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Decolorization and degradation of azo dye – Reactive Violet 5R by an acclimatized indigenous bacterial mixed cultures-SB4 isolated from anthropogenic dye contaminated soil

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ABSTRACT

Azo dyes an important group of synthetic compounds are recalcitrant xenobiotics. Conventional technologies are unsuccessful to efficiently remove these compounds from contaminated environment. However, consorted metabolic functioning of innate microbial communities is a promising approach for bioremediation of polluted environment. Bacterial mixed cultures SB4 proficient in complete decolorization of azo dye – Reactive Violet 5R was developed through culture enrichment technique. Bacterial community composition based on 16S rRNA gene analysis revealed that mixed cultures SB4 composed of six bacterial strains namely *Bacillus* sp. V1DMK, *Lysinibacillus* sp. V3DMK, *Bacillus* sp. V5DMK, *Bacillus* sp. V7DMK, *Ochrobacterium* sp. V10DMK, *Bacillus* sp. V12DMK. SB4 grew well in minimal medium containing low amount of glucose and yeast extract (YE) (1 g/L) and decolorized 200 mg/L of RV5 within 18 h under static condition. Mixed cultures SB4 decolorized wide range of azo dyes and maximum rate of decolorization was observed at 37 °C and pH 7.0. Decolorization efficiency was found to be unaltered under high RV5 and salt concentration where 1500 mg/L of RV5 was decolorized in presence of 20 g/L NaCl. We propose the asymmetric cleavage of RV5 and Fourier transformed infrared (FTIR), NMR and gas chromatography–mass spectrometry (GC–MS) confirmed the formation of four intermediatory compounds 1-diazo-2-naphthol, 4-hydroxybenzenesulphonic acid, 2-naphthol and benzenesulphonic acid.

1. Introduction

The production of synthetic compounds and their applications for human welfare is essential but an uncontrolled and undesirable discharge into the environment is of great concern. Different dyes are commonly found in industrial effluents. Amongst various dyes manufactured annually, azo dyes are the largest class of synthetic dyes used worldwide in industries ranging from textile to pharmaceuticals [1–5].

They are characterized by the presence of one or more azo bonds (—N=N—) with aromatic rings. Different substitutions on aromatic nucleus give structurally diverse and most versatile group of compounds which makes them recalcitrant and xenobiotic [6,7]. Due to their xenobiotic characteristic such compounds prove to be toxic, mutagenic and carcinogenic [8].

Therefore, to comply with the strict environmental legislation restricting the untreated discharge into the environment, industries are employing various technologies including physical, chemical and biological methods for the wastewater remediation [9]. But, conventional wastewater treatment technologies employing physico-chemical methods are unsuccessful to remove these recalcitrant dyestuffs from effluents. Moreover because of their high operational cost, lower efficiency, in-applicability to a wide variety of dyes and generation of byproducts which in-turn are toxic and require further processing [10]. On the contrary, bioremediation proved to be most viable, cost effective and feasible green technology. The general approach is to enhance the ability of native microorganisms from anthropogenic polluted environment which are well adapted and competent to clean such sites [4,11,12].

Decolorization process begins with reductive cleavage of azo group generating corresponding aromatic amines in anaerobic environment. These intermediatory aromatic amines are reported to be mutagenic and carcinogenic [3,13–16]. Therefore, complete mineralization of azo compounds for effective remediation is essential. Such amines are further degraded under aerobic condition [9,17].

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Several studies demonstrate the use of pure cultures for decolorization of azo dyes [1,11,13,18,19]. However, individual species have limited metabolic capability to mineralize dye completely and in many cases it has been clearly observed that mainly due to lack of catabolic pathway aromatic amines are not further degraded. Catabolic and synthrophic interactions of indigenous species lead to complete degradation of azo dyes [12,16].

In light of serious environmental threat and due to lack of complete metabolism cascade in pure cultures, our study was focused on characterization of enriched bacterial mixed cultures SB4. Various nutritional and environmental parameters were optimized for efficient dye decolorization. Further, an attempt have been made to understand the biodegradation pattern of RV5 by mixed cultures SB4 using ultraviolet–visible (UV–vis) and Fourier transformed infrared (FTIR) spectroscopy, ^{1}H nuclear magnetic resonance (^{1}H NMR) spectrometry and gas chromatography–mass spectrometry (GC–MS) analysis of degraded products. To our knowledge, this is the first study demonstrating complete degradation of azo dye –Reactive Violet 5R.

2. Materials and methods

2.1. Dyes and culture media

Azo dye – Reactive Violet 5R (RV5) has been very widely used in a large scale by several textile and dyeing industries. Moreover, it is a sulfonated polycyclic aromatic compound (detailed structure is described in Section 3.10) and hence it was selected as a model dye in the study. RV5 and other synthetic dyes were procured from Mantung Dyestuff and Intermediates, Ahmedabad, Gujarat, India.

A minimal Bushnell Haas medium (BHM) amended with varying concentration of glucose and yeast extract (YE) (0.1–5 g/L) (BGY) and 100 mg/Lof RV5 was used for isolation of mixed cultures SB4 (henceforth SB4).

Decolorization of RV5 in the presence of various co-substrates was studied by substituting glucose with 1 g/L of sucrose, lactose, pyruvate and starch in BHM containing 1 g/L YE. Similarly, decolorization efficiency of SB4 was studied by replacing YE by 1 g/L peptone, nitrate salts of ammonium, sodium and potassium and urea in BHM containing 1 g/L glucose.

Glucose and YE at varying concentrations (0.1–5 g/L) were supplemented in BHM to optimize their concentrations for efficient decolorization of RV5.

2.2. Development and enrichment of mixed cultures SB4

Soil samples collected from Kharicut canal (22°57′ 878″ N; 72° 38′ 478″ E) receiving industrial effluents from various dye manufacturing units in the Vatva Industrial Estate, GIDC, Ahmedabad, Gujarat, India were used to develop RV5 decolorizing SB4 by enrichment culture technique. Briefly, BHM medium containing different concentration of various carbon and nitrogen sources amended with 100 mg/L of RV5 was inoculated with soil suspension (10 g/L) and incubated under static condition for complete decolorizatoin of dye. Upon complete decolorization and repeated sub-culturing in fresh BHM medium containing glucose and YE (BGY) amended with 100 mg/L of RV5, SB4 exhibited consistent dye decolorization and growth.

