration on decolorization performance and growth of *R. radiobacter.* Dye concentration (mg/L): 100 (■), 200 (◆), 300 (▲), 400 (●), 500 (×). Biomass (g/L) at dye concentration (mg/L):: 100 (□), 200 (◇), 300 (△), 400 (○), 500 (×).

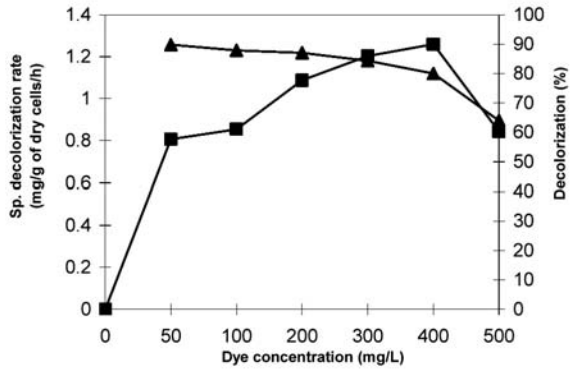

Figure 3. Effect of dye concentration on specific decolorization rate (■) and equilibrium conversion (▲) of Reactive Red 141 by *R. radiobacter.*

at 300 to 500 mg/L.[34] The time required for decolorization was proportional to dye concentration. The increase in dye concentration has no adverse effect on the growth of an organism (Figure 2). After 24 h, the growth of an organism increases with increase in dye concentration. The relation between substrate dependent specific decolorization rate and equilibrium conversion was shown in Figure 3.

The kinetic constants estimated from the experimental data were 1.26 mg/g cell /h for $v_{dye,\ max}$ and 50 mg/L for $K_{m.}$ The specific decolorization rate significantly increases with increase in dye concentration (more than 50%) and equilibrium conversion was above the 80% (50 to 400 mg/L). With further increase in dye concentration, the specific decolorization rate decreased and the equilibrium conversion was above 60% at 500 mg/L of dye concentration, similar results were reported in case of azo dye decolorization by *Rhodopseudomonas palustris.*[24]

There was increase in decolorization rate with rise in inoculum concentration. The maximum rate of color removal was observed at a 20% inoculum concentration with 90% decolorization efficiency. Beyond the 20% inoculum decolorization rate was not significantly increased, similar result was reported earlier.[35]

3. 4. Decolorization of Repeated Addition of Dye Aliquots

The intention of this study was to check the ability of organism for the repeated decolorization of dye. The *R. radiobacter* decolorized the repeated addition of Reactive Red 141 dye up to six cycles with variable decolorization rate (74–90%), whereas the mixed bacterial culture decolorized the azo dye up to three cycles.[36] In first cycle 90% decolorization of first dye aliquot required 48 h, 79% decolorization within 12 h in second and third cycles, the percent decolorization (74%) and time (6 h) remains constant up to last cycle (Figure 4).

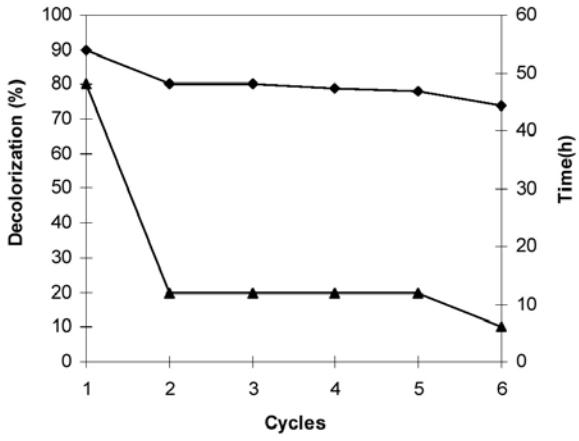

Figure 4. Fed batch decolorization of Reactive Red 141. % decolorization (◆), Time (h) (▲).

