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Biodegradative decolorization of Reactive Red 195-A by an isolated bacteria *Staphylococcus* sp: studies on metabolites and toxicity

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ABSTRACT

The study is focussed on complete decolorization and degradation of an azo chromophore- based dye CI Reactive Red 195-A (RR 195-A) within 6 h of growth of a newly isolated bacterial species, *Staphylococcus hominis* subsp. *hominis* DSM 20328, under optimized conditions of pH 7, temperature 37°C and salt concentration (50 mgL^{-1}). Induction of intracellular azoreductase, and NADH-DCIP reductase indicated their contribution in the biodegradation. The decolorization kinetics was studied and the kinetic parameters evaluated as $r_{dye\ max}$ = 19.158 mg L^{-1} h^{-1} and k_m = 347.28 mgL^{-1}. Ring opening of the dye during biodegradation produced carbon source for the growth of the bacterium. The products of biodegradation were monitored using UV- vis spectroscopy and characterized using FTIR, HPLC and GCMS. The proposed metabolic pathway with respect to the GCMS analysis revealed the formation of naphthalene, naphthalen-1-ol, 1, 3, 5 triazinan-2-one, α – ketoglutaric acid, aniline and benzene. The toxicity studies, namely, phytotoxicity, microtoxicity and cytotoxicity revealed that the metabolites produced are less toxic as compared to the parent dye. The newly isolated bacterium is non-pathogenic and has exhibited the potential to decolorize and degrade different dyes that could be present in the textile effluent showing its usefulness for possible industrial application.

Keywords: Azo dye; *Staphylococcus hominis* subsp. *hominis* DSM 20328; Azoreductase; Biodegradation; Toxicity studies

1. Introduction

Wastewater from the textile and dyestuff industries contains color that reduces the aesthetics as well as the photosynthesis. Some of the dyes and their degradation products can be mutagenic and carcinogenic [1]. The wastewater containing such dyes may alter the composition of the microbial communities and the enzyme activities of the soil, if discharged into the agricultural field [2]. The azo chromophore based dyes represent a major group (nearly 80%) causing environmental concern because of their presence in the wastewater, bio recalcitrance and potential toxicity to human beings, animals, and plants [3]. The nitro and sulfonic acid groups in the dyes are recalcitrant to aerobic biodegradation. Biological treatments for dye degradation can mineralize pollutants and are usually cheaper [4]. Various microorganisms that include bacteria, fungi and algae are capable of decolorizing a wide range of dyes with high efficiency [5,6].

The genus *Staphylococcus* includes more than 40 species, which are non-pathogenic and normally reside on the skin and mucous membranes of humans and animals [7]. These species are capable of degrading several aromatic amines, which include synthetic high molecular polyurethanes [8], 1, 2-dichlorobenzene [9], phenanthrene [10], etc., and are also used in the treatment of wastewater and sewage plants [11,12]. Adsorption of Cd (II) and Cr (VI) ions from their individual solutions by *Staphylococcus xylosus* and *Pseudomonas* sp [13] and of Cr (VI), Pb (II) and Cu (II) ions by *Staphylococcus saprophyticus* [14] has been reported. Recent studies on dye decolorization include use of *Staphylococcus saprophyticus* for Navy N5RL1 dye [15], *Staphylococcus gallinarum* for CI Acid Black 172, Acid Yellow 42, Direct Black 22, Reactive

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Yellow 84, Reactive Green 19, Reactive Blue 171, Reactive Blue 222, Reactive Red 141, Reactive Red 198, Reactive Black 5, Reactive Blue 160 [16], *Staphylococcus aureus* were used for decolorization of Orange II and Sudan III azo dyes [17], *Staphylococcus epidermidis* for triphenylmethane dyes [3] and *Staphylococcus arlettae* strain VN-11 I for CI Reactive Yellow 107, Black 5 and Red 198 and CI Direct Blue 71 [18].

This paper describes the isolation and characterization of a bacterial culture SH, which is capable of decolorizing dyes based on different chromophores. The phylogenetic placement of the isolate on the basis of 16S rRNA gene sequence confirmed the bacteria to be *Staphylococcus hominis* subsp. *hominis* DSM 20328. It was used for detailed study on biodegradation and decolorization of a reactive dye RR 195-A (azo dye). The study includes enzymatic status before and after the dye decolorization, the kinetics and the pathway of biodegradation and the phytotoxicity, microtoxicity and cytotoxicity of the metabolites formed after decolorization.

2. Materials and methods

2.1. Dyes and chemicals

Widely used commercial Reactive dyes containing different chromophores namely, RR 195-A (mono azo), Reactive Blue 222 (dis azo), Reactive Blue 198 (triphenyldioxazine) and Reactive Blue 19 (anthraquinone) were procured from Atul Pvt. Ltd. India and used for initial decolorization studies. Out of these, the dye RR 195-A was selected as a model azo dye for optimization of the decolorization parameters. Nutrient broth, nutrient agar and the biochemical test kit were obtained from Himedia (Mumbai, India). ABTS and NADH was purchased from Sigma Aldrich, Mumbai while, tartaric acid, n-propane and other chemicals were obtained from SD Fine Chemicals, India. DCIP was obtained from Molychem, Mumbai. All the chemicals used were of Analytical Reagent grade. The *Allium cepa* (onion) bulbs were obtained from the local market. Microbial cultures of *Escherichia coli* ATCC 35218, *Staphylococcus aeureus* ATCC 25923, *Azotobacter chroococcum* ATCC 9043 were obtained from NCIM, Pune. The composition of the nutrient medium was (gL^{-1}): peptone 5, beef extract 3, and NaCl 5.

2.2. Acclimatization, screening of microorganism and culture conditions

The sludge collected from the dye-contaminated site around Alok Textile Industries, Gujarat, India was used for screening of the dye-decolorizing bacterial strains. One gram sludge was added to 100 ml nutrient broth containing nutrient medium along with dye RR 195-A (50 mgL^{-1}) and incubated at 37°C under shaking (60 rpm) as well as static condition. The culture showing decolorization at static condition was acclimatized by transferring 5 ml aliquots from the decolorized flask to the fresh dye-containing nutrient medium and inoculated with 0.1 ml suspension from these flasks. The isolated colonies were transferred to this inoculum and selected on the basis of rapid decolorization. The species was identified on the basis of morphological colony, gram staining and biochemical test according to the Bergey's manual and on the basis of 16S rRNA gene sequence analysis as *Staphylococcus hominis* subsp. *hominis* DSM 20328 (SH). The pure culture was preserved and maintained on nutrient agar slants containing RR 195-A dye (50 mgL^{-1}) at 4°C.

2.3. rRNA sequencing

The 16S rRNA sequence of isolated bacteria was analyzed at NCCS, Pune, India (http://www.nccs.res.in/dnaseq.html). The nucleotide sequencing analysis was done using EZ-Taxon Database at BLAST-n site of NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The corresponding alignment of the sequences was refined and submitted in *FASTA* format. The Phylogenetic tree was constructed by using the aligned sequences by the Neighbor-Joining method [19] using the MEGA 4.0 software programme [20].

