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Original research article

Biodegradation of Reactive Black 5 by *Aeromonas hydrophila* strain isolated from dye-contaminated textile wastewater

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ABSTRACT

Aeromonas hydrophila isolate showed the best decolorization of Reactive Black 5 (RB5) at the concentration of 100 mg L^{-1} on modified mineral salt medium among 42 bacterial isolates. Optimization of parameters for RB5 dye decolourization was studied under static condition. Under optimized condition, decolorization efficiency of RB5 by *A. hydrophila* was found to be 76% at 100 mg L^{-1} within 24 h. The optimum pH and temperature for the decolorization was 7 and 35 °C respectively. Biodegradation and decolorization of RB5, was monitored by UV–Vis spectrophotometry, Thin Layer Chromatography, Fourier Transform Infrared Spectroscopy and Gas Chromatography Mass Spectrometry analysis. The study has confirmed the potential of *A. hydrophila* isolated from textile effluent in degradation of RB5 and opened scope for future analysis in the treatment of textile effluent.

1. Introduction

One of the important classes of the pollutants is dyes that are considered to be particularly dangerous organic compounds for the environment [1]. Once the dyes entered the water, it is difficult to treat such as dyes have a synthetic origin and a complex molecular structure which makes it more stable and difficult to be degraded. Dye molecules comprise of two key components: the chromophores, responsible for producing the color, and the auxochromes, which can not only supplement the chromophore but also render the molecule soluble in water and so give enhanced affinity toward the tissue [2,3]. Dyes may be classified on the basis of their solubility: soluble dyes which include acid, mordant, metal complex, direct, basic and reactive dyes; and insoluble dyes including azoic, sulfur, vat and disperse dyes.

Significant proportions of these dyes enter in the environment through wastewater. Discharge of colored textile effluents into drainages and lakes results in reduced dissolved oxygen concentration and creates toxic conditions to aquatic flora and fauna [4]. Among many classes of synthetic dyes used in the textile and dyeing industries, reactive dyes are used widely in many industries due to their bright color, excellent colorfastness and ease of application [5]. Reactive dyes are typically azo-based chromophores combined with different reactive groups. They differ from all other dye classes in that they bind to the textile fiber, such as cotton, through covalent bonds; and thus are highly recalcitrant to conventional wastewater treatment processes. One of the main sources with severe pollution problems worldwide is the textile industry and its dye-containing wastewaters. In particular, the discharge of dye-containing effluents into the water environment is undesirable, because of their color, and se huge amount of dyes dissolved in large volumes of water both in the dye bath and also during the rinsing step. Without adequate treatment these dyes can remain in the environment for a long period of time. However, even lesser concentrations of dyes may have significant environmental impacts [6]. Therefore, the treatment of dye contaminated effluents is currently an environmental concern [7].

Reactive Black 5 (RB5) dye is one of the most common used synthetic reactive dyes in the dyeing industry. This type of dye is highly soluble in water and has reactive groups which can form covalent bonds between dye and fiber [2,3]. RB5 is the most commonly used to dye cotton and other cellulosic fibers, wool and nylon [8]. RB5 forms a covalent bond with the fiber and contains chromophoric groups such as azo, anthraquinone, triarylmethane, phthalocyanine, formazan, oxazine, etc. Large amounts of these

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dyes are discharged in the water courses in developing countries such as Egypt that can have adverse effects on water and human bodies. Thus removing color from wastewater is as important as treating other colorless organics. There is an urgent need for textile industry to develop effective methods of treatment because small amounts of dye are clearly visible and detrimental to the water environment [8].

RB5 was selected as a model compound, due to the incomplete fixation reaction on cellulose. The reason is the competition between the reaction of the reactive vinylsulphone groups with the fiber and the hydrolysis of the vinylsulphone groups yielding the 2-hydroxyethylsulphone groups. The 2-hydroxyethylsulphone groups do not react with the fibers resulting in low efficiency of the dyeing process [9].

Although there are many methods for the removal of the dyes, it is difficult to treat the wastewater by using traditional methods, because most of the synthetic dyes are stable to light, chemicals and biological treatment [10]. Among these methods, biodegradation can be thought to be the most efficient process for treating industrial effluents due to it is a cost-effective and eco-friendly technique as well as its ease of operation. For this reason, biodegradation has the ability not only to decolorize dyes but also to detoxify [11].

In this study, biodegradation was used for the bacterial decolorization of RB5 in batch mode studies. The effects of experimental conditions such as initial dye concentration, temperature and pH were investigated to obtain information on dye removal.