2.3. Inoculum development

10 mL of 24 h grown SB4 (in BGY medium amended with 100 mg/L of RV5) was harvested at 3000 × *g* for 5 min at 4 °C followed by resuspending the cell pellet in 1 mL of sterile distilled water and subsequently used as an inoculum to confer the effect of various nutritional and environmental conditions on RV5 decolorization.

2.4. Isolation of pure cultures and bacterial identification

Serial dilutions of SB4 were plated onto Luria agar and Reasonar's Agar and incubated at 37 and 20 °C. Isolated colonies with distinct morphology were screened to obtain different culturable bacteria present in the mixed cultures.

Genomic DNA of all the isolates was extracted following Ausubel et al. [20]. 16S rRNA gene was amplified using universal eubacterial primers 8F and 1492R following Desai et al. [21]. The purified amplified products were sequenced by automated DNA Analyzer 3730 using ABI PRISM BigDye™ Terminator Cycler Sequencing v3.1 Chemistry (Applied Biosystem, USA). 16S rRNA gene sequence was analyzed using BLAST(n) programme at NCBI server to identify bacterial strains.

2.5. Decolorization assay

Aliquots of 2.0 mL from experimental and control medium were withdrawn at regular intervals of 6 h upto 48 h or till complete decolorization was observed and centrifuged at 6000 × *g* for 15 min at 4 °C to obtain clear supernatant. RV5 decolorization was monitored at 558 nm (λ_{max}) by Double Beam Specord® 210 BU UV–vis spectrophotometer (Analytica Jena AG, Germany) against media blank and growth was monitored turbidometrically by resuspending the cell pellet in distilled water and measuring absorbance at 600 nm. RV5 decolorization was studied under static environment at 37 °C and pH 7.0, unless specified. Uninoculated media with 100 mg/L of RV5 served as abiotic control. All the experiments were performed in triplicates.

2.6. Effect of different environmental conditions

RV5 decolorization was studied at different pH (5–10), temperature (4–55 °C), NaCl concentration (0–40 g/L), different initial RV5 concentration (100–2000 mg/L) and in presence of different dyes (100 mg/L), where decolorization efficiency was determined at their respective absorbance maxima.

2.7. Biodegradation analysis

SB4 was grown at 37 °C under static condition till complete decolorization of RV5 in BGY medium. Biodegradation was studied by extracting metabolites from culture supernatant with equal volumes of ethyl acetate and evaporating it to dryness in a SpeedVac (Thermo Electron Corporation, Waltham, MA). The extracted metabolites were mixed with HPLC grade potassium bromide (KBr) in the ratio of 5:95 and analysed at mid IR region (400–4000 cm^{-1}) by FTIR using PerkinElmer, spectrum GX spectrophotometer (PerkinElmer, USA). For ^{1}H NMR spectrometry studies, extracted metabolites were dissolved in appropriate volume of D_2O and were analysed by Bruker ^{13}C NMR-400 MHz (Bruker, USA). GC–MS analysis of metabolites was performed using Auto-System XL (PerkinElmer, USA). Gas chromatography was conducted using capillary column TES–MS (0.25 μm × 0.25 μm × 30 m) with helium as a carrier gas at a flow rate of 1 mL/min with a speed ratio of 1:40. The initial column temperature was maintained at 70 °C for 4 min and linearly increased at 10 °C/min to 290 °C and held for 4 min. The temperature of injection port was 250 °C and GC–MS interface was maintained at 220 °C. Compounds were identified on the basis of their mass spectra and NIST library.

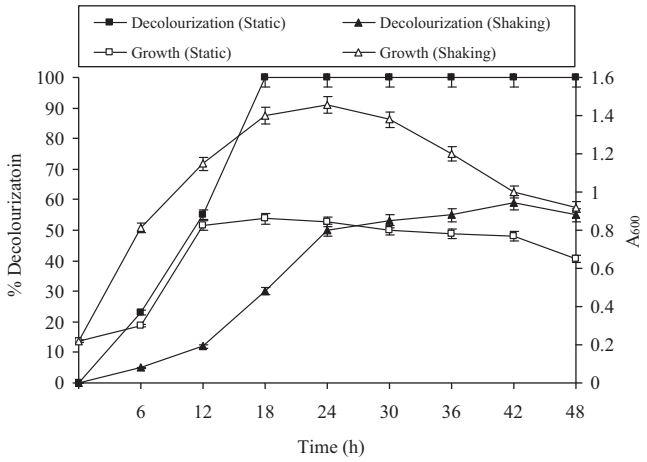

Fig. 1. Decolorization of RV5 (100 mg/L) by mixed cultures SB4 in BGY medium under static and shaking condition (110 rpm) determined as a function of time at 37 °C.

3. Results and discussion

3.1. Development and identification of bacterial mixed cultures SB4

Over the years it has been observed that microorganisms indigenous to the contaminated environment are efficient in degrading xenobiotic/recalcitrant compounds. But with a limited catabolic pathway individual microbial strain is incapable for complete mineralization; however synergistically in mixed population they are efficient in degrading environmental pollutants.

Kharicut canal in Vatva Industrial Estate, Ahmedabad, has been receiving waste water from various dye manufacturing industries for several years and thus became pertinent site for obtaining well acclimatized indigenous microorganisms capable of catabolising various dyes. SB4 competent in decolorizing and degrading a Reactive Violet 5 (RV5) was developed by culture enrichment method from above mentioned soil samples exhibiting consistent growth and decolorization during each successive transfer.

The bacterial community structure analysis on the basis of 16S rRNA gene sequencing study showed that SB4 composed of six bacterial species namely *Bacillus* sp. V1DMK, *Lysinibacillus* sp. V3DMK, *Bacillus* sp. V5DMK, *Bacillus sp.* V7DMK, *Ochrobacterium* sp. V10DMK, *Bacillus* sp. V12DMK having NCBI accession no. JF975597, JF975599, JF975601, JF975603, JF975605, JF975607 representing two bacterial phyla Firmicutes and Proteobacteria.

