

Biotreatment of the Wastewater Containing Insoluble Pigment by Halomonas Strain G_b

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ABSTRACT

Industrial dyeing processes produce a large amount of wastewater that contains many organic compounds such as different type of dyes and pigments, dispersing agents, surfactants which are difficult to treat. Considering that little studies have been done on biodegradation of oil-soluble azo dyes, the lake of this issue is completely felt. In this study, biotreatment of Toluidine Red (TR), an oil-soluble azo dye, was optimized under different environmental conditions. Halomonas strain G_b was capable of decolorizing TR at a pH range of 6.5-9.5 and temperature range of 25-40 °C. The optimum condition was 25 mg/L dye, pH=6.5, temperature 35 °C and 5% (w/v) NaCl. UV-Vis spectrophotometric method, high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometer (GC-MS) analyses confirmed that biodegradation of TR was accrued successfully. According to the results, Halomonas strain G_b can be recommended as practical bacteria for the treatment of industrial wastewaters containing azo dyes with different water solubility. *Prog. Color Colorants Coat.* 13 (2020), 53-62 © Institute for Color Science and Technology.

1. Introduction

Synthetic dyes are used in large quantities in the textile industries, paper printing, food, cosmetics, and pharmaceuticals. There is more than 7×10^5 tons of dye produced annually worldwide. Dyes are recalcitrant organic molecules that impart strong color to wastewater also contributing to the organic load and toxicity or the wastewater. Approximately, 15% of the total dyes used in different industries, discharged to the wastewater causing extensive pollution [1]. Azo dyes, one of the most important groups of synthetic dyes are characterized by aromatic moieties linked together with chromophoric azo groups (-N=N-). They cover about 70% of total dyes produced worldwide annually and

have extensive applications in various industries. The release of azo dyes into wastewaters may cause serious environmental problems not only for aesthetic reasons but also for their breakdown products (aromatic amines) and their toxic and/or mutagenic effects [2]. A number of physicochemical methods are available for the treatment of textile dyeing effluents containing azo dyes [3]. Although these methods are effective but they produce large quantities of sludge, whose secondary treatment increase total cost of the process. Many microorganisms including gr⁺ and gr⁻ bacteria and fungi are capable of decolorizing the azo dyes. Bacterial decolorization of azo dyes is generally based on the azoreductase activity which is produced by

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different trophic groups of bacteria. These enzymes catalyzed the reductive cleavage of the azo bond which leads to the decolorization of the azo dyes into their colorless aromatic byproducts [4]. Further degradation of aromatic amines in biological systems generally needs a sequential anaerobic-aerobic process. In many cases, the industrial wastewater effluents contain high salinity thereby causing problems for conventional biotreatments. Halotolerant and halophilic bacteria usually tolerate noticeable amounts of toxic metals in their environment thus are used for bioremediation of oil and oxyanion pollution [5]. Although there are some reports about the ability of halophilic bacteria for biodegradation of water-soluble azo dyes, little is available for biotreatment of oil-soluble ones. In this study, we report the ability of halotolerant bacteria; for degradation of an oil-soluble azo dye, for the first time. Various environmental parameters were optimized by traditional stepwise strategy, one factor at a time method. Analytical techniques such as UV-Vis, GC-MS, and HPLC were used for analyzing dye degradation intermediates and a plausible pathway of degradation proposed.

2. Materials and Methods

2.1. Dyes and chemicals

Toluidine Red (Pigment Red 3, C.I. No. 12120) was from Ciba Geigy GmbH representative in Iran (CIBA) and was used without any purification. The stock solution of dye was made by dissolving dye powder in dimethyl formamide (DMF) as solvent (1g/L^{-1}), using an ultrasonic bath due to the insolubility of TR. Also, to reduce the adverse effects of insolubility of dye, 0.25% (v/v) Tween 80 was added as the surfactant to dye stock solution. Acetonitrile used in HPLC analysis was of HPLC grade. All other chemicals and reagents were of analytical grade and were procured from Merck (Darmstadt, Germany) unless otherwise specified. Chemical structure of TR is shown in Figure 1.

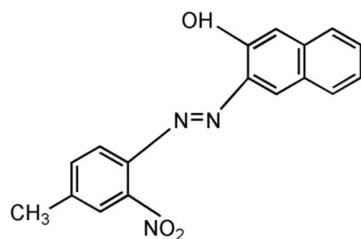


Figure 1: Chemical structure of TR.