3. 5. Effect of Carbon and Nitrogen Sources

The reduction of azo dyes depends on the presence and availability of a co-substrate[37] because it acts as an electron donor for the azo dye reduction. The rate of azo-reduction process also depends on the type of co-substrate used and chemical structure of the azo dyes.[38] Many different co-substrates were found to suite as electron donor, like glucose[14] and yeast extract.[39] Reactive Red 141 was decolorized up to 2% in control (BHM medium), whereas, up to 85.71% decolorization was observed in presence of yeast extract (0.5%) and urea (0.5%) in BHM. When only yeast extract was supplemented as sole carbon and nitrogen source, 56.52% decolorization observed. Similar results were reported in Bacterium RvM 11.1 in presence of glucose and yeast extract.[40] In order to check the influence of other carbon sources on the decolorization pattern of Reactive Red 141, the yeast extract was supplemented with glucose, mannitol, sucrose, starch and sodium citrate. There was significant growth with reduced decolorization rate in presence of these effective carbon and energy sources, 11.42% decolorization was observed in glucose (0.5%) as carbon source, 36.58% in mannitol (0.5%), 17.50% in sucrose (0.5%) and 22.22% in sodium citrate (0.5%) (Table 2).

Table 2. Effect of carbon and nitrogen source on decolorization of Reactive Red 141 by *R. radiobacter* MTCC 8161.

Carbon and Nitrogen source	Decolorization (%)	Dry cell weight (g/L)
BHM (Control)	02	0.72±0.02
BHM + Glucose + Yeast extract	11	1.36±0.07
BHM + Glucose + Urea	02	1.54±0.015
BHM + Mannitol + Yeast extract	36	1.27±0.07
BHM + Sucrose + Yeast extract	17	0.45±0.06
BHM +Starch + Yeast extract	10	0.94±0.02
BHM + Sodium citrate + Yeast extract	22	1.27±0.03
BHM + Yeast extract	56	1.45±0.01
BHM + Peptone	07	1.72±0.01
BHM + Peptone + Yeast extract	27	2.18±0.04
BHM + Urea	27	1.09±0.02
BHM + Urea + Yeast extract	85	1.63±0.05
BHM + Ammonium chloride	10	0.36±0.03
BHM + Ammonium chloride + Yeast extract	20	0.23±0.01
BHM + Ammonium acetate	02	0.27±0.04
BHM + Ammonium acetate + Yeast extract	13	0.18±0.06

BHM = Bushnell Hass Medium.

The effect of various nitrogen sources on the decolorization performance *R. radiobacter* was tested by using organic (each of 0.5%; urea, yeast extract and peptone) as well as inorganic (each of 0.5%; ammonium acetate and ammonium chloride) nitrogen sources (Table 2). The or-

ganic nitrogen source supported decolorization, mainly urea, yeast extract and peptone. The inorganic nitrogen sources give poor growth and low decolorization efficiency. Urea and yeast extract are effective co-substrates for better decolorization by *R. radiobacter*, where the Bacterium RVM 11.1 and mixed bacterial culture showed maximum decolorization in presence of peptone and yeast extract as co-substrate.[36–40]

Table 3. Enzyme activity of control (0 h) and Induced state (48 h decolorization).

Enzyme assay	Control	Induced
azo reductase[a]	1.05 ± 0.07	1.86 ± 0.08
DCIP reductase[b]	7.67 ± 0.24	8.62 ± 0.20***
MG reductasec	9.91 ± 0.31	32.70 ± 1.00***
Lignin peroxidase (Intracellular)[d]	3.63 ± 0.1	4.33 ± 0.7
Lignin peroxidase (Extracellular)[d]	11 ± 0.3	12 ± 0.97
Aminopyrine *N*-demethylase[f]	0.104 ± 0.02	0.123 ± 0.04

[a] µmoles of Methyl red reduced/mg of protein/minute.
[b] µg of DCIP reduced/mg of protein/minute.
[c] µg of MG reduced/mg of protein/minute.
[d] Enzyme activity – Units /mg of protein/minute.
[f] nmoles of formaldehyde released/mg of protein/minute.