Bacterial strains of phyla Firmicutes has been recently reported forming a major portion of acclimatized textile sludge capable of decolorizing Direct Black 38 [22]. We have recently established the dominance of Firmicutes at anthropogenic polluted environment; where several *Bacillus* sp. were isolated having an ability for simultaneous detoxification of RV5 and heavy metal Cr(VI) in a continuous bioreactor system [23]. Moreover, *Bacillus* sp. is well documented for its dye decolorizing potential in several studies [14,24,25].

3.2. Effect of dissolved oxygen (DO) on decolorization

Since bacterial decolorization is a reductive process, presence of molecular oxygen in a medium may diminish its reduction potential. We have studied the decolorization and growth profile of the SB4 at 37 °C in BGY medium amended with 100 mg/L of RV5 under agitation of 110 rpm and static condition (Fig. 1). There was a 3.3 fold increase in decolorization potential of SB4 as DO level dropped from 6.1 mg/L to nearly 0.4 mg/L under static condition. During this process it was observed that while having relatively faster growth and higher cell density (A_{600} 1.3) at oxygen rich environment as compare to static condition (A_{600} 0.8), decolorzation was much less due to presence of oxygen. At the same time, SB4 under static growth exhibited complete decolorization within 18 h. In a similar study by Chang et al. [26] decolorization of Reactive Red 22 by *Pseudomonas luteola* significantly increased when DO dropped to 0.08 mg/L under static condition from 4.4 mg/L. During dye decolorization in aerobic environment electrons released by oxidation of electron donors are preferentially utilized to reduce free oxygen rather than azo dyes [16].

Since SB4 has shown decolorization under microaerophilic environment, the demand for oxygen is very less and that will save great deal of kinetic energy require in aeration system and will reduce operational cost.

3.3. Effect of carbon and nitrogen sources

SB4 was able to decolorize only 4% of 100 mg/L of RV5 within 18 h without any carbon or nitrogen source, whereas on supplementing glucose and YE in a medium, decolorization was enhanced by 25 fold (Fig. 2a). And complete decolorization was observed within 18 h at 37 °C under static condition. However, in absence of YE only 11% RV5 was decolorized within 18 h; whereas SB4 without glucose exhibited 55% decolorization. Hence, preliminary studies revealed the obligatory requirement of nitrogen source for metabolism of RV5 [3]. Prerequisite of carbon and nitrogen sources for better decolorization of azo dyes was demonstrated in several studies [3,12,27].

The effect of various carbon sources on RV5 decolorization was studied in BHM medium along with 1 g/L YE by substituting glucose. When sucrose and lactose were used as carbon source decolorization was 42% and 21%, respectively within 18 h (Fig. 2a). Decolorization in presence of pyruvate and starch was only 30% and 40%; while glucose conferred complete decolorization of RV5 within 18 h. Glucose being the simplest carbohydrate was preferred carbon source in RV5 decolorization by SB4.

With progressive increase in glucose concentration from 0.1 to 1 g/L, rate of decolorization also increased. Complete decolorization was observed in presence of 1 g/L of glucose concentration (Fig. 2c). But, surprisingly upon further increase in glucose concentration to 5 g/L efficiency of RV5 decolorization decreased and only 85% of dye was decolorized within 24 h (Fig. 2c). Parshetti et al. [28] observed that *Kocuria rosea* MTCC 1532 fail to decolorized Malachite Green in the presence of 10 g/L glucose and only 31% decolorization was observed even at 30 g/L initial glucose concentration. Explaining such a phenomenon of negative effect of glucose on decolorzation, Chang et al. [26] stated that it might be related to metabolic regulation known as glucose/catabolic repression. Further, they added that during such repression there is a high possibility of inhibiting the transcription of cyclic-AMP-dependent genes (due to presence or at higher concentration of glucose), few of them might be involving in dye decolorization, encoding for azoreductase [26]. Henceforth, 1 g/L glucose concentration was used as optimal carbon source for further characterization of the SB4.

The efficiency of SB4 in the presence of various nitrogen sources was studied in BHM medium along with 1 g/L glucose at 37 °C under static condition by substituting YE. On providing different salts of nitrate, ammonium nitrate exhibited only 20% decolorization of the dye, whereas sodium and potassium nitrate decolorized 35% and 30% respectively within 18 h (Fig. 2b). Likewise, in presence of urea and peptone 16% and 70% of RV5 was decolorized respectively. In accordance with the other studies [11,12,19,27], YE confer complete decolorization of RV5 within 18 h providing better decolorization efficiency compare to other nitrogen sources. Kapdan et al. [29] reported YE as dual source of carbon and nitrogen