2.2. Organism and culture

Halomonas strain G_b (GenBank accession no. DQ489548) was obtained from extremophiles laboratory, department of microbiology, school of biology, University of Tehran, Iran. Details of the isolation and identification of this strain are reported thoroughly by Asad et al. [5]. According to their report, the isolate shared the main phenotypic features of the genus Halomonas and was 98% similar to Halomonas salina. It was grown on nutrient agar plates supplemented with 7.5% (w/v) NaCl at $35\text{ }^\circ\text{C}$, maintained at $4\text{ }^\circ\text{C}$ and subcultured once in two weeks.

2.3. Decolorization medium

Minimal medium was used to study dye decolorization and consisted of the following components per liter: KNO_3 : 5 g; $(\text{NH}_4)_2\text{SO}_4$: 1g; $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$: 0.87 g; KH_2PO_4 : 0.54 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$: 0.2g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$: 0.02g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$: 0.01g; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$: 0.005g. 2.5%-10% NaCl was added to the medium and dye was used as the sole carbon source. This medium is generally used for denitrifying bacteria, and its preparation method is explained in detail in the Handbook of microbiological media [6].

2.4. Effect of different parameters on decolorization

A loop full of microbial culture (Halomonas strain G_b) was cultivated in the static condition to 35 mL of glass tubes containing 15 mL of sterilized minimal medium. The optimization under different culture conditions including initial dye concentration (10 to 25 mg/L), initial pHs in the range of 4.5 to 10.5, NaCl percents (2.5 to 10 % (w/v)) and incubation temperatures (25 to $40\text{ }^\circ\text{C}$) was investigated using one-factor-at-a-time method (OFAT) method with the basic condition of 25 mg/L TR, $\text{pH}=8.5$, 5% (w/v) NaCl and temperature $35\text{ }^\circ\text{C}$. This method is well studied and has been accepted by researchers [7], based on changing one parameter at a time and maintaining the pre-optimized at a constant level. pH was adjusted using NaOH and HCl 1N. Incubation was carried out at static condition. The stock solution of dye was sterilized separately. Different concentrations of Tween 80 (% 0.25, % 0.3, % 0.5 and % 0.75 (v/v)) were used for preparation of dye stock solution. The best solubility of the dye in the decolorization medium was observed at 0.25% (v/v).

2.5. Decolorization measurement

To determine decolorization efficiency, tubes containing decolorization medium were well agitated before sampling due to the insolubility of dye, and then aliquots (1.5 mL) of the culture media were withdrawn at regular time intervals. Clarification was done with centrifuging of the samples at 7500 rpm for 5 min with a micro refrigerated centrifuge (Hettich, Micro 200R, Germany) at 20 °C to separate bacterial cell mass. Decolorization was calculated at the corresponding maximum absorbance wavelength ($\lambda_{\max}=490$ nm) of the samples. Uninoculated culture media were used as controls. The rate of decolorization was obtained using the following equation (Eq.1):

$$(\%) \text{ Decolorization} = \frac{\text{Initial absorbance} - \text{final absorbance}}{\text{initial absorbance}} \times 100 \quad (1)$$

2.6. UV-Vis spectral analysis

Decolorization was monitored qualitatively using UV-Vis spectra of TR from 200-800 nm. Samples from incubated medium were withdrawn at regular time intervals, and after centrifuging at 7500 rpm for 5 min in the micro refrigerated centrifuge, the supernatants scanned using Varian Cary 100 spectrophotometer equipped with quartz cells (1 cm³). Decolorization manner of Halomonas strain G_b recognized through comparing control and sample spectrum to detect any transformation of medium compounds qualitatively.

2.7. Decolorization and degradation analysis

Biodegradation was analyzed using the HPLC method. Intermediates produced during biodegradation of TR were identified with GC-MS analysis. HPLC and GC-MS analysis were carried out at optimum condition after 10 days of incubation. The extraction of the metabolites was done using two equal volume of ethyl acetate.

Resulted extracts lost water in a rotary evaporator and the remaining solvent was dried overnight at room temperature. The residue was dissolved in 0.5 mL HPLC grade Acetonitrile and used for HPLC and GC-MS analysis. High performance liquid chromatography (HPLC) analysis was carried out on C₁₈ column (symmetry, 406 mm×250 mm, Knauer, K 2500, Germany). The mobile phase was Acetonitrile: water containing 0.1 % formic acid (50:50 v/v) with 1 mL/min flow rate for 10 min at 254 nm. An HP6890 GC coupled with an HP-5973 MS detector (Hewlett Packard, USA)

was used to perform GC-MS analysis of the metabolites. Gas chromatography was done with an HP-5MS column (30 m long×0.25 mm i.d.) in a temperature programming mode. The chromatographic condition was: 60 °C as the initial column temperature kept constant for 3 min. 250 °C as the final temperature held for 3 min. 10°C /min as temperature increasing rate. 250 °C as the injection port temperature. The carrier gas used in the analysis was Helium at a flow rate of 1 mL/min. The ionization voltage was 70 eV. Degradation products were identified on the basis of mass spectra and using the Wiley 275 spectral library stored in the computer software (ChemStation) of the GC/MS.