3. 6. Enzymatic Analysis

The term azo dye reduction may involve different mechanisms or locations like enzymatic,[41] non-enzymatic,[21] mediated,[42] intracellular[43] and various combinations of these mechanisms and locations. Oxidative biodegradation takes place upon action of enzymes such as peroxidases and laccases. The involvement of fungal peroxidases and laccases for the oxidation of sulfonated azo dyes has been reported earlier.[44] Bacterial extracellular azo dye oxidizing peroxidases have been characterized in *Streptomyces chromofuscu.*[45] Lignin peroxidase catalyzes the oxidative breakdown of azo dye Disperse Orange 3,

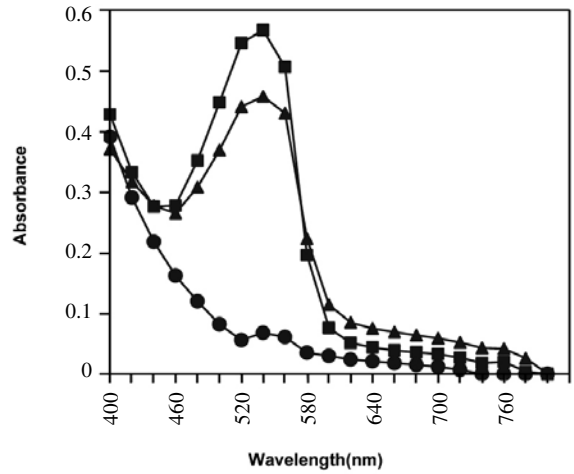

Figure 5. Spectrophotometer analysis of decolorization of Reactive Red 141. 0 h spectra (■), 24 h spectra (▲), 48 h spectra (●).

which results into the formation of nitrobenzene as a major product[47]. The involvement of intracellular lignin peroxidase in decolorization of dyes and xenobiotic compounds was reported earlier.[11,24,46] The induction in the activity of intracellular lignin peroxidase (119%), extracellular lignin peroxidase (109%), azo reductase (177%), MG reductase (330%) and DCIP reductase (112%) was observed when compared with the control (0 h) (Table 3). In this study, induction of lignin peroxidase (extracellular and intracellular), azo reductase, MG reductase and DCIP reductase strongly indicate that Reactive Red 141 can be oxidatively and reductively cleaved into the simple metabolites.

3. 7. Analysis of Biodegraded Products

The difference in Rf value of dye (0.846) and decolorized product (0.615) indicates biodegradation of Reac-

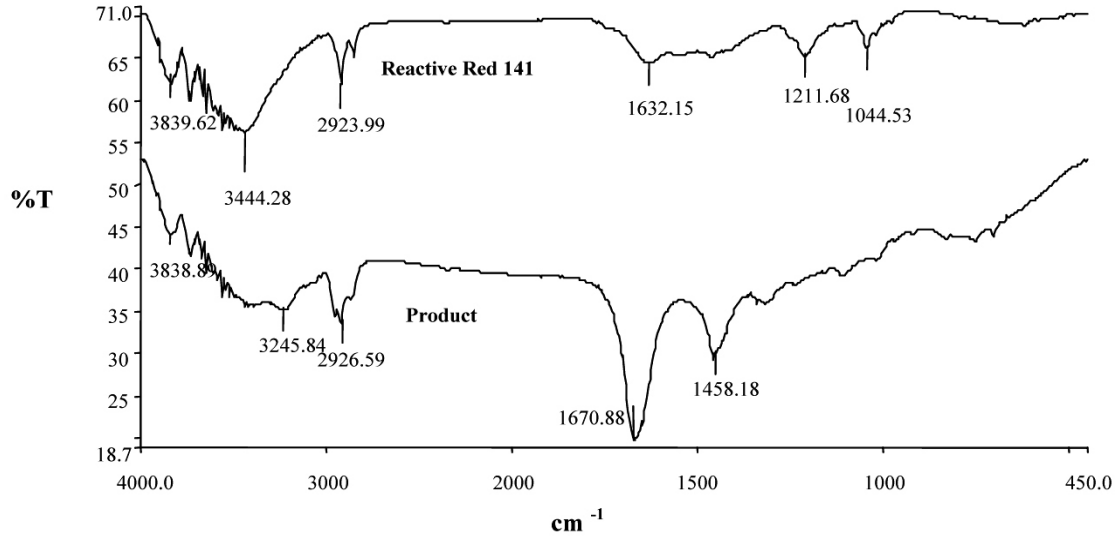

Figure 6. FTIR spectra of Reactive Red 141 and its degradation metabolites.

tive Red 141. Decolorized sample did not show absorption peak at 530 nm (Figure 5).