2.4. Preparation of dye solutions and estimation of % decolorization

The dye powders were dissolved in distilled water. The absorbance of a dye at its λ_{max} was measured using UV-vis spectrophotometer (Shimadzu UV 1800 ENG, Japan) to estimate the percent decolorization (D), which was calculated by using the equation:

$$D, \% = \frac{A_0 - A}{A_0} \times 100 \quad (1)$$

where A_0 and A are initial and final absorbance values of the dye solution at its λ_{max}.

2.5. Decolorization experiment

The nutrient broth was inoculated in 250 ml Erlenmeyer flask with the isolated bacterial strain by addition of uniform cell density (O.D.$_{600}$ 1.0, approximately 4.6×10^8 cells, 1 ml) for 12 h in shaking condition (60 rpm). The mean count of the bacterial culture was maintained between 10^8 and 10^9 cells mL^{-1}. Unless otherwise mentioned, all the experiments were performed in 100 ml nutrient broth (pH 7.0) as growth medium and 10% inoculum having ~10^8 cells mL^{-1}. Decolorization study was carried out under static condition at 37°C ± 2°C along with the dye RR 195-A (50 mgL^{-1}, 50 ml). Five ml samples were drawn at different time intervals, centrifuged at 10,000 rpm for 15 min and the clear supernatant was used to measure the decolorization of the dye as in sec. 2.4. Uninoculated controls were used to compare the color loss during the experiment. The capability of the bacterial isolate to decolorize different commercial textile dyes was also examined in a similar manner. All the decolorization experiments were performed in three sets, and an average was taken.

The decolorization parameters such as pH (5–10), temperature (30°C–80°C), initial dye concentration (50–1,000 mgL^{-1}) and NaCl concentration (0–1,000 mgL^{-1}) were optimized along with the effect of agitation (60 rpm) and static condition on decolorization of RR 195-A dye. Repeated use of the isolated bacteria for decolorization was done at 37°C under static condition with the dye concentration of 50 mgL^{-1}. The process was repeated up to 6 cycles in the same nutrient medium.

2.6. Preparation of cell free extract

SH was incubated without and with the dye (50 mgL^{-1}) for 12 h and centrifuged at 10,000 rpm, 4°C for 15 min to prepare cell-free extract and cell pellets for estimating extracellular and intracellular enzyme activity. Cell pellets were suspended in 50 mM potassium phosphate buffer (pH 7), chilled at 4°C and sonicated keeping the sonifier output at 40 amp, giving 12 strokes each of 30 s at 1 min interval (Sonapros PR-1000 MP, Oscar ultrasonics, India). The sonicated cells were centrifuged and the supernatant was used for intracellular enzyme assay while the cell-free extract supernatant was used for extracellular enzyme assay.

2.7. Enzyme assay

The activities of different enzymes were assayed spectrophotometrically in triplicate at room temperature, with the reference blank containing all the components except enzyme, and the average was calculated. One unit of enzyme activity was defined as a change in absorbance unit and the amount of enzyme required to convert 1 μmol of dye/substrate per min.

Azoreductase assay was performed by taking 100 μl of 2 mM NADH, 50 mM potassium phosphate buffer (pH 7), 150 μl (50 mgL^{-1}) dye, and 100 μl enzyme solution. The change in the color intensity was monitored at 540 nm by UV-vis spectrophotometer. The NADH-DCIP reductase activity was determined by the assay of the mixture containing 3 ml of 25 mM DCIP, 100 μl enzyme solution and 50 mM potassium phosphate buffer (pH 7), which was monitored at 500 nm by addition of 250 mM NADH [21]. LiP activity was measured by monitoring the formation of propanaldehyde at 300 nm in 3 ml of a reaction mixture containing 100 mM *n*-propanol, 250 mM tartaric acid and 10 mM H_2O_2 [22]. Laccase activity was determined by taking 10% ABTS in 0.1 M acetate buffer (pH 4.9) at room temperature and the volume of the reaction mixture was maintained at 3 ml. Oxidation of ABTS was measured at 420 nm [23]. Tyrosinase activity was determined by formation of catechol quinine at 410 nm in the reaction mixture at room temperature. The mixture contained 0.01% catechol in 50 mM phosphate buffer (pH 7.0) [24].

2.8. Decolorization and biodegradation studies

The decolorization was monitored by UV-vis spectroscopy whereas the biodegradation was monitored by HPLC (Hitachi L-24450, Japan) and FTIR (Shimadzu FTIR -8400S, Japan). Identification of the metabolites was carried out by the GCMS (Shimadzu GC-2010) analysis. After complete decolorization of the dye, the decolorized medium was centrifuged (10,000 rpm, 15 min) and the supernatant obtained was used to extract the metabolites with equal volume of Dicholoromethane (DCM) [25]. The extract was dried over anhydrous Na_2SO_4 and evaporated to dryness in the rotary evaporator. No DCM was left over. The powder was dissolved in a small volume of HPLC grade methanol and used for HPLC and GCMS analysis.

The FTIR analysis of the dye and the extracted metabolites was done in the mid IR region of 400–4,000 cm^{-1}. HPLC analysis was carried out on C18 column (symmetry, 4.6 × 250 mm) and UV detector at 260 nm by using the gradient of methanol with flow rate of 1 ml/min for 10 min. GC-MS analysis of the metabolites was carried out using Varian capillary column (25 mm, 0.25 mm, 0.25 μm) and the compounds were identified on the basis of mass spectra and by using the NIST library.

2.9. Toxicity assay

2.9.1. Phytotoxicity study

Ten seeds each of *Triticum aestivum* (monocot) and *Vigna radiate* (dicot) were used for the study. The test was carried out according to Jadhav et al. [26] with slight modifications. The seeds were watered every day separately with 20 ml solutions of parent dye and the decolorized aqueous dye solution containing metabolites. Control set was carried out using distilled water alone at the same time. Germination (%) and length of plumule and radical were recorded after 7 d.

2.9.2. Microbial toxicity

The microbial toxicity of RR195-A dye and the metabolites obtained after its biodegradation was carried out using *E. coli* (Gram negative bacteria), *S. aeurus* (Gram positive bacteria) and *Azotobacter* (Nitrogen fixing bacteria). The mean of inhibition zone (diameter in cm) was recorded after 24 h of incubation at 37°C [3].