3. Results and Discussion

3.1. Effect of various concentrations of dye on decolorization

Decolorization ability of Halomonas strain G_b was investigated by testing the strain against different concentrations of dye (10-25 mg/L, pH=8.5, temperature 35 °C, 5% (w/v) NaCl) up to 210 h of incubation by using dye as the sole carbon source. Maximum dye removal at 10 and 15 mg/L concentrations was 42.5 and 50 % respectively. At 20 and 25 mg/L dye concentrations, maximum decolorization rate was 72 and 80 % respectively and it decreased to 55 % at 40 mg/L (Figure 2). The amount of time required to reach the maximum decolorization extent at 10, 15, 20 and 25 mg/L dye concentrations over the same period of incubation was 48, 96, 96 and 240 h respectively. Results clearly showed that decolorization raised by increasing initial dye concentration and higher concentrations of dye required more time to reach the maximum percentage of decolorization. No decolorization was observed at 1.5 and 5 mg/L TR due to the poor carbon source for bacterial activation. A direct relationship between dye concentration and color removal could be attributed to the fact that bacterial strain used dye as the sole carbon source for its growth and activity. Similar observations were reported earlier for other strains and dyes [8, 9]. El Bouraie and Walaa [10], reported that decolorization of RG5 sharply increased up to 100 mg/L of dye concentration and then there was a gradual decrease in dye decolorization. Obtained results indicated that the best concentration of TR for color removal was 25 mg/L, so this concentration was used for further decolorization experiments.

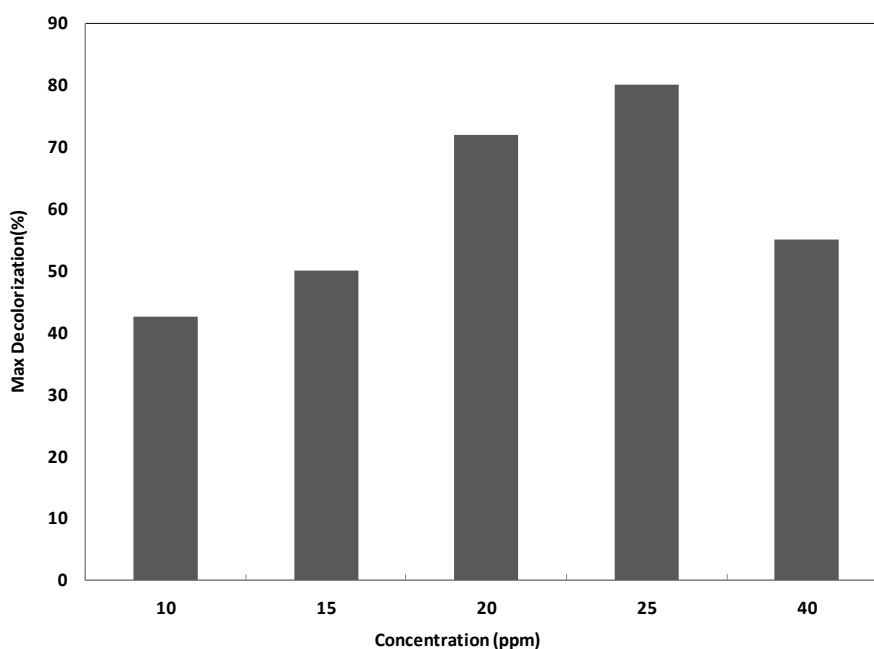


Figure 2: The effect of dye concentration on the percentage of decolorization.

3.2. Effect of pH on dye decolorization

The effect of pH at 25 mg/L dye, 35 °C, 5% (w/v) NaCl on decolorization of TR was tested in the range of 4.5-10.5 at static condition up to 240 h of incubation (Figure 3). Sodium hydroxide and hydrochloric acid were autoclaved separately before adding to decolorization medium. pH was measured with Metrohem 827 (Swiss made) pH meter. Decolorization percentage reached to its maximum extent (77 %) at pH 6.5 after 96 h of incubation. This could be due to the fact that bacterial cultures generally show maximum decolorization rate at pH values near neutrality or slightly acidic [10]. It was illustrated from the results that decolorization of TR took more time to reach its maximum extent as pH was increased. At strongly acidic or alkaline conditions (pH=4.5 and 10.5 respectively), the amount of decolorization was very low (20 % at pH=4.5 and 30% at pH=10.5). At lower pH value, the H⁺ ions compete effectively with dye cations, causing a decrease in color removal efficiency. Furthermore, at higher pH above this point charge, the surface of biomass gets negatively charged, which enhances the positively charged dye cations through the electrostatic force of attraction. Our results are similar to those presented earlier by Ayed et al. [11],

who reported that maximum decolorization of Crystal violete by *Basillus* sp. was occurred at pH=7.