2. Materials and methods

2.1. Chemicals

The RB5 dye used for this decolorization study was obtained from the local textile industry in Nasr Company for Spinning and Dyeing, El Mahala El Koubra, Egypt. Heavily dye-contaminated wastewater samples were obtained from surrounding areas of the textile industries and wastewater treatment plant in Nasr Company for bacterial isolation. Solutions of RB5 were simulated from the commercial product (Sigma–Aldrich, St. Louis, USA) according to real concentrations found in textile effluents. Generally, dye concentrations in textile effluents vary from 10 to 25 mg L^{-1}, although concentrations about 100 mg L^{-1} have also been found. A concentration of 100 mg L^{-1} was selected as the initial concentration of dye solutions for this work. Fig. 1 shows the structure of RB5 (Color index: 20505, formula: $C_{26}H_{21}N_5Na_4O_{19}S_6$, molecular weight: 991.8 and λ_{max}: 597 nm), where the main functional groups of the molecule, reactive groups and chromophore groups, are marked. All chemicals were of the highest purity and of an analytical grade.

2.2. Isolation of bacteria by enrichment method

The wastewater samples collected were subjected to enrichment culture technique. The enrichment was carried out by adding 10 mL of wastewater sample separately in 100 mL nutrient broth medium (5 g L^{-1} peptone, 1 g L^{-1} meat extract, 2 g L^{-1} yeast extract and 5 g L^{-1} NaCl at pH 7), to prepare concentration of RB5 dye to be 100 mg L^{-1} in 250 mL Erlenmeyer flasks. The flasks were then incubated in a rotary shaker at 50 rpm. After 3 d of incubation, a loop-full of medium was streaked onto sterile nutrient agar plates with same concentrations of ingredients and incubated 35 °C for 24 h. At the end of incubation the individual different isolated colonies were noted and re-streaked on nutrient agar plates for identification.

2.3. Screening of efficient dye decolorizing isolates

Screening was done to find out the most efficient bacterial isolates capable of decolorizing RB5 dye using modified mineral salt medium (MSM) (3 g L^{-1} glucose, 2 g L^{-1} $(NH_4)_2SO_4$, 1 g L^{-1} KH_2PO_4, 10 g L^{-1} K_2HPO_4, 0.1 g L^{-1} $MgSO_4 \cdot 7H_2O$ and 5 g L^{-1} NaCl) containing 100 mg L^{-1} RB5 dye [12]. For this, the morphologically different isolates isolated from the effluent were inoculated in modified MSM and incubated at 35 °C for 24 h. The decolorization activity was expressed in term of decolorization percentage (%) of dye. Aliquot (3 mL) was withdrawn aseptically and centrifuged at 10,000 rpm for 10 min and residual dye content in the supernatant was measured at λ_{max} 597 nm using UV–Vis spectrophotometer (Perkin Elmer Lambda 35). All assays were performed in triplicate and uninoculated mineral salt media supplemented with same concentration of dye was used as control [13]. Decolorization percentage was expressed by Eq. (1):

$$Decolorization(\%) = \frac{(I - F)}{I} \times 100 \quad (1)$$

where I = initial absorbance; F = final absorbance of decolorized medium. *Aeromonas hydrophila* isolate was found to possess more than 60% decolorization of the selected dye (RB5) in modified MSM containing 100 mg L^{-1} RB5 dye and incubated at 35 °C for 24 h. This isolate was selected for further study and stored at −20 °C in nutrient broth containing 20% (v/v) glycerol. Working culture was maintained by sub-culturing every two weeks on nutrient agar slants.

2.4. Phylogenetic analysis

The identification of *A. hydrophila* was based on standard morphological and biochemical methods and 16s rRNA gene sequence analysis. The polymerase chain reaction (PCR) amplification and DNA sequencing of the 16s rRNA gene was carried out as described earlier [14]. Almost the full length of 16S rRNA gene was amplified by PCR using universal primers forward 5′-AGAGTTTGATMTGGCTCAG-3′ and 5′-CGGYTACCTTGTTACGACTT-3′ corresponding to the positions 9 to 27 and 1525 to 1545, respectively, in the 16S rRNA gene sequence. PCR products were sequenced directly using ABI PRISM Big Dye Terminator Cycle Sequencing Kit on an ABI 3100 DNA sequencer following the manufacturer's instruction. Performed multiple alignments of the sequences, and a neighbor

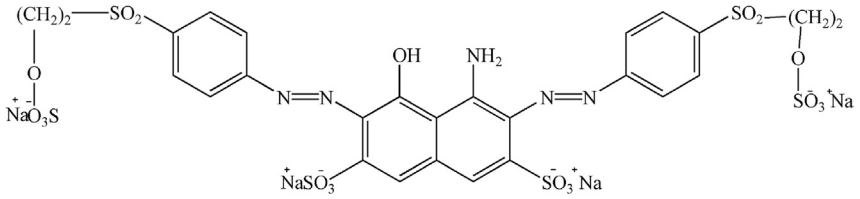

Fig. 1. Chemical structure of Reactive Black 5.