2.9.3. Cytotoxicity study (Allium assay)

Allium assay is a test for genotoxicity study where *Allium cepa* (onion bulbs) is used as a biomarker for environmental monitoring. No information is available on the effects of RR 195-A dye on root growth and cell division in root tip cells of *Allium cepa.* Therefore, our objective was to study the effects of dye and its metabolites on the root growth, cell division and its potential to induce micronuclei formation and other cell alterations in root tips of *Allium cepa.* Small bulbs of *A. cepa* with uniform size and shape were exposed to water initially for the development of roots. When the roots reached about 2 cm in length, they were grouped into three sets, followed by exposure to dye solution (RR 195-A), the metabolites of the dye obtained by biodegradation using SH and water (control) until 5 d. The bulbs were exposed at room temperature (30°C) under a 12 h light/dark cycle. On day 5, the bulbs were removed, thoroughly washed under running tap water and used for further studies. The micro slides were prepared for all sets following Saffranin squash technique. The root tips were kept in 1 M HCl for about 6 min followed by staining with 40%–45% Saffranin for about 5 min. The slides were analyzed at ×1,000 magnification under Leica Microsystems DM6 FS, India for cytological changes [27]. The % mitotic index was calculated as the number of dividing cells per number of observed cells [28].

$$\%\,\mathrm{MI} = \frac{\text{Number of dividing cells}}{\text{Number of observed cells}} \times 100 \quad (2)$$

2.10. Statistical analysis

All the experiments were conducted in triplicate and the results presented here are the mean of triplicate ± standard error. Results of each experiment were interpreted depending on their probabilities. Data were analyzed by one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparisons test. The *p*-values of less than 0.05 were considered to be statistically significant while those less than 0.001 were considered to be highly significant.

3. Results and discussion

3.1. Isolation, screening and identification of dye decolorizing bacteria

Potential organism was selected on the basis of the minimum time required for decolorization of the dye RR 195-A. Table 1 presents morphological characters of the bacteria along with the results on the biochemical and sugar utilization tests.

3.2. Phylogenetic position of isolates

In the phylogenetic tree (Fig. 1), the isolate strain SH was clade with the species *Staphylococcus hominis* subsp. novobiosepticus, *Staphylococcus caprae*, *Staphylococcus capitis* subsp. capitis, *Staphylococcus epidermidis*, *Staphylococcus saccharolyticus* and *Staphylococcus pasteuri* within the family Staphylococcaceae of Bacilli. Within this family, it showed a lineage distinct from species *Staphylococcus caprae*. The same branch also contained *Staphylococcus hominis* subsp. novobiosepticus and *Staphylococcus hominis* subsp. hominis. The separation of the isolate DSM 20328 from this genus was supported by bootstrap value of 99.4%–99.91%. This bacterial species belongs to the phylum Firmicutes and studies have shown that these species possess dye decolorizing property [29,30].

3.3. Optimization of nutrient parameters

Although glucose is generally used as the sole carbon and energy source for growth of microorganisms, the novelty of this study is that the species SH can grow substantially by consuming the dye RR 195-A itself. Very few bacteria have been reported to grow on azo compounds as a sole source of carbon [31,32]. Complete decolorization of the dye occurred in the nutrient broth alone without addition of supplements. This is because the azo dyes are polyaromatic, rich in carbon and nitrogen.

3.4. Decolorization of reactive dyes having different chromophores

Dyes of varied structures are often used in textile coloration for getting different colors on fabric and therefore, a number of dyes may be present in the colored wastewater simultaneously demanding a nonspecific biological process for their degradation. As seen in Table 2, the bacterial isolate SH under study was able to cause more than 80% decolorization of all the four dyes having different chromophoric systems, although the time required for each was different. Out of these, the dye RR 195-A was used in all the studies as it is a mono azo, mono chlorotriazine, multisulfonated, bi- naphthyl azo dye. Multisulfonated azo dyes and the triazine and naphthalene structures are known to be recalcitrant to biodegradation [33,34].

Table 1
Morphology and biochemical characterization of the bacterial isolate

Characteristics	DECO 10
Cell morphology	
Gram staining	Gram positive
Shape	Circular, in clusters
Color	Creamish, glossy
Consistency	Smooth
Opacity	Opaque
Spore formation	Non sporing
Motility	Non motile
Biochemical tests	
Ornithine utilization	+
Catalase	+
Lipase	+
Coagulase	–
Indole test	–
Methyl red	–
Starch hydrolysis	–
Citrate utilization	–
Lysine decarboxylation	–
Urease	+
Phenylalanine deamination	–
Nitrate reduction	–
H_2S production	–
Sugar utilization test	
Glucose	+
Fructose	+
Maltose	+
Sucrose	+
Manitol	–
Lactose	+
Arabinose	–
Sorbitol	–
Probable identify	*Staphylococcus*

3.5. Effect of physiochemical parameters on the decolorization performance

The temperature, pH, dye concentration, as well as the presence of oxygen and salt in the dye solution affect the decolorization efficiency of the microorganisms. Thus, keeping 1% inoculum under static condition, the temperature (30°C–70°C), pH (5–10), dye (RR 195-A) concentration (50–1,000 mgL^{-1}), shaking condition (30–100 rpm), and NaCl concentration (0–1,000 mgL^{-1}) were varied.

3.5.1. Effect of temperature

In the range of 30°C–70°C, the decolorization was complete at 37°C, which decreased by varying the temperature on

