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Aerobic biodegradation of azo dye Acid Black-24 by *Bacillus halodurans*

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Abstract

Bacillus halodurans MTCC 865 was employed for decolorization of textile azo dye, Acid Black-24 (AB-24). Thousand mgl^{-1} of AB-24 was decolorized with 90% efficiency by the strain within 6 hrs at pH 9 and 37°C with 5% NaCl under static conditions in screening medium. Decolorization was evaluated by measuring the periodic decrease in absorbance at 557nm (λ_{max}). Biodegradation of Acid Black-24 was determined by FTIR and HPLC. The FTIR spectrum of the AB-24 dye suggests the presence of azo bond (–N=N–) peak at 1618.28 cm^{-1}. Absence of the azo bond in the degraded sample spectrum indicates biodegradation of the dye. Formation of metabolites with different retention times in HPLC analysis further confirmed degradation of the azo dye, Acid Black-24 by *Bacillus halodurans*.

Key words

Acid black-24, *Bacillus halodurans* MTCC 865, Biodegradation, Decolorization

Introduction

Dyeing textiles has been practiced for thousands of years with the first record of the use of dyestuff dated at 2600 B.C in China. Among various industries, the textile industry utilises a multitude of chemical products containing organic surfactants, dispersants, alkalis, solvents, humectants, acids, salts and residual dyes (Tchobanoglous and Burton, 1995) thus, releasing a significant amount of wastewater from its various processing operations. The dyeing processes are an integral part of the textile industry, with consumption of large amount of fresh water and release of equally large volume of coloured effluent. Majority of the dyes employed in textile industries are synthetic, and of these approximately 60–70% are azo dyes. Azo dyes are aromatic moieties linked together by azo (–N=N–) bond and represent the largest class of dyes used in textile industries (Stolz, 2001). Dyes are detected in water at concentrations as low as 1 mg l^{-1} (Ramalho *et al*., 2004). Nearly 15–20% of these azo dyes fail to adhere to the fabric and are released into the sewage treatment systems or into the environment (Lewis, 1999; Reisch, 1996), causing aesthetic problems severely affecting the photosynthetic function due to low light penetration. Color is the first contaminant to be recognized in wastewater and has to be removed before discharging into water bodies. A number of physico-chemical methods (coagulation, adsorption, membrane filtration and ion exchange) are available for decolorization and detoxification of textile dyeing wastewaters (Churchley, 1994). However, almost all of these treatment methods have serious drawbacks such as high cost, huge chemical sludge production and does not remove all dyes (Krull *et al*., 1998; Sen and Demirer, 2003). For these reasons, biological methods of wastewater treatment are considered as the best alternative as they are environmental friendly and can lead to complete mineralization of organic pollutants at low cost. Azo dyes are generally considered xenobiotic compounds that are recalcitrant to degradation because of their synthetic origin and complex aromatic molecular structures (Banat *et al*., 1996). Several microorganisms (bacteria, fungi, algae and yeast) have been reported to possess the ability to decolorize and mineralize different kinds of azo dyes under various environmental conditions (Pandey *et al*., 2007; Maximo *et al*., 2003). Among these microorganisms, bacteria are most commonly used for various bioremediation processes. Extensive studies have been carried out to determine the potential of diverse bacteria in decolorizing various structurally different azo dyes (Yu *et al*., 2001; Chang *et al*., 2004; Chen *et al*., 2003; Hong *et al*., 2008). The initial step in bacterial degradation of azo dyes,

in either anaerobic or aerobic conditions, is the breakdown of –N=N– bond. Anaerobic azo dye reduction has been thoroughly investigated and found to be favourable but with generation of aromatic amines which are toxic, carcinogenic and mutagenic in nature and reoxidize in certain environmental conditions (Pinheiro *et al*., 2004; Myslak and Bolt, 1998). Bacteria are seldom able to decolorize azo compounds in the presence of oxygen , as a result very few reports exists on the aerobic decolorization of azo dyes (Coughlin *et al*., 2002; Chang *et al*., 2001; Buitron *et al*., 2004; Sarayu and Sandhya, 2010). Since, dyeing effluents are characterized by high levels of alkalinity and salinity, application of microorganisms able to sustain stringent conditions of effluent is indispensable for biodegradation. The aim of the present work was to evaluate the potential of *Bacillus halodurans* MTCC 865 to decolorize azo dye, Acid Black 24 (AB-24) aerobically at different pH, temperature and salinity using UV-Vis spectrophotometer, Fourier Transform Infrared Spectroscopy (FTIR) and High Performance Liquid Chromatography (HPLC).

Materials and Methods

Azo dye, Acid Black-24 (AB-24) with maximum absorbance at 557 nm (λ_{max}), was a generous gift from a local textile mill in Coimbatore, Tamil Nadu, India. HPLC-grade methanol was purchased from Sigma. Stock solution of azo dye was prepared by dissolving 5 gl^{-1} dye in distilled water and filtered through 0.45µm membrane filter. *Bacillus halodurans* MTCC 865 was procured from Institute of Microbial Technology, Chandigarh, India. The pure strain was routinely maintained at 4°C on Alkaline Bacillus Medium with the following composition. 10gl^{-1} Peptone, 5 gl^{-1}Yeast Extract, 10 gl^{-1} Glucose, 4 gl^{-1} K_2HPO_4, 15 gl^{-1} Agar and pH 9. Dye decolorization studies were carried out in screening medium consisting of 10 gl^{-1} Peptone, 10 gl^{-1} Meat Extract, 5 gl^{-1} NaCl and pH 9 under static conditions. Decolorization was spectro photometrically assayed by UV-visible spectro-photometer (UV- 2401 PC Shimadzu), biodegradation was analysed by FTIR (Thermo Nicolet AVATAR 330) and HPLC (Waters, Model No.501).