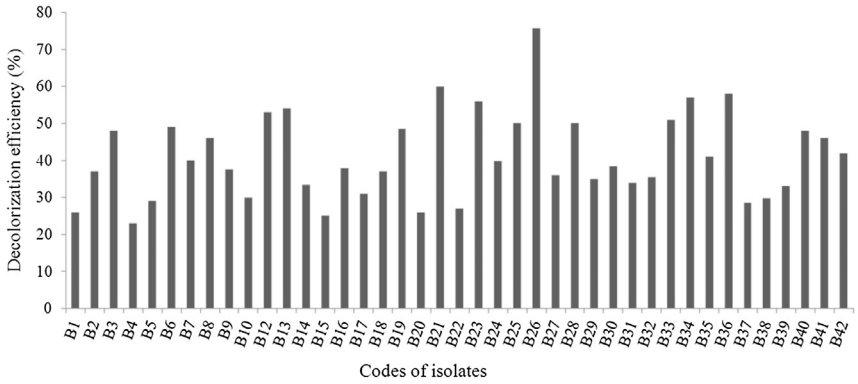

Fig. 2. Decolorization percentage of 42 bacterial isolates were isolated from dye-contaminated textile wastewater samples.

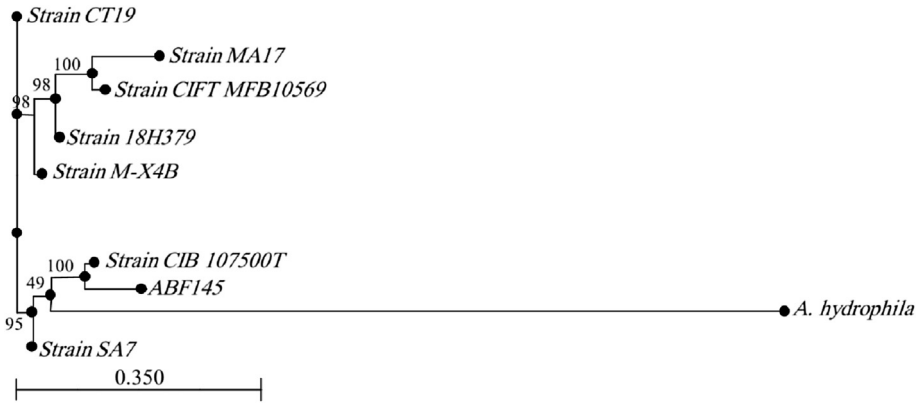

Fig. 3. Phylogram (neighbor-joining method) showing genetic relationship between strain *Aeromonas hydrophila* and other related reference microorganisms based on the 16S rRNA gene sequence analysis.

joining phylogenetic tree was constructed using the latest version (ver. 1.8) of the CLUSTAL W program [15]. Similarity values of the sequences were calculated by using the GENETYX computer program [16].

2.5. Optimization of environmental factors for efficient decolorization

Optimization studies included various concentrations of dye (50–250 mg L^{-1}), pH values (3–11) and temperatures (25–45 °C); they were experimented individually with shaking and static conditions. In the experimentations for optimization MSM medium was used along with the 100 mg L^{-1} of RB 5 azo dye and uninoculated medium supplemented with the same concentration of dye was used as control. After incubation the suspensions were centrifuged at 10,000 rpm for 10 min and residual dye in supernatant was measured using UV–Vis spectrophotometer.

2.6. Analysis of degraded metabolite

UV spectral analysis was done by using a UV–Vis spectrophotometer. Fourier transform infrared spectroscopy (FT-IR) analysis was carried out using Shimadzu 8400s spectrophotometer in the mid – infrared of 400–4000 cm^{-1}. Thin Layer Chromatography (TLC) was used for separation of degraded product of azo dye. The solvent system used was ethanol: water: methanol (3:1:3). The pH of the solution was measured by using digital pH meter (pHPLUS DIRECT Meter, LaMotte). A VKS-75 mechanical shaker operated at 120 U min^{-1} was used to shake solutions. Hewlett Packard 6890 gas chromatograph (GC) with a 30 m (length), 0.25 mm (diameter) and 0.25 μm (film thickness) HP-5MS capillary column coupled with a Hewlett Packard 5973 mass spectrometer (MS, Hewlett Packard, US) was used for GC–MS analysis. The GC–MS analysis was carried out using the following temperature program: 100 °C for 2 min, 15 °C min^{-1} up to 300 °C and held for 2 min. The temperature of the injector and detector were 260 and 230 °C, respectively.

2.7. Statistical analysis

The experiments were performed in The results obtained from each set of data have been expressed in terms of mean ± standard error and analyzed by one-way analysis of variance. Readings were considered significant when P was ≤ 0.05.