Aeromonas veronii GRI removed the color of Methyl Orange over a wide range of pH (7–9) with the optimum at pH=7 [12]. *Halomonas* strain G_b could tolerate a wide pH range (6.5-9.5) in contrast with common decolorizing bacteria that have a narrow pH range [13, 14]. The optimum achieved pH (6.5) was chosen for further decolorization experiments with *Halomonas* strain G_b.

3.3. Effect of salt concentration on decolorization of azo dye

Decolorization of TR by *Halomonas* strain G_b was investigated at various salt concentrations (2.5-10% (w/v) with 2.5 % Interval), keeping constant dye concentration (25 mg/L), pH (6.5) and temperature (35°C). It could be inferred from Figure 4 that the decolorization rate was significant at 5-7.5 % (w/v) NaCl and then it leveled off at 10 % (w/v). Guo et al. [15] reported that with raising NaCl amount from 5 to 15 %, decolorization increased and in the presence of more than 15% sodium chloride (15 to 30 %) it was decreased.

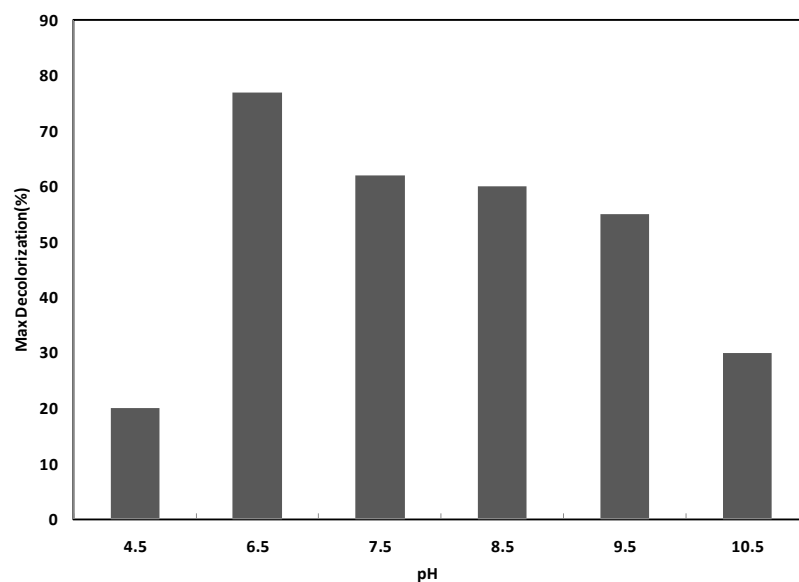


Figure 3: The effect of pH on the percentage of decolorization.

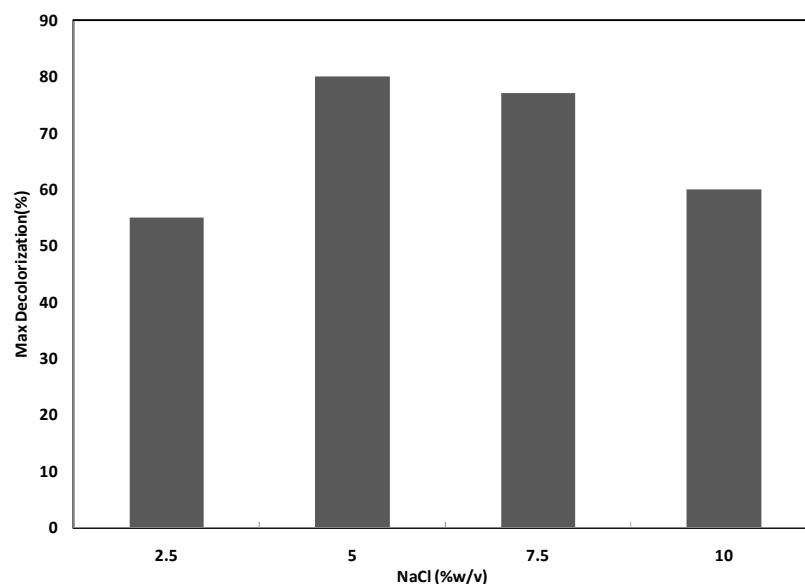


Figure 4: The effect of salt concentrations on the percentage of decolorization.