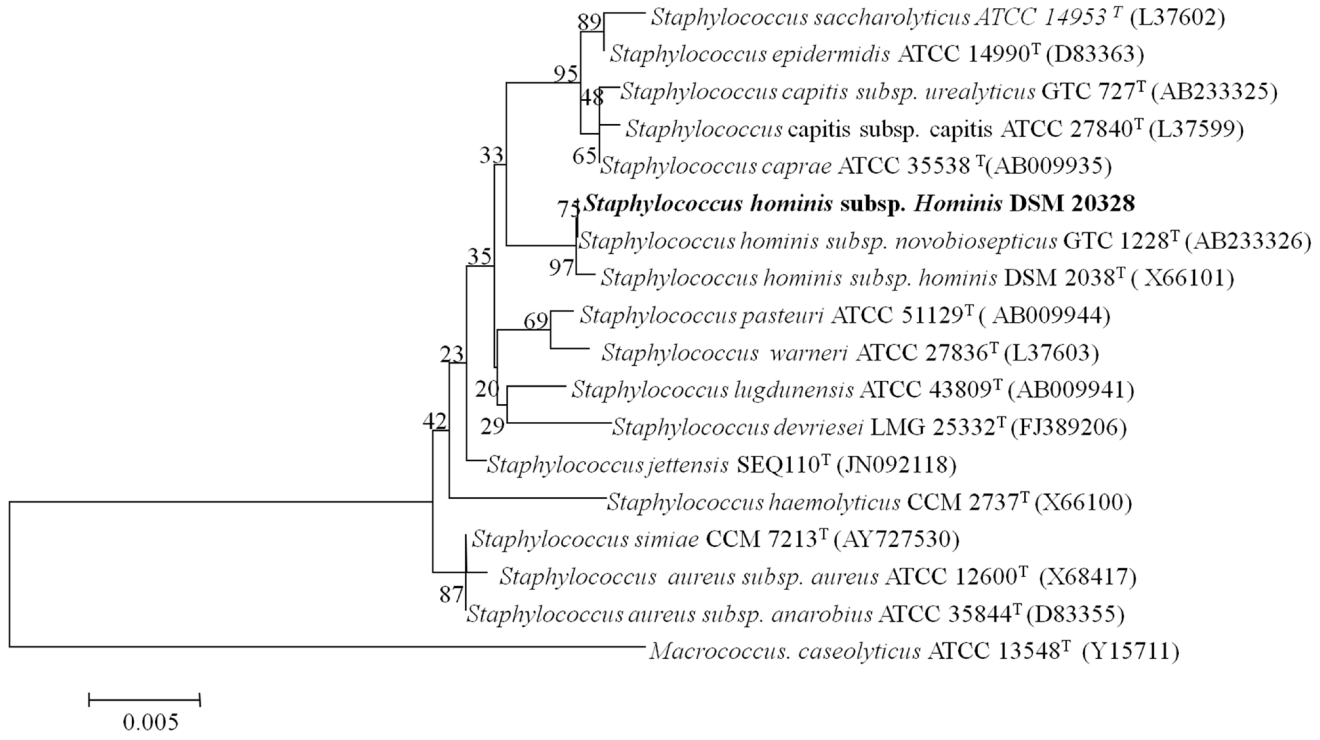

Fig. 1. Phylogenetic tree of the isolated bacteria *Staphylococcus hominis* subsp. *hominis* DSM 20328 and related organisms aligned on the basis of 16S rRNA sequences (neighbour-joining tree).

Table 2
Reactive dyes with different chromophores and their decolorization by SH

Reactive dye	Chromophore	Molecular weight	λ_{max} (nm)	Decolorization results	
				D (%)	Time (h)
Red 195-A	Mono azo	1136.32	540	99.19	6
Blue 222	Disazo	1357.49	610	96.58	6
Blue 198	Triphenodiaxazine	1304.80	625	89.87	10
Blue 19	Anthraquinone	626.35	590	83.94	12

either side (Fig. 2(a)). This may be due to the fact that the low temperature does not activate, whereas the high temperature deactivates the enzyme. Similar results have been reported by Kumari et al. [15] for azo based CI Acid Red 1. At higher temperatures of 60°C and 70°C, the decolorization observed was 25% and 21%, respectively, i.e., very poor, which could be mainly due to the adsorption of the dye onto the bacterial biomass. One-Way ANOVA Tukey–Kramer multiple comparisons test showed significantly higher decolorization at 37°C (p-value < 0.0001) as compared to that at other temperatures (30°C, p-value = 0.058, 50°C; p-value = 0.049). Therefore, temperature of 37°C was adopted as the optimum to investigate the bacterial decolorization in subsequent experiments.

3.5.2. Effect of pH

The bacterial cultures generally exhibit maximum decolorization at pH values near 7.0. SH shows activity in the pH range of 5–10 (Fig. 2(b)) with 100% decolorization of the dye RR 195-A at pH 7. The decolourization decreased at any pH lower or higher than 7, although no adverse effect on the growth of SH was observed in this pH range. Though the decolorization profiles as shown in Fig. 2(b) at different pH look similar, they are statistically different. This was confirmed by one-way ANOVA, which showed a significant difference (p-value < 0.0059) in the decolorization when carried out at different pH 5–10. The t test showed significantly higher decolorization at pH 7 as compared to that at pH 5 (p-value = 0.0061) and pH 10 (p-value = 0.0232). Therefore, pH 7 was adopted to investigate bacterial decolorization in the subsequent experiments.

3.5.3. Effect of dye concentration

The concentration of dyes in the effluent from a process house generally varies between 10 and 250 mgL^{-1} [35]

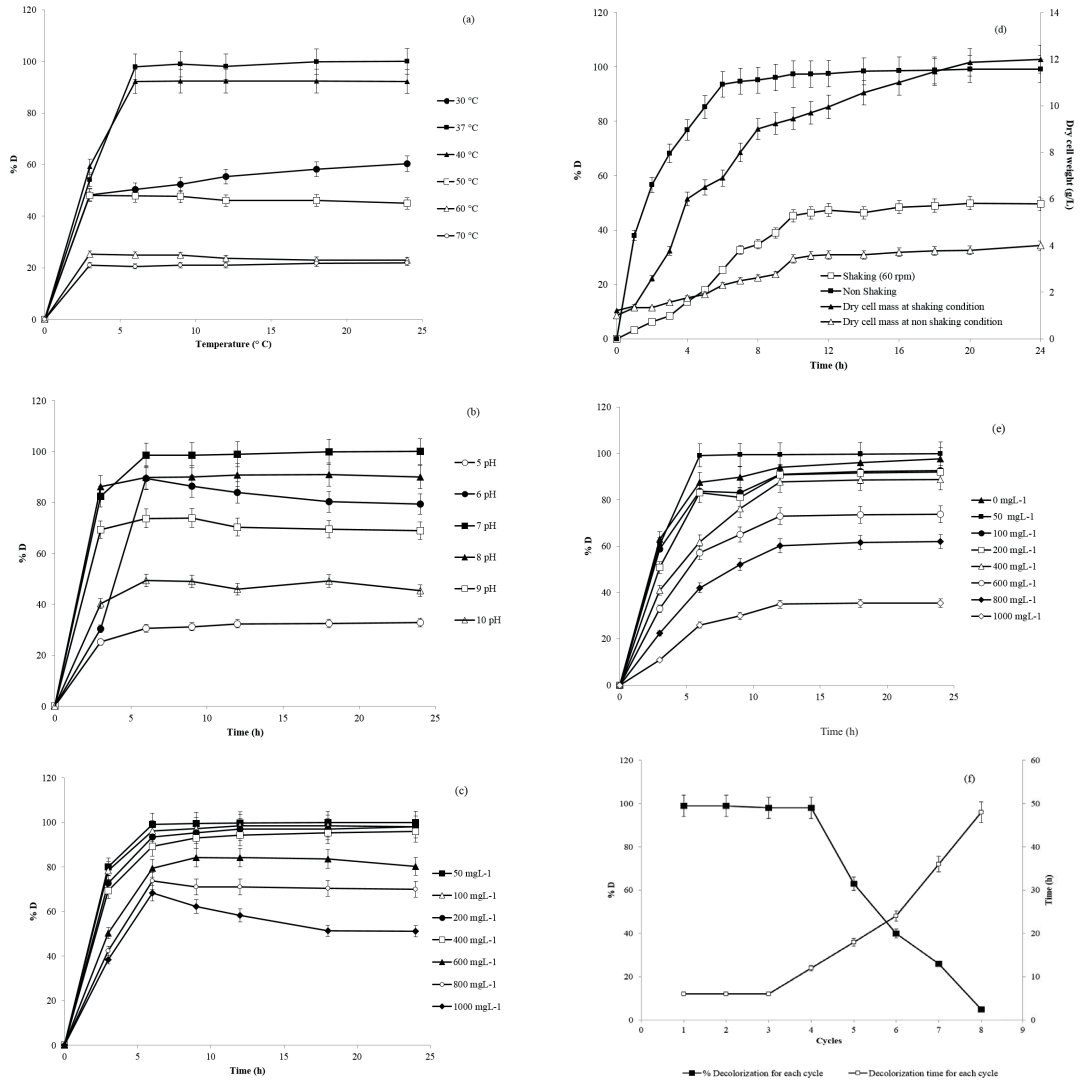

Fig. 2. Effect of various parameters on RR 195-A dye degradation by SH (a) temperature, (b) pH, (c) dye concentration, (d) presence of oxygen, (e) salt concentration (f) repeated batch process.