3. Results and discussion

3.1. Isolation and screening of bacterial isolates

The selective enrichment of liquid effluent collected from the local textile industry led to the isolation of 42 morphologically different bacterial isolates. In Fig. 2 all 42 isolates (isolate codes from B1 to B42) isolated from wastewater samples were tested individually for their ability to decolorize RB5 at the concentration of 100 mg L^{-1}. The decolorization percentages of isolates were ranged from 23 to 60%, except isolate number B26 (*A. hydrophila*) as shown in Fig. 2. *A. hydrophila* isolate was chosen for further evaluation based on its best decolorization efficiency (76%) in liquid medium containing 100 mg L^{-1} of dye. Numerous researchers have

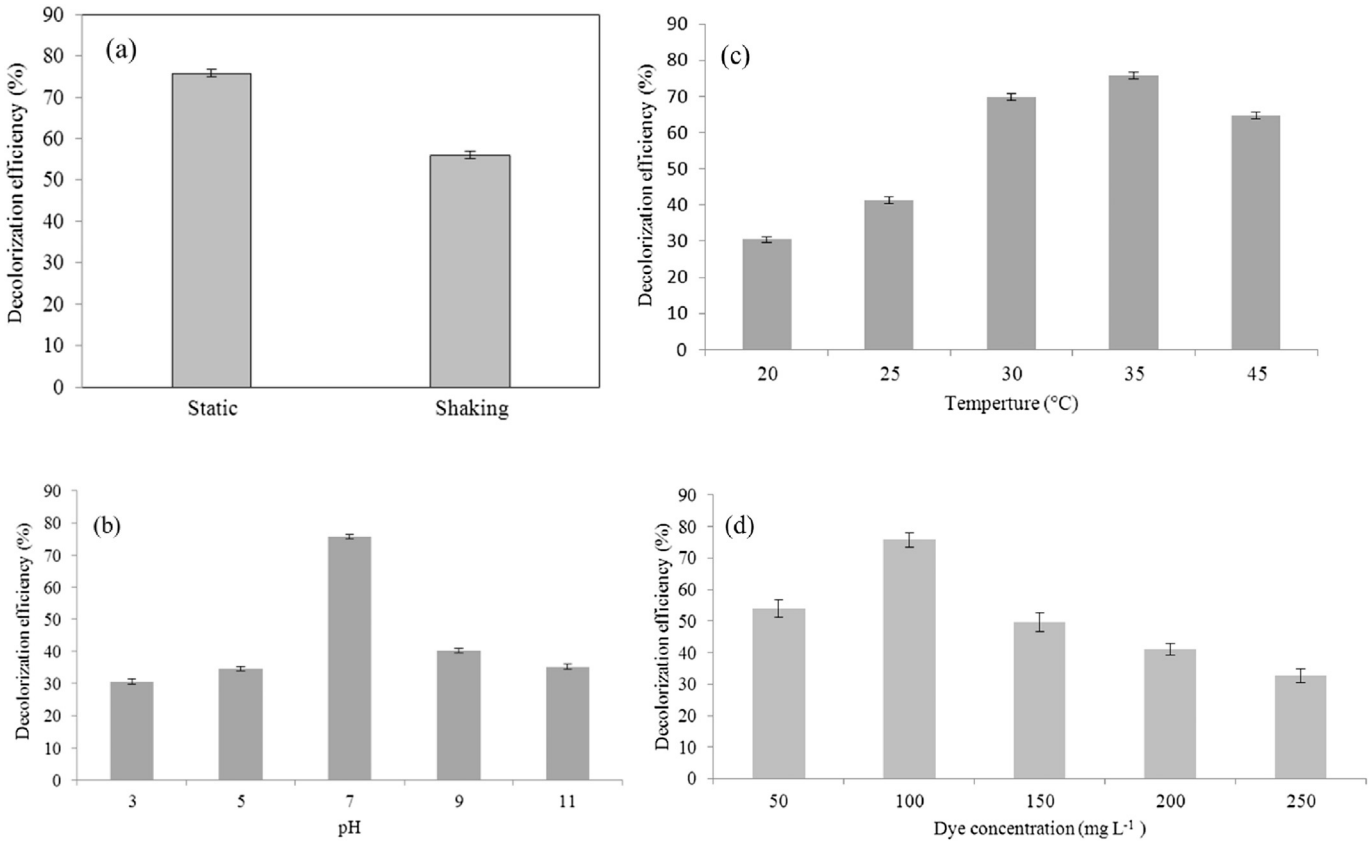

Fig. 4. Effect of experimental conditions on RB5 decolourization by *Aeromonas hydrophila*. (a) Shaking and static conditions, (b) Initial pH, (c) Temperature (d) Initial dye concentration.

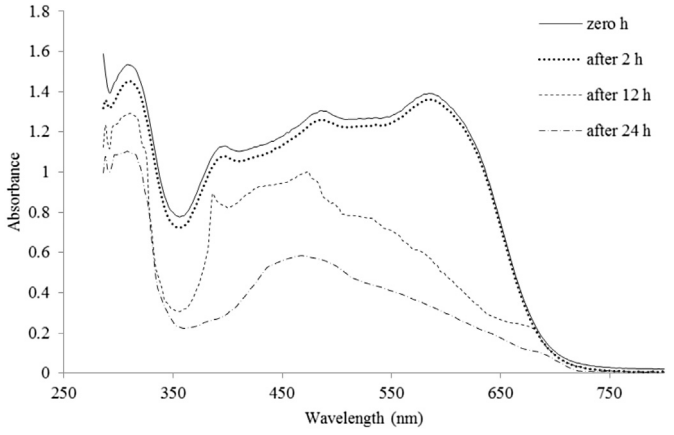

Fig. 5. Variation in the UV–Vis spectra of Reactive Black 5 before and after decolorization by *Aeromonas hydrophila*.