According to the study of Meng et al. [16] on AO27 decolorization by *Shewanella aquimarina*, azoreductase activity and decolorization rate increased up to 3 % NaCl, a further increase in NaCl concentration led to decreasing enzyme activity and color removal rate. Also, Ogugbue et al. [17] reported a sharp decrease in color removal with salt concentrations upper than 6 % (w/v) in decolorization of polar red b by *Bacillus firmus*. Our results indicated that the optimum sodium chloride concentration for color removal was 5 % (w/v) with 80 % decolorization rate. Maximum decolorization

extent at 2.5 %, 7.5 % and 10 % (w/v) NaCl was 55%, 77 % and 59 %, respectively. Data showed that increases in salt concentration from 2.5 % to 7.5 % led to high color elimination percentage and at concentrations above 7.5 %, decolorization suppressed. Reduction in decolorization at NaCl concentrations upper than 7.5 % (w/v) could be due to the loss of cell activity or their plasmolysis. In another words inhibition effect at high salt concentration (above 7.5 %) was due to the negative influence of salt on permeability of cell membrane for dye ions and relative competition between chloride and

dye molecules for active sites of *Halomonas* strain G_b at high salinity [17, 18]. According to this experiment *Halomonas* strain G_b was capable of dye decolorization at salty condition (up to 7.5 %), so it was a halotolerant organism and could be used for biotreatment of effluents containing salt and metal ions.

3.4. Effect of temperature on Decolorization

Decolorization ability of *Halomonas* strain G_b was studied in the range of 25-40 °C at pH= 6.5, 5% (w/v) salt concentration and 25 mg/L dye. Obtained data is shown in Figure 5. Decolorization rate of TR with *Halomonas* strain G_b was 40 % at 25 °C, 46 % at 30 °C, 80 % at 35 °C and 68 % at 40 °C which was achieved at 2nd, 2nd, 10th and 4th day of incubation, respectively. Results suggested that the optimum temperature for decolorization was 35 °C. Decolorization rate sharply increased from 30 °C to 35°C and then decreased slowly from 35 to 40 °C. This observation could be due to the loss of bacterial growth and the deactivation of the enzymes responsible for decolorization. Our observation could be compared to the results reported earlier by other researchers for other microorganisms and azo dyes [19] also Guadie et al. [20] reported that the optimum decolorization efficiency of the strain CH12 in the removal of the azo dye named RR 239 was found at 25–35 °C.

3.5. Decolorization mechanism of *Halomonas* strain G_b

Colorants possess color because of:

- Absorbing light in the visible range of spectrum (400–700 nm)
- Having at least one color-bearing group which is named chromophore
- Having a conjugated system (a structure with alternating double and single bonds)
- Exhibiting resonance of electrons which acts as a stabilizing force in organic compounds

If anyone of the above-mentioned cases does not exist in the molecular structure, the color is lost. The wavelength spectrum of absorbed light, which determines the color of the matter, is affected by its chemical structure consisting of components such as the chromophores and auxochromes [21]. Decolorization mechanism of *Halomonas* strain G_b was monitored qualitatively using a UV-Vis spectrophotometer. Figure 6 Shows the UV-Vis spectra (200-800 nm) of the supernatants at the 4th day of incubation at optimum condition (25 mg/L dye, pH=6.5, temperature 35 °C and 5 % (w/v) NaCl). Compared to the control sample, it was speculated that after decolorization of TR, its maximum absorbance peak at 490 nm in visible range was diminished entirely and only the 203 nm peak in the UV-region remained. Decolorization of azo dyes may be due to adsorption or biodegradation mechanisms.

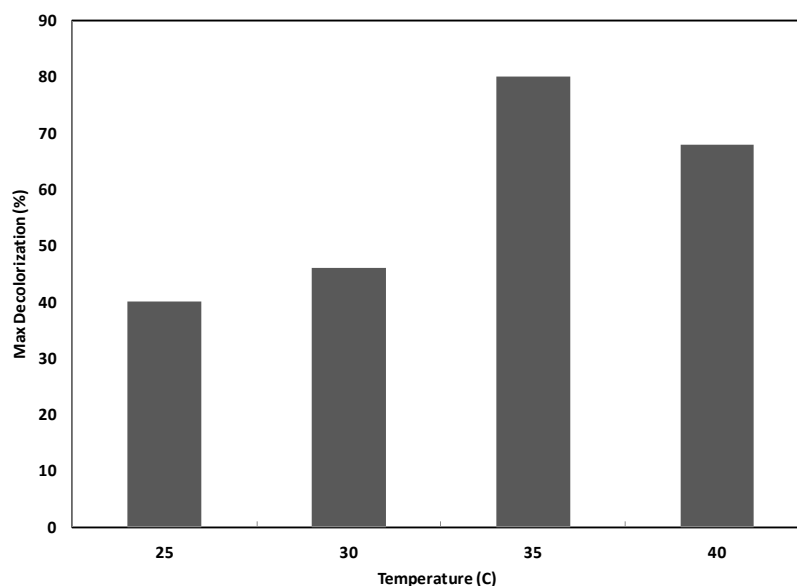


Figure 5: The effect of temperature on the percentage of decolorization.