whereas that from the dye manufacturing industry will be much higher. Therefore, the decolorization study was undertaken in the range of dye concentrations 50–1,000 mgL^{-1} and the results shown in Fig. 2(c). At 50 mgL^{-1} dye concentration, 99.19% decolorization was observed in 6 h at 37°C, which decreased with the increase in initial dye concentration. At 200 and 400 mgL^{-1} dye concentrations, 98% and 96% decolorization was observed with time extended to 24 h, whereas at 600 mgL^{-1} and beyond, the decolorization declined to less than 80% even after 24 h. The slower decolorization rate may be due to the presence of inhibitory groups like $–NO_2$ and $–SO_3Na$ in the dye or may even be due to higher dye concentration that contributes its toxicity to reduce the efficiency of the bacterial species. The increased number of metabolites produced after decolorization may also cause adverse effect on the enzyme activity. One way ANOVA followed by t test showed that there is a significant difference (p-value < 0.05) in the percent decolorization with varying dye concentration.

3.5.4. Effect of shaking and static condition

As the dye decolorization is a function of time, complete decolorization of RR 195- A (50 mgL^{-1}) was observed in 6 h under static condition (less oxygen) in nutrient medium at 37°C. Under shaking condition, since the dissolved oxygen is much higher, SH was able to decolorize merely 25% dye in the same time (Fig. 2(d)). The growth of bacteria in shaking condition (250 ± 6.22 mgL^{-1}) was, however, much better as compared to that in static condition (158 ± 6.94 mgL^{-1}) due to easy availability of the molecular oxygen [36]. Since decolorization of azo dye is known to take place through reductive mechanism, the presence of molecular oxygen inhibits azoreductase activity. Static condition was therefore adopted for all the experiments.

3.5.5. Effect of salt concentration

Sodium chloride is commonly used to enhance the dye uptake on cotton and once the dyeing is complete, the salt

gets discharged into wastewater [37]. To investigate the effect of salt on the decolorization efficiency of SH, NaCl was added to the dye solution up to 1,000 mgL^{-1}. This was similar to the synthetic dye bath, identical to the actual one. With increase in the NaCl concentration, the dye decolorization efficiency decreased (Fig. 2(e)). With 50 mgL^{-1} of salt added, 85% decolorization was observed in 6 h, which increased to 94% after 12 h. However, increase in the salt concentration beyond 400 mgL^{-1} caused significant decrease in the decolorization from 73% (600 mgL^{-1}) to 35% (1,000 mgL^{-1}) with incubation time of 12 h. One way ANOVA followed by t test showed that there is significant difference (p-value < 0.0240) in the decolorization with varying salt concentrations, when experimented with the salt concentrations between 0 and 1,000 mgL^{-1}.

3.5.6. Repeated batch dye decolorization study

As a normal practice, the colored wastewater is periodically discharged (batch operations) from the dyeing industry, therefore the consequence of repeated addition of dye lots on the possibility of continuous decolorization was investigated by making six repeated additions of 50 mgL^{-1} of RR 195-A (Fig. 2(f)). In the first addition, 99% dye was decolorized in 6 h. The subsequent additions also resulted in faster decolorization till the fourth addition under optimized conditions, without replenishment of any nutrient. However, during the fifth and sixth dye additions, the decolorization efficiency gradually reduced.

The constant decolorization rate observed up to 4th dye addition can be due to the increase in the bacterial mass with the additional dye. After this, however, the decolorization efficiency of the organism decreased and required more time, which may be due to the decrease in the number of viable cells in the system or depletion of the nutrients in the medium as well as the inhibition of enzymes, responsible for decolourization, by the metabolites formed. Similar observations have been recorded previously for the decolorization of reactive dyes like Red BLI by *Pseudomonas sp.* SUK1 [38] and Red 141 by *Rhizobium radiobacter* MTCC 8161 [39]. Thus, the species SH shows a substantial ability to decolorize repeated additions of the dye suggesting its commercial significance.

3.6. Effect of initial dye concentration on dye decolorization rate

The concentration of dye can influence the efficiency of the dye decolorization through a combination of factors including the toxicity of the dye at higher concentrations and the ability of the enzyme to recognize the dye efficiently at very low concentrations. Higher concentrations of the dye RR 195-A beyond 600 mgL^{-1} adversely affected the decolorization rate. Similar results have been reported for azo dyes [34,40–42].

Michaelis–Menten kinetic model has been widely used for the kinetics of dye degradation by enzyme and/or by living cells. The Michaelis constant, k_m, and the maximum decolorization rate, $r_{dye\ max}$ can be readily derived from the rates of catalytic reaction measured at various dye concentrations (50–1,000 mgL^{-1}). The constants of the equation are determined from the Lineweaver–Burk plot, $1/[r_{dye}]$ (x) vs. 1/[Dye] (y), which yields a straight line with an intercept $1/r_{dye\ max}$ and a slope $k_m/r_{dye\ max}$ (Fig. S1). The high-degree of linearity ($r^2 = 0.9264$) between the two variables gives reliable estimations which for $r_{dye\ max}$ should usually be between 10 and 100 mgL^{-1} dye h^{-1} and for k_m between 600 and 5,000 mgL^{-1} [43]. The values obtained were, $r_{dye\ max}$ = 19.158 mgL^{-1} dye h^{-1} and k_m = 347.28 mgL^{-1}.

3.7. Enzymatic activity before and after dye degradation

Decolorization of different textile dyes is due to the action of different enzymes present in the bacterial strain. Several bacterial enzymes that can be used in bioremediation belong to oxidative and reductive types. The enzymes present in the bacterial strain SH and their activities before and after decolorization are summarized in Table 3. The presence of the extracellular and intracellular enzyme activities was also analyzed during the decolorization of the dye RR 195-A. Increased amounts of azoreductase, NADH-DCIP reductase, Laccase and LiP were seen during the time course of the dye decolorization, whereas the activity of tyrosinase was high before dye decolorization. The role of intracellular azoreductase was most prominent in the decolorization process followed by NADH-DCIP reductase, Laccase and LiP.