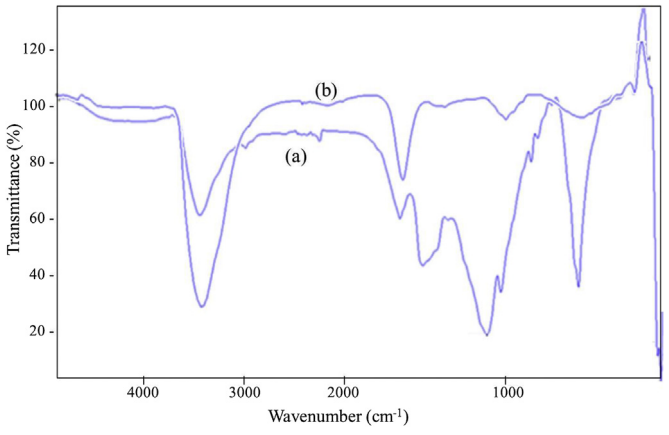

Fig. 6. FT-IR analysis of biodegraded dye sample a) Control dye sample, b) Biodegraded dye sample.

isolated efficient dye decolorizing bacteria from the textile dye effluent [7–9,17], which indicate the natural adaptation of these isolates to high dye concentration and their survival in the presence of toxic dyes [18].

3.2. Identification of A. hydrophila isolate

The 16S rDNA gene sequence was used to carry out BLAST with the database of NCBI Genbank. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using GENETYX computer program. Evolutionary history was inferred using the neighbor-joining method [16]. The tree was drawn to the scale, with branch lengths in the same units as those of the evolutionary distance used to infer the phylogenetic tree [19]. Fig. 3 shows the phylogenetic relationship between the isolated bacterium and other related bacteria found in the GenBank database. The homology indicates that the most efficient bacterial strain capable of decolorizing RB5 is in the phylogenetic branch of the genus *Aeromonas*. This isolate was identified as *A. hydrophila*.

Fig. 7. TLC analysis of biodegraded dye sample.

The sequence analysis of 16S rDNA shows that isolate *A. hydrophila* has the highest similarity with the species *Aeromonas* (97%) which has been proved to have dye decolorizing ability against the moniliformin or structurally related to mycotoxins [20].

3.3. Optimization of decolorization

3.3.1. Effect of static and shaking conditions

The *A. hydrophila* isolate was able to decolorize 76% of RB5 dye (100 mg L^{-1}) within 24 h at static condition, whereas the decolorization was significantly decreased up to 56% at shaking conditions (Fig. 4a). Hence, the static condition was adopted to investigate bacterial dye decolorization in further experiments [21].

3.3.2. Effect of pH

The effect of different pH was carried out on RB5 dye (100 mg L^{-1}) decolorization. Initially with the increase in pH value from 3 to 7, decolorization efficiency increased and maximum occurred at pH 7. Further increase in pH from 7 to 9 exhibited negative effect on decolorization. The decolorization efficiency was above 31% at all pH range (pH 3–11), with the highest 76% observed at pH 7 (Fig. 4b). Thus the pH has a major effect on the efficiency of dye decolorization, and the optimal pH for color removal is often between 6 and 9 [22].

3.3.3. Effect of temperature

Temperature is another important parameter carried out on RB5 dye (100 mg L^{-1}) decolorization. The selected isolate was mesophilic bacterium because it showed better decolorization ability in the temperature range of 25–35 °C (Fig. 4c). Although, the decolorization at 20 °C was only 30%, it gradually increased with increase in temperature from 70% at 30 °C and 76% at 35 °C. The increase in temperature up to 45 °C, however, decreased the efficiency to 65% due to the loss of cell viability or deactivation of the enzymes responsible for decolorization [23]. Similar results were also reported by Wang et al. [22]. The mesophilic range was used by Varel et al. [24] since it was generally thought that maintaining high temperature would be uneconomical and degradation within the psychrophilic range (at low temperature) was too slow.

Table 1
Dye degradation products identified using GC–MS.