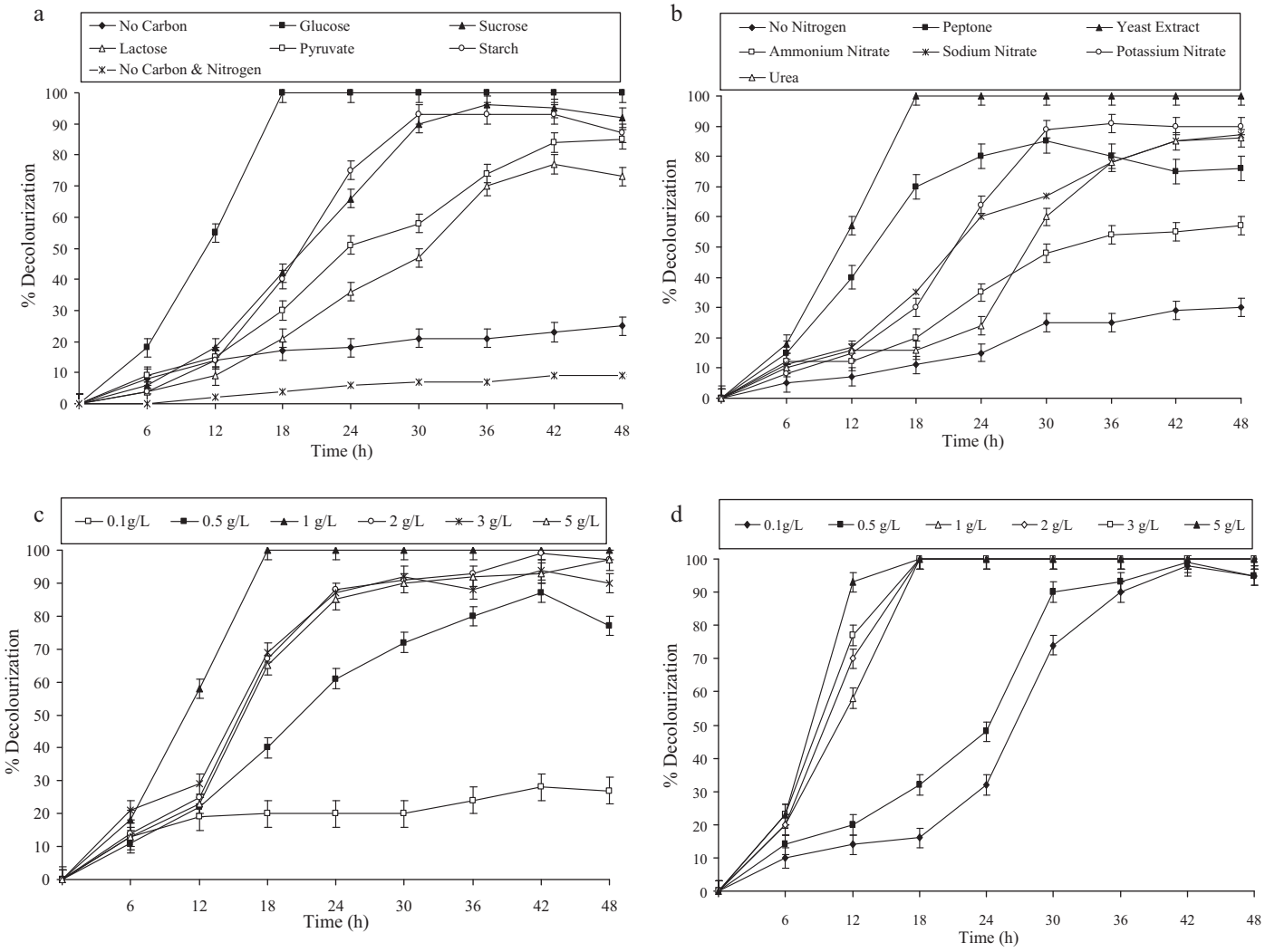

Fig. 2. Effect of co-metabolites on dye decolorization by mixed cultures SB4 under static condition at 37 °C, pH 7.0: (a) Effect of various carbon sources (1 g/L) on RV5 (100 mg/L) decolorization in BHM along with 1 g/L yeast extract. (b) Effect of various inorganic and organic nitrogen sources (1 g/L) on RV5 decolorization in BHM along with 1 g/L glucose. (c) RV5 decolorization by mixed cultures at increasing concentration of glucose in BHM along with 1 g/L yeast extract. (d) RV5 decolorization by mixed cultures at increasing concentration of yeast extract in BHM along with 1 g/L glucose.

in decolorization of various dyes by mixed bacterial consortium PDW.

With increase in YE concentration (0.1–5 g/L) efficiency of decolorization remains unaltered as observed from Fig. 2d. Therefore, 1 g/L YE concentration was used as optimal nitrogen source along with 1 g/L glucose in BHM for further characterization of SB4. Organic nitrogen sources such as YE are considered essential for regeneration of NADH which act as electron donor in azo bond reduction [11,12,14].

3.4. Effect of temperature and pH

SB4 exhibited more than 85% decolorization over a broad range of pH 5–8.5. Maximum decolorization was obtained at neutral pH 7.0 (Fig. 3a). However, apparent decrease in decolorization was observed towards more alkaline pH and it decreased by 3.3 fold at pH 10. Further, pH between 2 and 6 found to be inhibitory for *Bacillus* sp. VUS during decolorization of Brown 3REL [25]. But, on contrary to above study we obtained comparative decolorization at lower pH (Fig. 3a). *Clostridium bifermentas* SL186 optimally decolorized Reactive Red 3B-A in alkaline pH of 10, whereas *Bacillus firmus* exhibited better decolorization in pH 6.0 [9,30].

With increase in incubation temperature we observed linear increase in dye decolorization. SB4 exhibited maximum decolorization of RV5 at 37 °C (Fig. 3b). At higher temperature of 55 °C, the rate of decolorization was relatively stable with 70% of dye was decolorized within 48 h. *Bacillus* sp. VUS showed maximum decolorization activity at 40 °C, whereas lower temperature of 30 °C was found to be optimum for halophilic bacterium *Halomonas* sp. GTW [25,31].

3.5. Effect of initial dye concentration

Decolorization of various initial RV5 concentrations exhibited distinct pattern of dye decolorization, where 1200 mg/L of dye was completely metabolized within 24 h. Less than 18 h was required by the SB4 to degrade 200 mg/L of RV5 as observed from Fig. 5. Upon further increase in dye concentration to 1500 mg/L, complete degradation of RV5 was observed within 42 h (Fig. 3c). Moreover it was observed that cell density simultaneously decreased from A_{600} 0.8 to A_{600} 0.2 with an increase in dye concentration (200 mg/L to 1500 mg/L). Thus, decline in decolorization efficiency at higher concentration of dye may be attributed to the presence of four