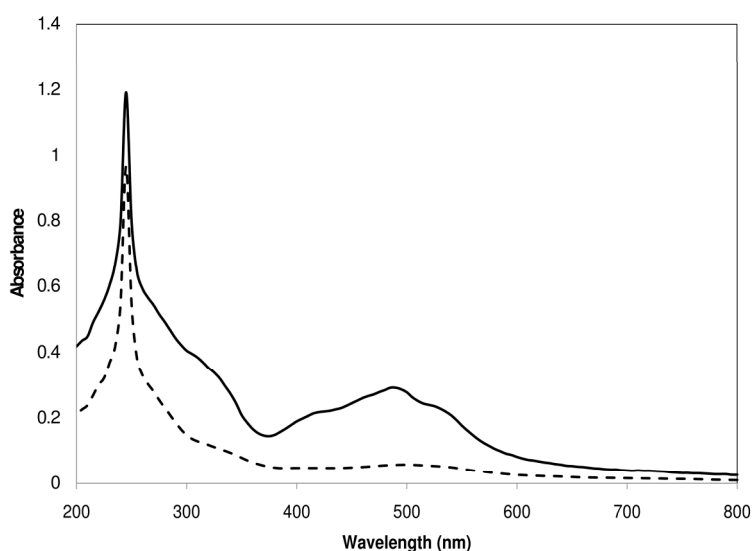


Figure 6: Variation in the absorption spectra of TR (490nm) before (—) and after (----) decolorization of TR by *Halomonas* strain G_b at optimum condition.

In case of adsorption, the UV-Vis absorption peaks decrease approximately in proportion to each other whereas if dye removal is due to biodegradation, either the major visible light absorbance peak disappears, or a new peak appears [22]. According to obtained results, since the disappearance of visible peak occurred in UV-Vis spectra of TR, decolorization was attributed to biodegradation rather than surface adsorption and new metabolites were produced in culture supernatant because of altering the chemical structure of azo dye due to the enzymatic activity of *Halomonas* strain G_b .

3.6. Biodegradation analysis

Biodegradation metabolites of TR were monitored with HPLC analysis. The appearance of new peaks at different retention times compared to control sample confirmed degradation mechanism due to the enzymatic activity of *Halomonas* strain G_b rather than visual observation of dye removal. HPLC analysis of the beginning sample showed two major peaks at retention time 1.290 min and 1.911 min and one minor peak at retention time 2.887 min. At the end of decolorization, some new peaks were observed; three major peaks at 1.315 min, 2.123 min and 3.471 min and two minor peaks at 2.528 min and 2.916 min (Figure 7). Verification of degraded metabolites was carried out with GC-MS analysis. Extracted sample with ethyl acetate was injected into a GC-MS after 10

days of incubation. Ion chromatogram of compounds produced from TR degradation is indicated in Figure 8. Figure 9 illustrates the proposed pathway of azo dye degradation. As shown in figures, the peak having a retention time of 7.76 min was identified as para methyl benzene diazonium with molecular ion peak at m/z 121 resulted from the main degradation of TR and then missing NO_2 group. The protonated molecule at m/z 121 was fragmented to give rise a mass peak (m/z) 105 after the breakage of the methyl bond. Finally, benzene fragment at m/z 77 has been obtained from benzene diazonium (m/z 105) by cleavage of azo group. Cleavage of azo dyes can take place symmetrically or asymmetrically. The main degradation of TR in this study occurred asymmetrically by breakage the bond between the nitrogen of azo group and the carbon of 2-naphthol ring. The peak with retention time 18 min corresponds to 1,2-benzene dicarboxylic acid with the mass peak (m/z) 167 and 3-Phenyl-acrylic acid with m/z 149 ± 1 which were the fragmentation products of 2-(2-carboxy vinyl) benzoic acid with m/z 193. It was possible to propose a metabolic pathway as shown in Figure 8 to give this fragment [23]. Our results are comparable with Xu et al. [24] who identified degradation product of sudan II dye by *E. faecalis* as para methyl benzene diazonium at m/z 121 ± 1 . Also, Chen et al. [25] reported that in degradation of sudan III with *Lactobacillus acidophilus* and *L. fermentum*, benzene

fragment at m/z 77 ± 1 was formed. Based on obtained results, *Halomonas* strain G_b used dye as sole carbon and energy source for its growth and enzymatic activity. No mass peak was observed in mass spectra

regarding to degradation of Tween 80 so the only role of Tween 80 was to increase the solubility of dye and not to act as a co-substrate