Compound	Molecular weight	Retention time (min)
Oxalic acid	90	25.2
Phthalic anhydride	148	22.6
Phthalic acid	166	22.1
1,7-diamino-8-hydroxynaphthalene	175	21.7
3,6,8-trihydroxynaphthalene	178	19.5
1-sulphonic,2-(4-aminobenzenesulphonyl) ethanol	280	15.2
1,2,7-triamino-8-hydroxy-3,6-naphthalinedisulfonate	349	12.8
Reactive Black 5	991	10.2

3.3.4. Effect of dye concentration

It was evident in Fig. 4d that RB5 dye decolorization sharply increased up to 100 mg L^{-1} of dye concentration and maximum decolorization 76% was observed. Then there was a gradual decrease in dye decolorization. Investigations with different dye concentrations in other experiments also reported higher net color removal efficiencies at lower dye concentrations. Decrease in decolorization ability at high substrate concentration might be due to the toxicity of the dye [13]. Azo dyes generally contain one or more sulphonic-acid groups on aromatic rings which might inhibit the growth of microorganisms. Another reason of the toxicity at higher concentration may be due to the presence of heavy metals (metal-complex dyes) and/or the presence of nonhydrolyzed reactive groups which may retard the bacterial growth (reactive dyes). Similarly, reduction in decolorization at low concentration of the substrate might be due to the decrease in enzyme ability to recognize the substrate efficiently.

3.4. Identification of metabolic intermediates

3.4.1. UV–Vis spectra of RB5

From Fig. 5, the maximum absorbance of the dye (λ_{max} = 597 nm) was 1.39. After 24 h, the minimum absorbance of the dye was found to be 0.34. The *A. hydrophila* isolate was able to decolorize RB5 to 76% efficiency in 24 h under optimal conditions such as pH 7, 35 °C, static condition and with the dye concentration of 100 mg L^{-1} in mineral media. After biodegradation of RB5, the absorbance peaks in the visible region disappeared while the absorption peak in the UV range did not diminish.

3.4.2. Biodegradation assay via FT-IR spectrum

The FT-IR spectrum of RB5 dye (Fig. 6a) showed peaks at 3000–3718 cm^{-1} (–NH stretch), 2968–2888 cm^{-1} (CH_2), 2161 cm^{-1} (C–S and S–O stretch), 1626–1748 cm^{-1} (C=C stretch), 1387–1596 cm^{-1} (N–H), 1078–1256 cm^{-1} (O–C), 1011–1078 cm^{-1} (alkenes) and 386–644 cm^{-1} (secondary amines). After RB5 degradation, significant difference in FT-IR spectrum was observed in Fig. 6b. The peak of –NH stretch was reduced and the peak at 3000–3750 cm^{-1} was observed because of O–H stretch. The vibration range from 1750 to 2800 cm^{-1} was completely disappeared which was of aldehyde C–H. The peaks from 500 to 2000 cm^{-1} were decreased and the appearance of new peaks at 1728 cm^{-1} (C=O stretch), 1400–1500 cm^{-1} (C–C stretch), 373–807 cm^{-1} (C–N stretch). The peaks observed after decolorization were for O–H, C–H, C=O, C–C and C–N clearly indicating the removal of amine from the degradation product [25].

3.4.3. Biodegradation assay via TLC

After decolorization by *A. hydrophila* isolate, the decolorized medium was centrifuged at 8000 *g* for 10 min and the supernatant extracted with chloroform after alkalization to pH 8 to extract the biotransformed products. Then the extracted product was evaporated in a rotary evaporator. The concentrated extract was dissolved in 1 mL chloroform and used for TLC analysis. The mobile phase for