Table 3
Activity of different enzymes produced by SH

Enzyme	Enzyme activity after		
	0 h	6 h	12 h
Azoreductase (intracellular)[a]	35.67 ± 0.172	78.64 ± 0.473*	89.23 ± 0.135*
Azoreductase (extracellular)[a]	14.32 ± 0.109	16.71 ± 0.285*	18.95 ± 0.473*
NADH-DCIP reductase (intracellular)[b]	9.54 ± 0.232	15.87 ± 0.218*	20.98 ± 0.469*
NADH-DCIP reductase (extracellular)[b]	2.54 ± 0.277	2.98 ± 0.194*	3.87 ± 0.328*
Laccase (intracellular)[c]	0.52 ± 0.040	0.965 ± 0.007**	0.986 ± 0.217
Lignin Peroxidase (intracellular)[c]	0.276 ± 0.037	0.485 ± 0.018**	0.567 ± 0.033
Tyrosinase (intracellular)[c]	0.86 ± 0.040	0.349 ± 0.007	0.321 ± 0.217

Values are mean of three experiments (±) SEM.
Significantly different from control cells at *P < 0.05; **P < 0.001 by One way analysis of variance (ANOVA) with Tukey–Kramer comparison test.
Enzyme activity [a] = μM of RR 195-A reduced min^{-1} mg^{-1} protein.
Enzyme activity [b] = μg of DCIP reduced min^{-1} mg^{-1} protein.
Enzyme activity [c] = units min^{-1} mg^{-1} protein.

3.8. Degradation mechanism for the dye RR 195-A

Decolorization of a dye by bacteria could be due to its adsorption onto microbial cells or due to the biochemical reaction or both, leading to reduction in the color value of the dye solution. The reduction in color value was confirmed by visible spectrum (Fig. 3). If adsorption had played any major role in the decolorization, the spectrum would have shown all the peaks decreased approximately in the same proportion to each other [44]. As the major light absorbance peak at λ_{max} 540 completely disappeared, the dye removal was attributed to the biochemical degradation due to which the chromophore (–N=N–) was completely destroyed. The change occurring in the UV–vis spectra indicate that the molecular structure of RR 195-A evidently changed leading to mineralization after the decolorization.

In FTIR spectrum (Fig. 4(a)) of the dye RR 195-A, the presence of –NH stretching vibration of aromatic amines coupled with –OH stretching is seen at 3,433 cm^{-1}. The –N=N– group (the azo chromophore) is detected at 1,543 cm^{-1}. The peak at 1,618 cm^{-1} corresponds to –C=N– stretching and –NH– bending of the secondary amines. The symmetric stretching of $–SO_2$ is observed at 1,035 cm^{-1}. The presence of poly nuclear aromatic ring of RR 195-A is observed at the peak 758 cm^{-1}. The peak at 897 cm^{-1} indicates presence of C-Cl group in the dye RR 195-A.

The FTIR spectrum of the metabolite (Fig. 4(b)) formed clearly illustrates the decrease in the prominent peaks present in the dye spectrum. A new peak at 1,454 cm^{-1} represents –C=C– stretching while a peak at 1,234 cm^{-1} indicates presence of –CN– stretching which supports formation of primary aromatic amine. The peak at 1,753 cm^{-1}, belonging to the carboxylic group in the dye, disappeared. New peak at 1,637 cm^{-1} belongs to –N–H bending of the primary amine, 1,531 cm^{-1} to C–C stretching and 1,369 cm^{-1} belongs to –C–N stretching of aromatic amine, while 1,057 cm^{-1} belongs to aliphatic amines. Similar were the observations for FTIR characterization by [45,46] while carrying out biodegradation of RR 195-A dye with bacterial consortium AR1 and *Georgenia* sp. CC-NMPT-T3, respectively.

TLC analysis (data not shown) indicated appearance of the a spot with *Rf* value 0.9 for the dye RR 195-A, whereas the *Rf* values for the metabolites formed after 6 h of biodegradation were different (0.14, 0.35 and 0.58). This proves removal

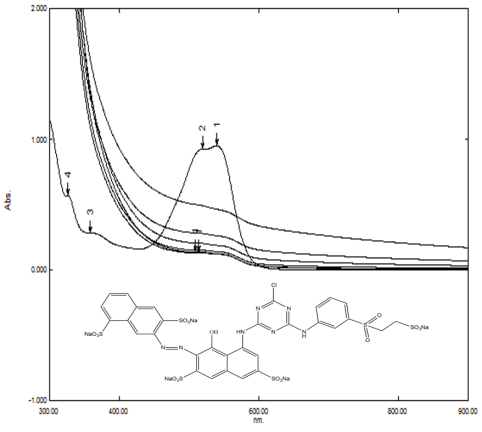

Fig. 3. Structure of the dye RR 195-A and its visible spectra before and after decolorization.

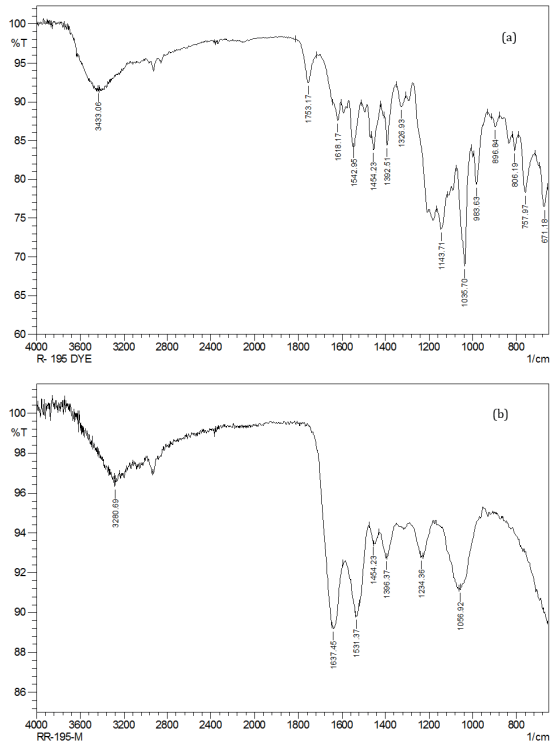

Fig. 4. FTIR spectrum of (a) RR 195-A dye and (b) its metabolites.

of color from the dye solution by biodegradation of the dye molecules.

The HPLC analysis of RR 195-A dye showed a single peak at retention time 4.23 min (Fig. S2). As the decolorization progressed, additional peaks appeared due to degradation of the parent dye (after 6 h) at retention time 2.820, 3.080 and 3.313.

The metabolites formed after decolorization were confirmed by GCMS analysis (Table S1) which depicts that the parent high molecular weight dye was degraded to low molecular weight aromatic compounds. With the help of GCMS analysis, a pathway of RR 195-A dye decolorization using SH is proposed in Fig. 5. Accordingly, cleavage of –N=N– chromophore led to initial fragmentation of RR 195-A dye into two fractions, one identified as sodium 2-aminonapthalene-1, 5-disulfonate (*m/z:* 349) and the other unknown. The former was further fragmented into naphthalene-1, 7-disulfonic acid (*m/z:* 284) which, after desulfonation, was converted to naphthalene (*m/z:* 125). The unknown metabolite could further fragment into aniline (*m/z:* 93), 1, 3, 5-triazine-2, 4(1 H, 1 H)-dione (*m/z:* 113), naphthalen-1-ol (*m/z:* 141), α – ketoglutaric acid (m/z: 149). The α – ketoglutaric acid is one of TCA cycles intermediates, which is commonly present in any organism [47,48]. Thus, complete mineralization of the dye RR 195-A by SH was achieved successfully.