The considerable difference in FTIR spectrum of Reactive Red 141 and degradation product indicate biodegradation (Figure 6). The FTIR spectra of control Reactive Red 141 showed the specific peaks in fingerprint region for disubstituted or multisubstituted benzene ring which is supporting the peak at 1044 cm^{-1} for –C–Cl– m-ortho substituted aromatic bending vibration and the peak at 1211.48 cm^{-1} asymmetric $–SO_{3-}$ stretching vibrations. The group frequency region shows specific peaks for functional groups, the peak at 1632 cm^{-1} for –N=N– stretching vibrations of azo group and the peaks at 2923.99 cm^{-1} and 3444.28 cm^{-1} for –OH– and –NH– stretching vibrations represents the presence of hydroxyl (–OH) and secondary amino (–NH–) groups in Reactive Red 141. The FTIR spectrum of extracted product shows peak at 1458 cm^{-1} for –N=O stretching vibrations, the peak at 1670.88 cm^{-1} –N=N– stretching vibrations supports the formation of naphthalene diazonium and p-dinitrobenzene and peak at 2926.59 cm^{-1} for –OH– stretching vibration revealed formation of the

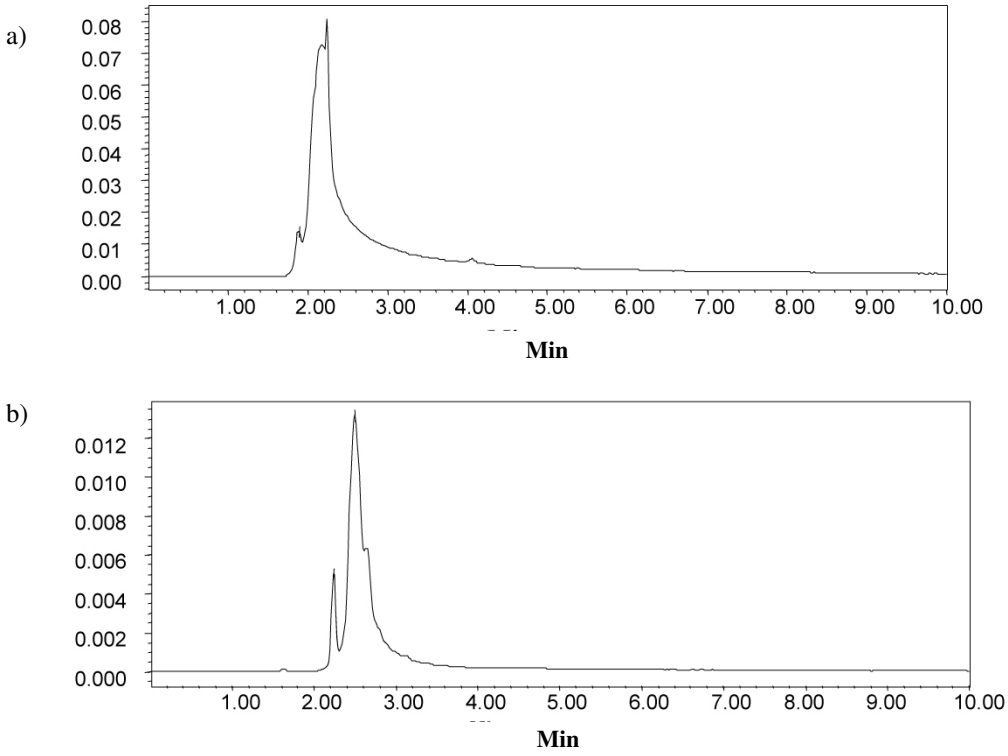

Figure 7. HPLC elution profile of Reactive red 141 (a) and its degradation metabolites (b).