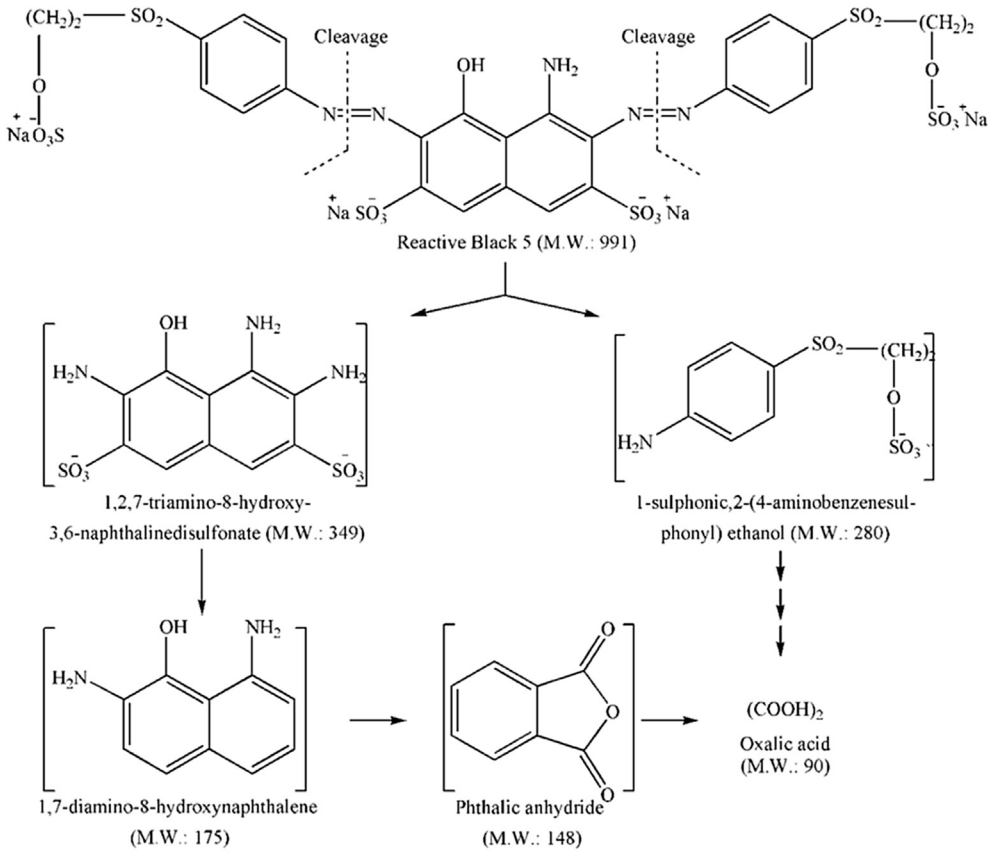

Fig. 8. Proposed biodegradation pathway of Reactive Black 5 by *Aeromonas hydrophila*.

the organic and the aqueous extracts was petroleum ether: chloroform: methanol (4:1:1). The bands of decolorization metabolite were observed under UV light [26]. The TLC chromatograms under UV light showed that the decolorized sample had two bands indicating that RB5 dye was cleaved into two fragments as shown in Fig. 7.

3.4.4. Biodegradation assay via GC–MS

After dye decolorization, the extraction of metabolic products was performed using the liquid–liquid extraction technique where dichloromethane was used as the extractable solvent and dried over anhydrous sodium sulfate. The solvent was evaporated and the samples were analyzed by GC–MS. The GC degradation products of RB5 dye showed the presence of several peaks. The structures of the detected compounds were assigned from the fragmentation pattern and *m/z* values obtained by GC–MS analysis (Table 1). The cleavage of azo bond in RB5 leads to the formation of 1,2,7-triamino-8-hydroxy-3,6-naphthalinedisulfonate (*m/z* 349) and 1-sulphonic,2-(4-aminobenzenesul-phonyl) ethanol (*m/z* 280) [27]. Further it gives rise to oxalic acid (*m/z* 90), which could be mineralized. Based on the intermediates identified by GC–MS, plausible pathway for biodegradation of RB5 dye has been proposed (Fig. 8). During the degradation, there is asymmetric cleavage of azo bonds in RB5 resulting in formation of 4-aminobenzenesulphonyl, while the naphthalene part of the dye was degraded resulting in the formation of smaller compounds [28]. Further biodegradation of naphthalene part with opening of ring, giving rise to the formation of carboxylic group, is confirmed from the FT-IR data. Thus, it is clear from the analytical methods used that the RB5 dye is degraded to intermediate compounds as a result of cleavage of azo bond (N=N). The intermediates identified, 4-aminobenzenesulphonyl, naphthalene derivatives and carboxylic, are devoid of any chromophores like azo group (N=N) and hence are colorless. Table 1 depicts the structures of the identified products formed from biodegradation of RB5 dye according to its retention time. These results suggest that the decolorization of dyes proceed via the cleavage of azo bonds resulting in the formation of aromatic amines as shown in Fig. 8.

In the present work, the degradation products of RB5 by *A. hydrophila* were analyzed by GC–MS as shown in Fig. 9. Analysis of RB5 before the degradation at zero h showed a peak at a retention time of 10.2 min, which corresponded to that of unmetabolized RB5 azo dye. This peak disappeared completely at the end of the biodegradation of RB5. Also, unidentified small peaks at retention times of 12.8 and 15.2 min were detected. The intensity of these peaks increased towards the end of degradation. Furthermore new peaks at retention times of 19.5, 21.7, 22.1, 22.6 and 25.2 min were detected. Interestingly, these peaks completely disappeared at the end of the aerobic treatment. Mane et al. [29] reported that the decolorization of azo dyes normally begins with an initial reduction or cleavage of azo bond then followed by complete degradation of aromatic amines under static conditions.

The GC–MS spectrum showed various retention time of peaks indicating the partial mineralization of RB5 after the degradation as in Fig. 9. The retention time of peaks and suggested molecular weight of these compounds indicated that in the biodegradation, RB5 was most likely broken down into compounds with lower molecular weight. It also suggested that decolorization was related to the breaking of azo bonds which are associated with chromophores, i.e. conjugated unsaturated bonds (–N=N–) in the molecule. In this stage, some aromatic amines intermediates, such as 1-sulphonic,2-(4-aminobenzenesulphonyl) ethanol and 1,7-diamino-