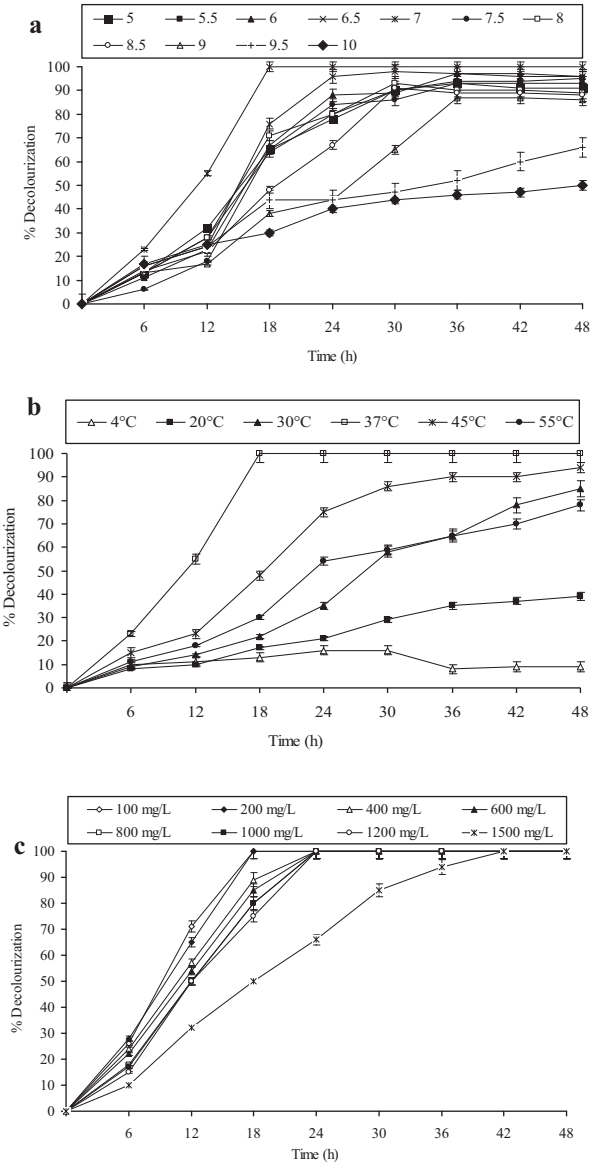

Fig. 3. RV5 decolorization by mixed cultures SB4 (a) at different temperature (b) varying initial pH (c) at different initial RV5 concentration in BGY medium under static condition.

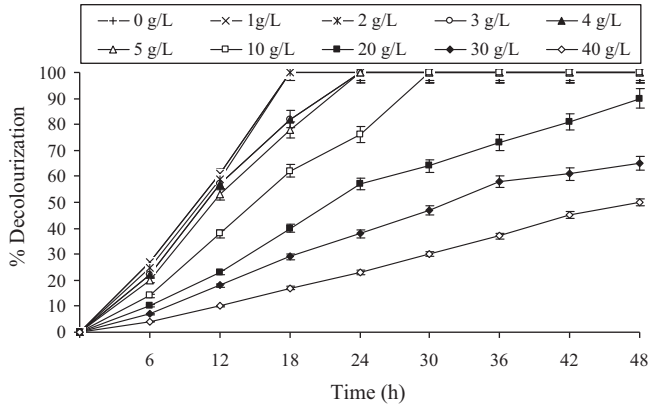

Fig. 4. Decolorization of RV5 (100 mg/L) by SB4 at varying initial NaCl concentration under static condition at 37 °C.

sulphonic acid groups on RV5 which acts as detergent exerting inhibitory effect on growth of SB4 [18].

3.6. Spectrum of dyes

Textile manufacturing and processing industries widely use several different synthetic dyes each of them are structurally diverse in their composition [24,32]. Thus, SB4 was assessed for its ability to decolorize 21 different dyes. Based on the obtained results it was apparent that more than 80% decolorization was observed within 24 h, for all the dyes used in study except Navidol Black MSR3 and Reactive Blue MR where decolorization was 39% and 35% respectively (Supplementary Fig. S1). Complete mineralization was observed in Reactive Red P6B, Reactive Magenta HBD, Reactive Orange H2R, Direct Red 81, Procion Orange H2R and RV5 after 24 h, which indicated that the SB4 was competent for bioremediation of textile effluent containing different kinds of dyes. Variation in efficiency for decolorization of different dyes was due to chemical composition of individual dye, type of substitution i.e., presence and position of groups like $-SO_3$, $-NO_2$ [12].

3.7. Effect of NaCl concentration

Textile effluent generally contains chloride salts of sodium and potassium which are frequently used for salting out of dyes and discharged into the effluent along with unused dyes. Hence, decolorizing efficiency of the SB4 over a range of NaCl concentrations (0 to 40 g/L) was assessed under static condition at 37 °C. Obtained results indicated that no inhibitory effect was noticed upto 2 g/L NaCl and complete degradation was observed within 18 h. At 5 g/L

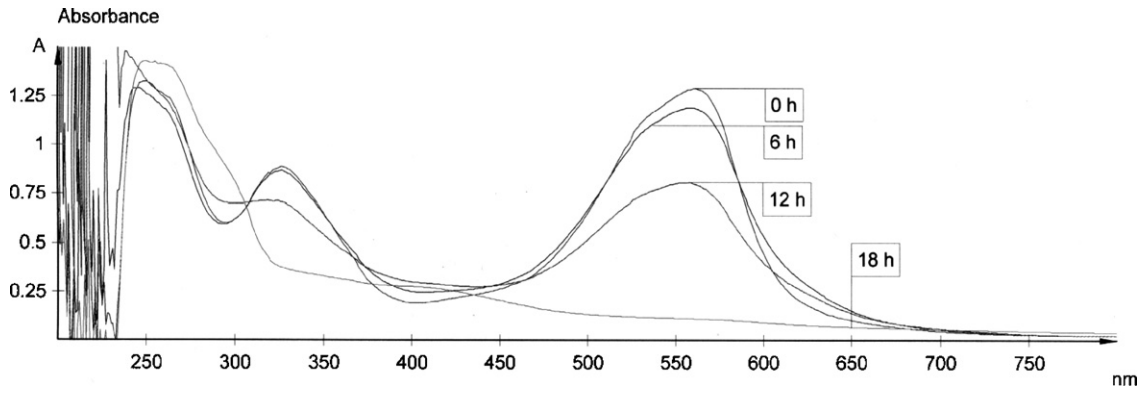

Fig. 5. UV–vis over-lay spectra of RV5 and ethyl acetate extracted degraded products of RV5 at varied time intervals under static condition at 37 °C by mixed cultures SB4.