3.9. Toxicity test

3.9.1. Phytotoxicity test

Despite the fact that the untreated dye-containing wastewater may cause serious environmental and health hazards, it may be disposed off in the water bodies that are used for irrigation purpose. Therefore, it was thought necessary to

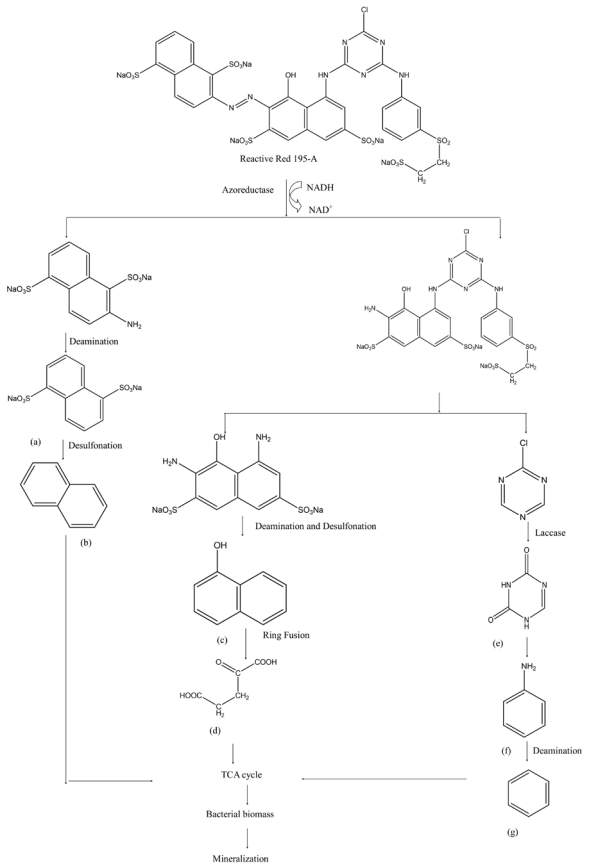

Fig. 5. Pathway of degradation of dye RR 195-A by isolated bacterial strain SH by GCMS analysis.

assess the toxicity of the dye before and after its degradation as it has a direct impact on fertility of soil [49]. The root and shoot lengths showed considerable decrease when irrigated with water containing the dye, whereas the presence of metabolites obtained after dye degradation had very little effect (Table 4) and the growth was comparable to the use of distilled water.

3.9.2. Microtoxicity test

In microtoxicity test, significant reduction in the zone of inhibition was observed surrounding the well containing metabolites formed after degradation of RR 195-A dye, when compared with the untreated dye (Table 4). This confirms the non-toxic nature of the products formed.

3.9.3. Cytotoxicity test (Allium assay)

Table 4 shows that the dye RR 195-A inhibits the root growth and decrease the frequency of division of cells that were statistically significant when compared to the control. No chromosomal aberration was observed in the metabolites. The mitotic index (MI) value for RR 195-A was 4% whereas for metabolites it was 15% when compared with control (16%). The cytotoxicity level can be determined by the decreased rate of the MI. Decrease in MI below 22% of the control causes sub lethal effects on the test organisms [50], while a decrease below 50% usually has lethal effects [51].

The comparison given in Table 5 clearly indicates that the decolorization of RR 195-A dye by SH is much better than reported by using different bacteria.

Table 4
Toxicity study of dye RR 195-A and its metabolites

Toxicity test	Parameters	Tested on	Control	RR 195-A	Metabolite
Microtoxicity	Zone of Inhibition (cm)	*E. coli*	0.75 ± 0.04	1.42 ± 0.03	0.74 ± 0.03
		S. aeurus	0.65 ± 0.03	1.8 ± 0.08	0.67 ± 0.03
		Azotobacter	0.72 ± 0.01	1.68 ± 0.01	0.76 ± 0.01
Cytotoxicity	Root Length (cm) Mean ± SD	On *Allium cepa* roots	3.68 ± 0.20	1.2 ± 0.17	3.28 ± 0.22
	Mitotic Index Mean ± SD		16 ± 3.5	6 ± 2.24	14.89 ± 0.69
	Prophase (%)		10.7 ± 4.0	4.33 ± 1.8	5.56 ± 2.19
	Metaphase (%)		3.6 ± 2.2	1.56 ± 1.33	3.56 ± 1.67
	Anaphase (%)		2.9 ± 2.3	1 ± 1	2 ± 1.41
	Telophase (%)		1.9 ± 2	0	1.11 ± 0.78
Phytotoxicity	% Germination	*Triticum aestivum*	100	40	80
	Mean shoot length (cm)		2.14	0.87 ± 0.239	1.57 ± 0.396
	Mean root length (cm)		6.86	4.17 ± 0.239	6.57 ± 0.396
		Vigna radiate	100	60	100
			8.30	5.13 ± 0.199	7.42 ± 0.207
			6.80	4.14 ± 0.247	6.48 ± 0.305

Values are mean of three experiments (±) SEM.
Percent of cells in Prophase among total number of cells counted.
Percent of cells in Metaphase among total number of cells counted.
Percent of cells in Anaphase among total number of cells counted.
Percent of cells in Telophase among total number of cells counted.

Table 5
Comparison of RR 195-A dye biodegradation results with those reported in literature

Organism used	Dye Conc. (mg/L)	Incubation period	Co-substrate	Final product	References
Enterobacter sp.	30	2 d	Glucose, peptone	Aromatic amines	[52]
Bacillus cereus (M1)	200	72 h	Maltose, peptone	–	[53]
Georgenia sp. CC-NMPT-T3	50	3 h	Peptone	Aromatic amines	[46]
Paenibacillus spp. *R2*	–	48 h	Glucose	Complete degradation	[48]
Bacterial consortium AR1	100	14 h	Maltose and proteose peptone	Aromatic amines	[45]
SH	50–400	6 h	–	Complete degradation	This study
SH	600–1,000	12 h	–	Complete degradation	This study

This confirms that the biodegradation of the dye RR 195-A by SH has taken place in a much better way.

4. Conclusion

The bacterial species *staphylococcus hominis* subsp. *hominis* dsm 20328, isolated from the sludge of a textile processing industry has shown the ability to decolorize structurally different dyes (mono azo, disazo, triphenodiaxazine, anthraquninone). It could decolorize an azo dye (RR 195-A) under broad range of dye and salt concentrations, and also up to four dye lot additions suggesting its usefulness in biological treatment of textile and dyestuff industry's wastewater. The dependence of the specific decolorization rate on RR 195-A concentration can be described by the lineweaver –burk equation. The intracellular azoreductase and NADP-DCIP reductase secreted by the bacterial strain SH played a major role in the dye degradation process. Decolorization was confirmed by UV analysis and the biodegradation into various metabolites was confirmed by FTIR and GCMS analysis. The metabolites produced were also found to be less toxic, making the use of this non-pathogenic bacteria ecofriendly.