Table 4. Phytotoxicity of Reactive Red 141 and its degradation product.

	Triticum aestivum			*Oryza sativa*		
Parameters Studied.	**Water**	**Reactive Red 141[b]**	**Extracted metabolite[b]**	**Water**	**Reactive Red 141[b]**	**Extracted metabolite[b]**
Germination (%)	100	60	80	100	30	80
Plumule (cm)	16.25 ± 1.38	11.41 ± 1.10*	17.50 ± 0.84$^{\$}$	4.11 ± 0.29	0.0 ± 0.00***	3.16 ± 0.210$^{\$\$\$}$
Radical (cm)	3.63 ± 0.2	0.35 ± 0.05**	5.33 ± 0.60	3.33 ± 0.719	0.30 ± 0.04***	1.26 ± 0.27

[b] 1000 mg/L concentration phytotoxicity study of Reactive Red 141 and its metabolites formed after biodegradation, values are mean of germinated seeds of three experiments ± SEM, significantly different from the control (seeds germinated in water) at *P<0.05 **P <0.01, ***P <0.001. Significantly different from Reactive Red 141 treated group $ P <0.05 and $$$ P <0.001 by one-way analysis of variance (ANOVA) with Tukey-kramer multiple comparison test.

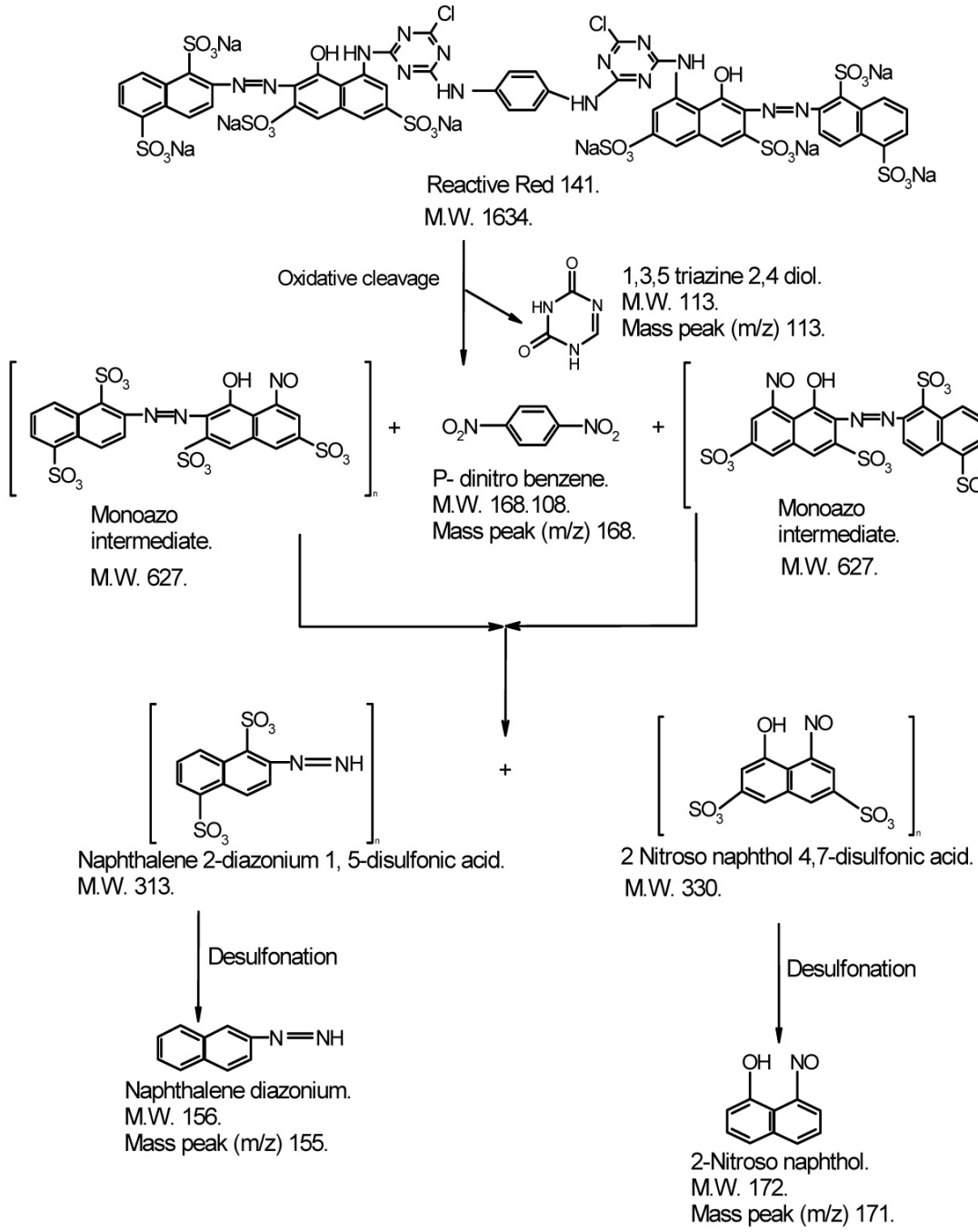

Figure 8. Proposed pathway of biodegradation of Reactive Red 141 by *R. radiobacter.*

naphthol. HPLC analysis of Reactive Red 141 showed a medium peak at 1.89 minute and sharp peak at 2.243 minute, the degradation metabolites has two sharp peaks at 2.241 minute, 2.498 minute and minor peaks at 4.80, 6.84 minutes (Figure 7). The GC-MS spectra showed conversion of Reactive Red 141 into naphthalene diazonium (23.648 minute, 34.9% area and 155 m/z), 1, 3, 5-triazine 2, 4-diol (21.322 minute, 5.6% area and 112 m/z), p-dinitrobenzene (21.328 minute, 4% area and 168 m/z) and 2-nitroso naphthol (26.133 minute, 14% area and 171 m/z) (Figure 8).

3. 8. Toxicity Study

Phytotoxicity revealed the toxic nature of Reactive Red 141 to the *Triticum aestivum* and *Oryza sativa* plants. Germination (%) of both the plants was less with Reactive Red 141 treatment as compared to their degradation pro-