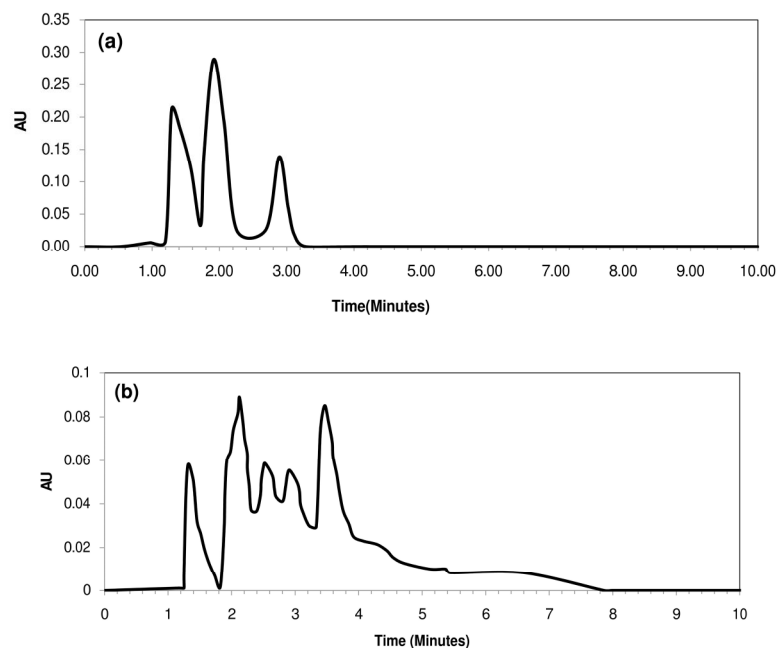


Figure 7: HPLC analysis of (a) control TR and (b) metabolites formed after degradation at optimum condition.

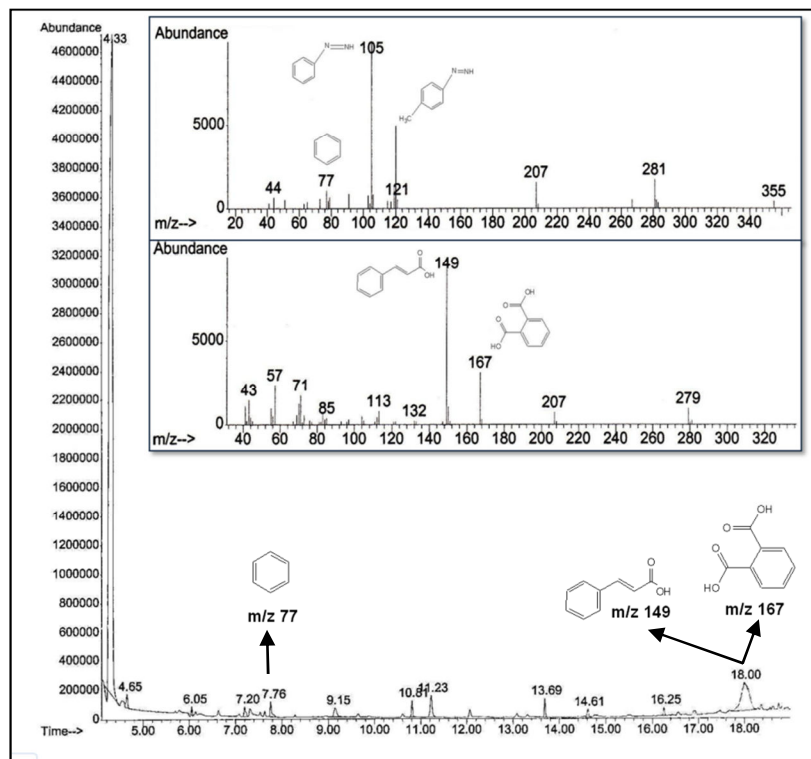


Figure 8: Decolorization products of TR (a) Total ion chromatogram of GC-MS analysis, (b) Benzene and (c) 1, 2-benzene dicarboxylic acid.