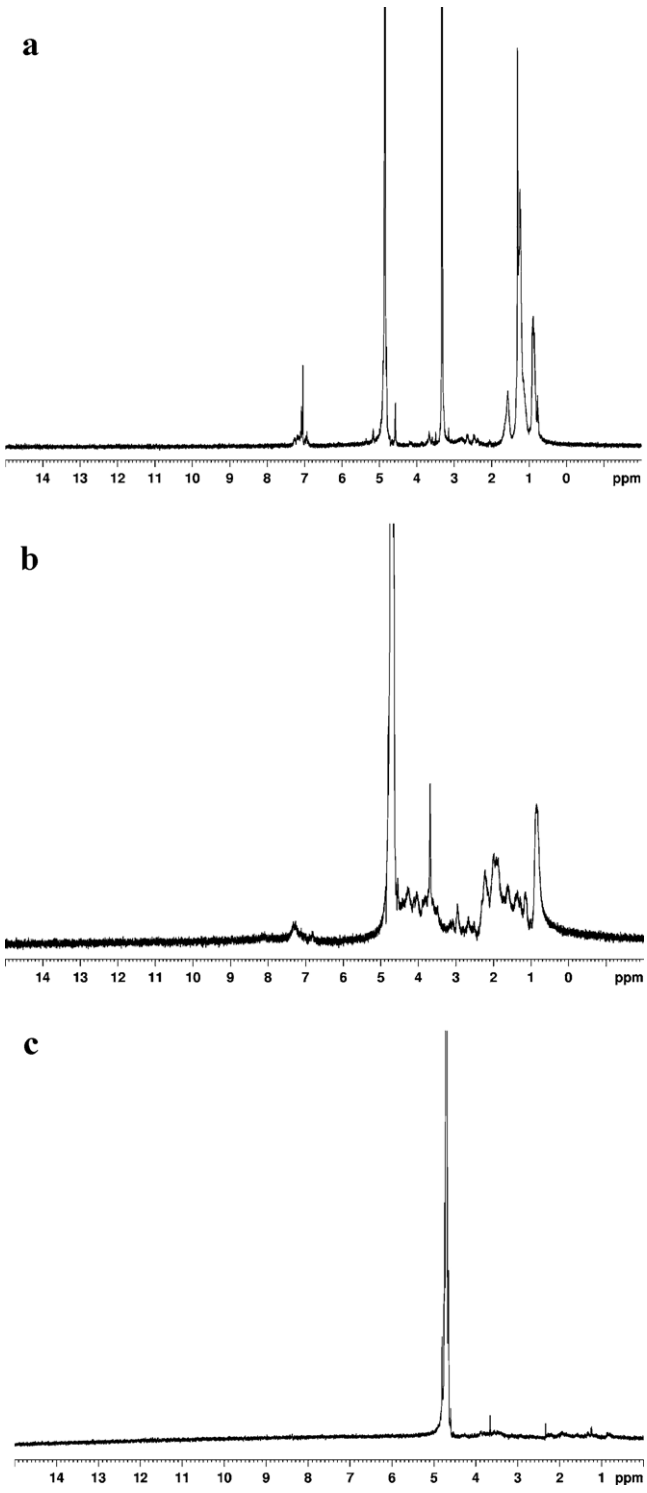

Fig. 6. ^{1}H NMR spectrum of (a) RV5 (b) ethyl acetate extracted metabolites after 9 h (c) complete decolorization (18 h) of RV5 by mixed cultures SB4.

NaCl concentration complete decolorization of 100 mg/L of RV5 was observed within 24 h (Fig. 4). Furthermore, it was stated that sodium concentration higher than 3 g/L can cause inhibition of most the bacterial metabolism [31]. But on contradictory, we observed 100% and 90% decolorization even at 10 and 20 g/L NaCl within 30 to 40 h respectively. Hence SB4 was competent in azo dye decolorization at higher salt concentration.

3.8. Decolorization of RV5 by pure cultures

Isolated pure cultures were tested for their potential to decolorize 100 mg/L of RV5 in BGY medium at 37 °C under static condition. Results obtained clearly demonstrated the incompetence of individual bacterial strain as they fail to decolorize 100 mg/L of dye even within 48 h, whereas SB4 decolorized RV5 completely in 18 h. *Bacillus* spp. V1DMK, V5DMK and V12DMK decolorized only 12, 14 and 18% of 100 mg/L RV5. *Ochrobacterium* sp. V10DMK, *Lysinibacillus* sp. V3DMK and *Bacillus* sp. V7DMK exhibited better decolorization potential by decolorizing 40, 58 and 65% dye respectively. Higher decolorization efficiency by SB4 could be attributed to the catabolic and synthrophic interactions of indigenous species leading to complete degradation of azo dye [12]. Different *Bacillus* sp. either as pure culture or in mixed form has been demonstrated previously in dye decolorization by several studies [14,24,25].

3.9. Decolorization and biodegradation analysis

Decolorization and biodegradation pattern of RV5 by the SB4 under static condition was studied using UV–vis and FTIR spectroscopy, NMR and GC–MS. UV–vis spectral analysis (200–800 nm) of RV5 showed single peak in visible region at 558 nm corresponding to its (λ_{max}) and two intense peaks in UV region near 250 and 325 nm respectively corresponds to phenyl and naphthyl rings of RV5 (Fig. 5) [33]. During decolorization π bond of conjugated chromophore [34] in RV5 were broken down and peak at 558 nm gradually decreased and completely disappeared within 18 h, without any shift in λ_{max}. Since the azo bonds were cleaved corresponding intermediates with phenyl and naphthyl rings having absorbance in UV region were accumulated in the medium. Further, with increase in time concentration of such compounds with naphthyl rings were also broken to form phenyl intermediates or completely cleaved to aliphatic hydrocarbons [33]. Thus, absorbance/concentration near 250 nm (corresponding to phenyl rings) should increase and we indeed observed that after 12 h peak got broaden up near 250 nm. As it would be discussed in subsequent results, the end products without any conjugated bonds were of lower molecular weight aliphatic hydrocarbons, free amides, alcohols, or even completely mineralized to CO_2 and H_2O which indicated the opening of all aromatic nuclei. Free SO_2, N_2 were also formed during decolorization.

Biodegradation of RV5 was analyzed by FTIR at mid-IR fingerprinting region (4000–400 cm^{-1}) of IR spectrum where the spectra of dye and its decolorized metabolites showed specific bands for multisubstituted benzene and degraded products (Supplementary Fig. S2). FTIR scan of RV5 showed characteristic bands at 1142.50, 1339.19, and 1186.07 cm^{-1} corresponding to the presence of two meta-substituted SO_3 groups and symmetric SO_2 group, respectively in RV5 —N=N— stretching vibration of a symmetrical trans-monoazo group of RV5 gave absorption band near 1548.56 cm^{-1}, whereas para-substituted azobenzene (phenyl ring) exhibits band near 1437.42 cm^{-1} [35]. RV5 is a metal containing azo dye where carbonic ion is bonded with a central copper metal ion giving rise to asymmetrical stretching which was observed near 1615 cm^{-1} [35]. Specific bands at 1650 cm^{-1} represent the primary amide of the parent structure of RV5. Moreover, —C—N— linkage and stretching corresponds to 1218.12 and 1403.75 cm^{-1}.