duct and distilled water. The Reactive Red 141 was significantly reducing the length of plumule and radical than its degradation product which indicated the less toxicity of the degraded products (Table 4).

4. Conclusion

Hence the present study indicates biodegradation of Reactive Red 141 through oxidative and reductive enzymes of *R. radiobacter* into different metabolites. We suggested the degradation pathway of Reactive Red 141 on the basis of GC-MS analysis. Azo dyes can be cleaved symmetrically or asymmetrically depending on the structure of substrate and active site of an enzyme. Initially asymmetric oxidative cleavage leads to formation of monoazo intermediates which can be further oxidatively and reductively cleaved into simple metabolites. The identified products were naphthalene diazonium, 1, 3, 5-triazine 2, 4-diol, p-dinitrobenzene and 2-nitroso naphthol. Due to involvement of various enzymes it is very difficult to define exact role of each enzyme in biodegradation, it require the further purification of enzymes. The strain has potential to degrade various dyes having structural hierarchies into less toxic compounds.

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Povzetek

Iz odpadne vode tekstilne industrije v Ichalkaranji, Indija, je bila izolirana bakterija *Rhizobium radiobacter* MTCC 8161. Ta bakterija je sposobna razbarvanja različnih azo, trifenilmetanskih, disperznih in reaktivnih barvil pri statičnih anoksičnih pogojih ter pri optimalni pH vrednosti 7,0 in temperature 30 °C do 80–95 % stopnje z 83,33 % redukcijo KPK. Poskusi so bili izvedeni z različnimi viri ogljika in dušika od katerih sta kvasni ekstrakt in sečnina dala najboljše rezultate. Razbarvanje lahko pripišemo različnim induciranim oksidativnim in reduktivnim encimom. Toksikološke študije so pokazale manj toksično naravo razgradnega produkta.