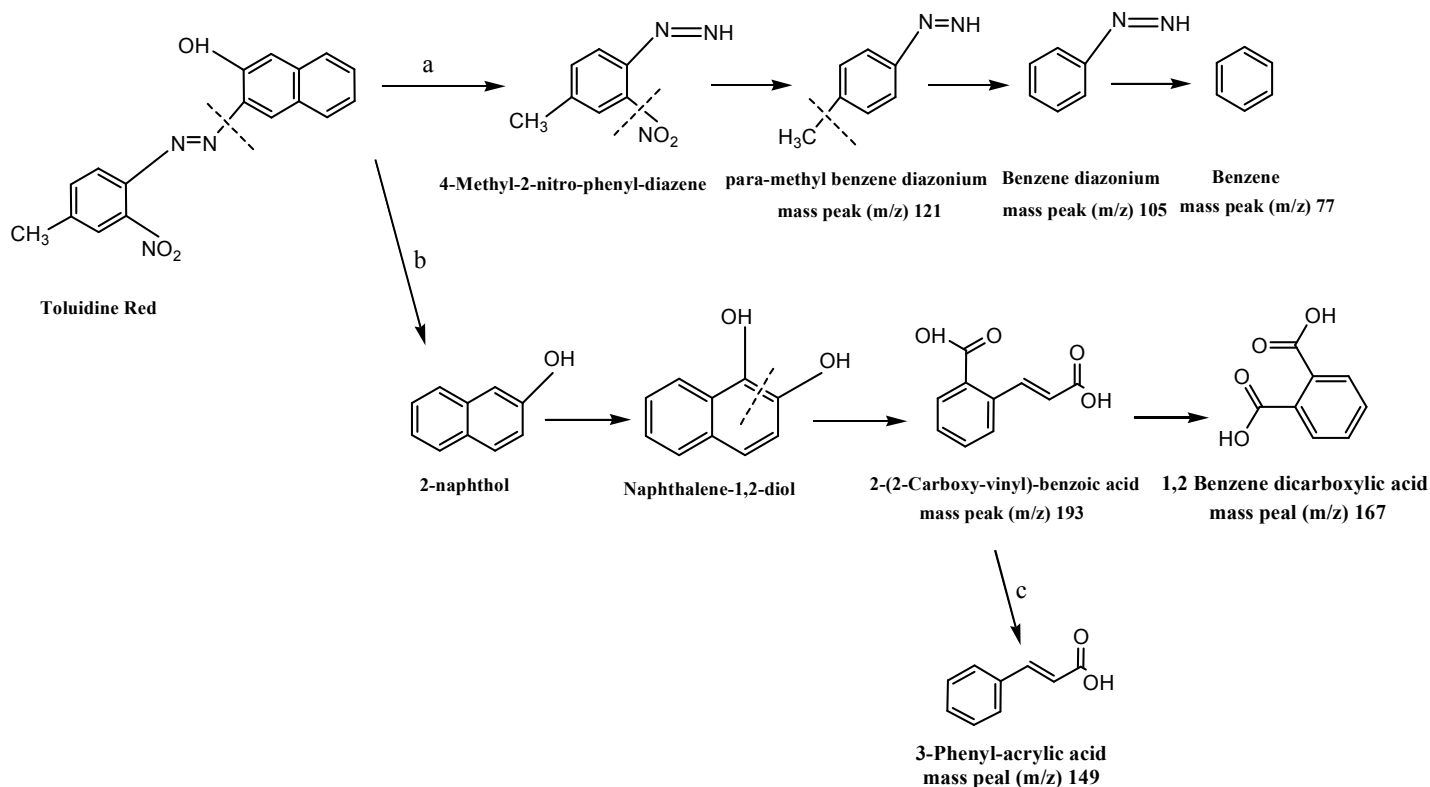


Figure 9: Proposed pathway for TR degradation by Halomonas strain G_b .

4. Conclusion

Halomonas strain G_b was tested for its ability of decolorization of TR, an oil-soluble diazo dye, in the presence of Tween 80 as the surfactant. Different parameters such as dye concentration, pH, NaCl percent (w/v) and temperature were optimized with one factor at a time method. UV-Vis, HPLC and GC-MS analysis showed that decolorization was done under degradation mechanism instead of inactive surface adsorption. Final compounds formed due dye degradation were identified

as benzene at m/z 77, 1,2-benzene dicarboxylic acid at m/z 167 and 3-Phenyl-acrylic acid at m/z 149 ± 1 with GC-MS analysis. It was possible to propose a metabolic pathway as shown in Figure 9 to give the fragments resulted from dye chemical structure breakage. The ability of Halomonas strain G_b to decolorize TR, at salty condition (up to 7.5% (w/v)) make it potential for biotreatment of effluents containing salt and metal complexes.

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