Acknowledgements

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Non-standard abbreviations

SH	—	*Staphylococcus hominis subsp. hominis DSM 20328*
RR 195-A	—	Reactive Red 195-A
DCM	—	dichloromethane
LiP	—	Lignin Peroxidase
NADH	—	Nicotineamide-Adenine-Dinucleotide-Hydrogen
ABTS	—	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
DCIP	—	2,6-Dichlorophenolindophenol

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Supplemental material

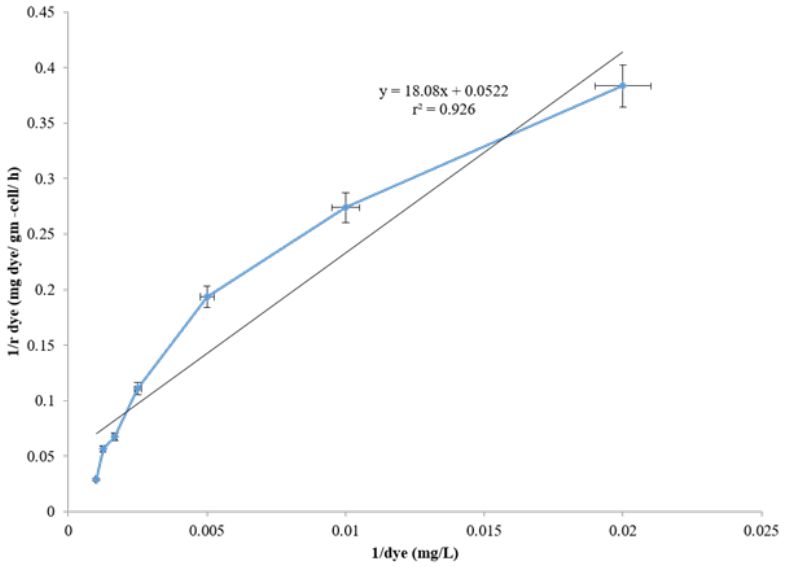


Fig. S1. Effect of initial dye concentration on decolorization rate, the double reciprocal plot of 1/ r_{dye} (mg dye/gm-cell/h) against 1/ dye (mg/L) to calculate $r_{dye\ max}$ and k_m.

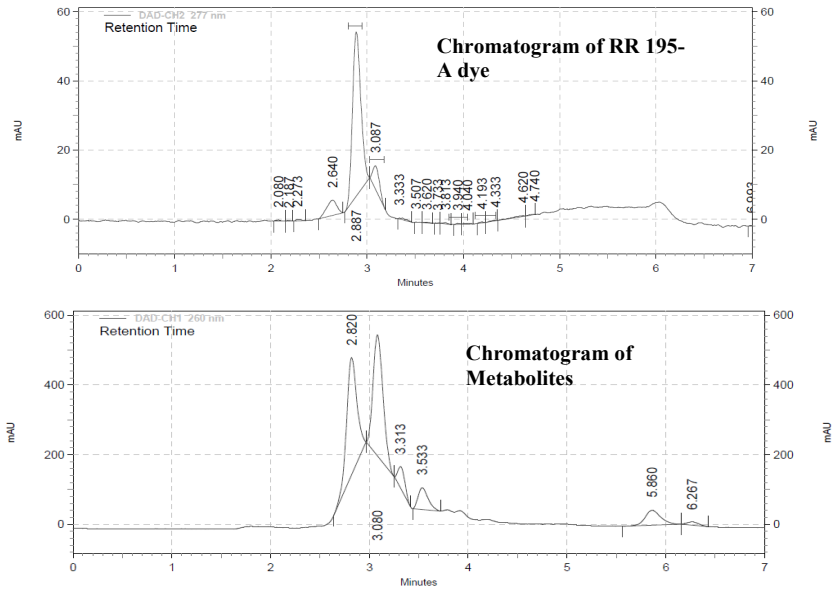
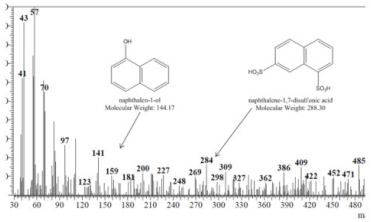
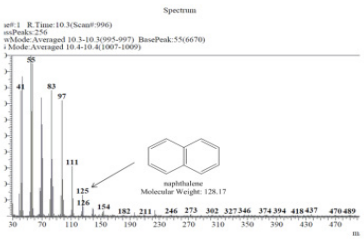
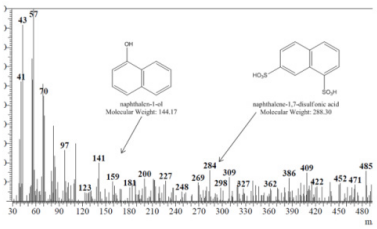
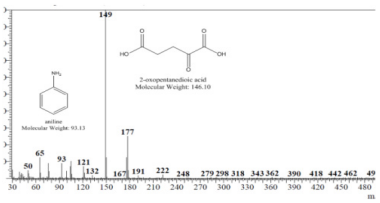
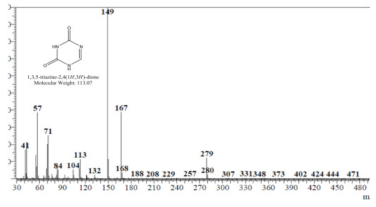
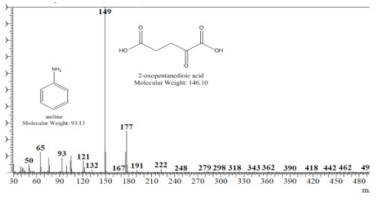


Fig. S2. HPLC–UV analysis of metabolite after decolorization of RR 195-A.

Table S1
GCMS analysis of RR 195-A metabolites

Sr. No.	Chemical name	Chemical formula	Molecular weight	GCMS (m/z)	Gas chromatograms
a	Sodium 2-aminonaptha-lene-1, 5-disulfonate	$C_{10}H_7NNa_2O_6S_2$	347.28	349	ND
b	Naphthalene-1,7-disulfonic acid	$C_{10}H_6Na_2O_6S_2$	288.30	284	
c	Naphthalene	$C_{10}H_8$	128.17	125	
d	Naphthalen-1-ol	$C_{10}H_8O$	144.17	141	
e	2- oxopentanedioic acid (α-ketoglutaric acid)	$C_5H_6O_5$	146.10	149	
f	1,3,5-tri-azine-2,4(1H,1H)-dione	$C_3H_3N_3O_2$	113.07	113	
g	Aniline	C_6H_7N	93.13	93	
h	Benzene	C_6H_6	78.11	73