Concurrently, as degradation of RV5 proceeds, cleavage of monoazo bond was evidently observed by disappearance of specific band near 1437.42 cm^{-1} and decrease in intensity of band at 1548.42 cm^{-1}. Formation of free —NH group was evident as band at 3412.35, 1646.92 and 1106.70 and 3424.05, 1639.29 and 1104.34 cm^{-1} shows —NH stretching of amides after complete

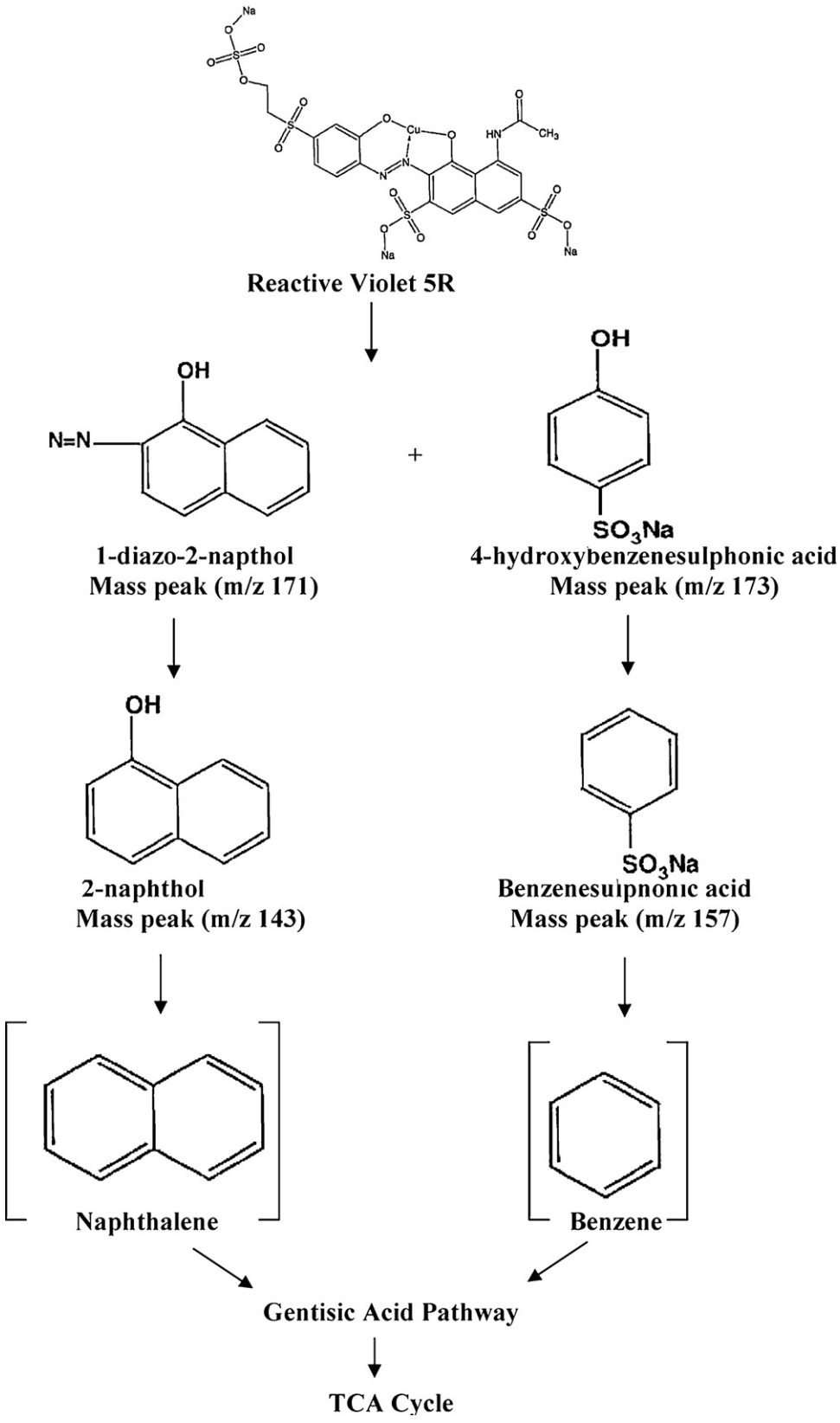

Fig. 7. Proposed degradation pathway of RV5 by mixed cultures SB4.

decolorization of RV5 indicated the production of primary or secondary amides. The S=O bonds of SO_2 and SO_3 groups were cleaved from the native structure of the dye which was clearly observed by the absence of the bands near 1140, 1180 and 1330 cm^{-1}. Mononuclear and polynuclear aromatic rings generally absorb strongly in low frequency range between 900 and 675 cm^{-1} which was apparent in IR spectra representing the presence of phenyl and napthelyl ring of RV5 (Supplementary Fig. S2). Moreover, the

substituted phenyl of RV5 absorb near 586.65 and 494.89 cm^{-1}. During degradation, decrease in intensity of bands between 900 and 675 cm^{-1} suggested the breakdown of aromatic rings. Correspondingly, absence of bands at 817, 763, 720 and 671 cm^{-1} after complete degradation of RV5 clearly demonstrated the loss of aromaticity or fission of benzene rings of the dye [35,36]. Bands at 2925 and 2929 cm^{-1} in RV5 spectrum and degraded products respectively indicated the asymmetrical stretching of C—H in CH_3.

Fig. 6 represents the ^1H NMR spectra of RV5 and degraded products. ^1H NMR spectra of RV5 showed that downshift signals between δ 6.9 and 7.2 are from hydrogen of naphthalene and benzene ring of dye molecule (Fig. 6a). After 9 h intensity of peaks/signals gradually decreased and couple of new peaks appeared. A spin network was observed by two resonances at δ 6.9 and 7.3. These signals might have appeared from the protons of 4-hydroxybenzenesulfonte [37]. Conversely, absence of corresponding peaks for aromatic protons in low field zone/higher frequency between δ 6.0 and 9.0 indicated the complete degradation and mineralization of RV5 [14,17].