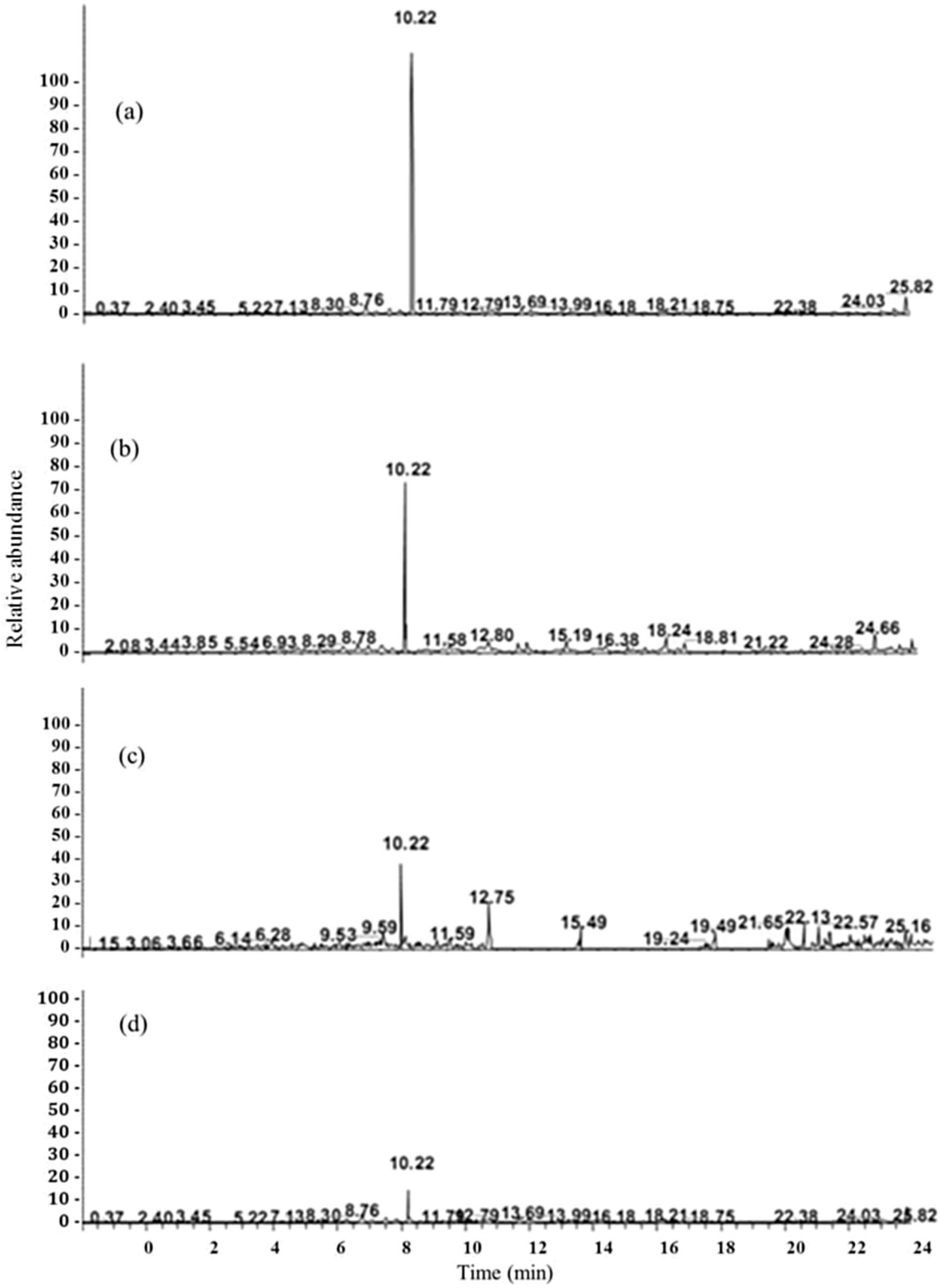

Fig. 9. GC–MS analysis of RB5 and its degradation metabolites resulting under static condition, Temp = 37 °C and pH = 7: (a) Untreated dye at zero h; (b) at the beginning of degradation after 2 h; (c) after 12 h; and (d) after 24 h.

8-hydroxynaphthalene, may have been produced, indicating partial mineralization of RB5. Junnarkar et al. [30] reported that complete dearomatization of dye is associated with biodegradation process. The cleavage of azo bonds and formation of aromatic amines with the amines are also reported by Junnarkar et al. [30].

4. Conclusions

The present study confirms the ability of isolated bacterial culture *A. hydrophila* to decolorize the textile dye RB5 with decolorization efficiency of 76%, thus suggesting its application for

decolorization of dye bearing industrial wastewaters. The decolorization of RB5 dye occurred as a result of cleavage of azo bond accompanied by the formation of oxalic acid. The ability of *A. hydrophila* isolate can be considered as potential bioremediation tool for the treatment of industrial wastewater. There is no information available in open literature concerning optimization of process parameters by applying statistical software for decolorization of RB5 by *A. hydrophila*. The results of this study could be used to design a suitable process to get higher percentage decolorization of RB5.

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