A spin network in high field zone observed as singlet, triplet and multiplet signals were from saturated/unsaturated aliphatic compounds (Fig. 6b). Signals in this field were almost doubled after 9 h due to unstable alkenes resulting from ring opening of naphthalene and benzene nucleus (Fig. 6c). But after complete degradation, only few signals were remained in the region of δ 1.0 to 3.0, which resulted due to lower molecular weight aliphatic hydrocarbons such as free $-CH_3$, etc.

To get further insight into the RV5 degradation mechanism, we have studied the GC–MS behavior of degraded products at different time intervals. RV5 being a multisulfonated, non-volatile, aromatic compound, it is difficult to obtain mass spectrum of dye [38]. Nevertheless based on the mass spectra of degraded products, its molecular weight and chemical structure of dye we could identify four intermediatory products. The base peak at m/z 171 and 173 were identified as 1-diazo-2-naphthol (1D2N) and 4-hydroxybenzenesulphonic acid (4HBSA). Both theses intermediates were further broken down into 2-naphthol and benzenesulphonic acid respectively to give corresponding peaks at m/z 143 and 157 (Fig. 7). Similar to our results, azo dye orange II were also broken down into 4HBSA and benzenesulphonic acid [38].

3.10. Proposed mechanism

In the present work, monoazo-RV5 dye was completely decolorized by SB4 under microaerophilic condition in the presence of glucose and YE as sole source of carbon and electron donor/redox mediator respectively. Water soluble redox mediators such as NAD/NADH accelerate the decolorization rate of azo dyes by shuttling electrons between biological oxidation of primary electron donor (i.e., carbon/glucose oxidation) to electron acceptor such as azo dye (here RV5) [39]. Thus, obligatory requirement of glucose and YE for dye decolorization by SB4 justify the requirement of co-substrates in RV5 decolorization and degradation.

It is well known that chemical structure and molecular weight of dye greatly influences its decolorization rate. Dye with simple structure and low molecular weight (with less substitution) exhibits higher rate of decolorization. On the other hand, dyes with more substitution and higher molecular mass are difficult to decolorize [39]. Furthermore, dyes with hydroxyl or ammonia substitution are more likely to degrade faster than dyes with methyl, sulfo, nitro or methoxy groups [16,39].

There are three benzene rings in RV5. The first one is on 'left side' which contains hydroxyl group conjugated with copper ion, ethyl sulfonyl group attached to another 'sulfonly group' (sulphonic group). The second benzene ring is the one in the naphthalene ring connecting an azo bond and has another hydroxyl group conjugated with copper ion and a sulfonic group. The third and last one is in naphthalene ring with fourth sulfonic group and an acetoamide group. All the three rings are substituted and thus we believed that it would be difficult to reduce RV5 and this justified that SB4 took longer time of 18 h for complete decolorization of 100 mg/L of dye.

This can be explain by justification given in Zimmerman et al. [40], that two hydroxyl group (i.e., charged group) conjugated with copper ion is in close proximity to the azo group which may hinder the decolorization process. Moreover, Hu [19] concluded that substrates with sulfonated groups exhibited a low Km; however reaction velocity decrease and sulfonic acid act as a detergent. RV5 has four sulfonic acid groups; collectively they all exerted the inhibitory effect on SB4 and render decolorization.

However, sulfonic acid being a strong electron withdrawing group, it should enhanced dye decolorization. Besides, Zimmerman et al. [40] observed that such groups on phenyl ring of dye molecule also accelerate the decolorization process. Two such electronegative substituents (sulfo groups) are present on phenyl ring of RV5 which can accelerate decolorization, presumably because azo group may have render more accessible to free electron [40]. In addition, sulfo group on phenyl ring present at para position and hence it can enhance the decolorization of RV5.

Various mechanisms for azo dye degradation by microorganisms have been proposed. All these, generally follow two main routes as proposed by several studies: symmetrical or asymmetrical cleavage of azo bonds. Also few authors suggested co-existence of both the pathways [37]. Based on the identification of intermediates it was obvious that SB4 has asymmetrically cleaved RV5.

In consequence of the analysis, based on FTIR, NMR and GC–MS we proposed four intermediary products of RV5 degradation. Presence of 4HBSA and of 1D2N in a reaction mixture clearly indicated the asymmetrical cleavage of azo linkage leading to the formation of these two compounds. Also presence of 1D2N is in consistent with the identification of 2-naphthol (m/z 143) in reaction mixture after the loss of N_2. Further, reduction of 4HBSA produced benzenesulphonic acid. In our study presence of 4HBSA was confirmed by both NMR and GC–MS.

Though free sulfonate group ($-SO_2$) was detected by FTIR, but in unambiguous way its presence was also confirmed by GC–MS. That is, loss of 64 amu from benzenesulphonic must have lead to the formation of benzene (m/z 78) and was went undetected in our study. Nevertheless its presence in a reaction mixture should not be neglected. A separate peak of 64 m/z was observed in MS spectrum. Likewise, 2-naphthol must have been converted to naphthalene which inturn or 2-naphthol directly might have entered gentisic acid degradation pathway [41] followed by TCA cycle and finally leading to complete mineralization of RV5.

4. Conclusions

In the present scenario of environmental pollution, its restoration is a mammoth challenge. The acquired knowledge of their hazards and the strict legislation has led for implementation of several strategies for cleaning up the environment. Inspite of having spent several decades we are still searching for an optimistic approach and in view of that present bioremediation strategy demonstrated the potency of enriched bacterial mixed cultures SB4 efficiently decolorizing and degrading high concentration of azo dye RV5 with minimal nutritional requirement. The ability to decolorize wide array of dyes under ambient conditions and complete mineralization of RV5 indicated the useful application of SB4 for *ex situ* bioremediation. The synergy of results obtained has forced us to study further and now our lab is working to isolate the genes

responsible for dye degradation from these mixed cultures with an aim to design a strain capable to degrading several dyes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2012.02.010.

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