Decolorization experiment : A loopfull of *B. halodurans* was inoculated in Luria Bertani broth and precultured at 30°C with shaking at 130 rpm overnight (Sambrook *et al*., 1999) until absorbance at 600nm reached 1.0 (about 10^9 cell ml^{-1}). An 10% inoculum (*v/v*) of pre-grown cells of *B. halodurans* were used to inoculate 100ml of screening medium in 250ml Erlenmeyer flasks amended with 1000 mgl^{-1} of Acid Black-24 dye. The experimental set up was incubated at pH 9 and 37°C under static conditions for the process of decolorization. Aliquot (3ml) of sample was withdrawn at regular intervals (0, 2, 4 and 6hr) and centrifuged at 8,000 × g for 15 min to remove suspended biomass. The supernatant obtained was analysed for decolorization by measuring the absorbance at 557 nm (λ_{max}). Uninoculated medium was always included as control. The reduction percentage in dye concentration was calculated from the following equation:

$$\text{Reduction percentage} = \frac{C_i - C_r}{C_i \times 100\%}$$

where C_i and C_r were initial and residual dye concentrations, respectively. All assays were carried out in triplicate.

Effect of physico-chemical parameters on decolorization : Decolorization of AB-24 by *B. halodurans* was assessed under various physico-chemical conditions. The effect of different temperatures (32, 37, 40, 45, 50 and 55°C) at pH 9, various pH (6, 7, 8, 9 and 10) at 37°C and salinity levels (2, 5, 7, 10 and 12% NaCl) at pH 9 and 37°C on dye decolorization was analysed in screening medium under static conditions.

Biodegradation analysis : Biodegradation of Acid Black-24 by *B. halodurans* was analysed by FTIR and HPLC. For this, 100ml of culture broth was taken at 0hr and 6hr (before and after decolorization), centrifuged at 10,000g for 15min and the degraded metabolites were extracted from supernatant using equal volumes of ethyl acetate. The extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness in rotary evaporator. The metabolites were characterized by FTIR in the mid IR region of 400–4000 cm^{-1} with 16 scan speed. The samples were mixed with spectroscopically pure KBr in 7 : 93 ratio, pellets were fixed in sample holder, and the analysis was carried out. For HPLC analysis, the evaporated metabolites were dissolved in small volume of HPLC grade methanol and run on C_{18} column (symmetry, 4.6×250mm) with a flow rate of 1ml min^{-1} and UV detector set at 557 nm. The mobile phase was methanol/water (50:50, v/v).

Statistical analysis : For analysis of data, one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparison test was used. Readings were considered significant when P was ≤ 0.05.

Results and Discussion

Decolorization of Acid Black-24 by *B. halodurans* MTCC 865 was achieved within a short time of 6 hrs under static aerobic conditions. Decolorization was monitored spectrophotometrically at 557 nm (λ_{max} of AB-24). The major dye peak at 0hr was reduced significantly by the end of 6hr (Fig. 1). Ninety percent of AB-24 (1000 mgl^{-1}) was decolorized within 6 hrs at pH 9 and 37°C under static conditions in screening medium. Disappearance of major dye peak at 557 nm with time, confirmed decolorization of AB-24.

Environmental factors are known to play a crucial role affecting the decolorization activity of microorganisms (Pearcea *et al*., 2003). The result of the effect of pH on AB-24 decolorization by *B. halodurans* is shown in Fig. 2a. *B. halodurans* decolorized AB-24 (1000 mg l^{-1}) over a wide range of pH (6–10). The strain

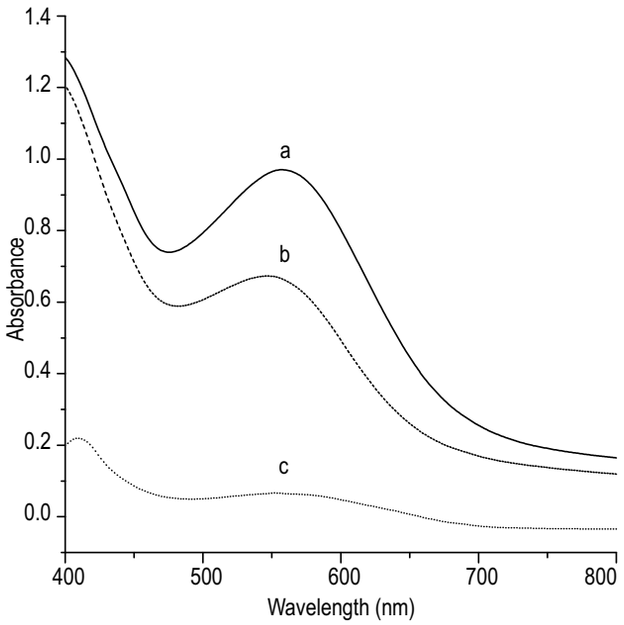

Fig. 1 : UV- Scan of culture supernatant extracted at different time during AB-24 decolorization. Spectra at (a) 0hr, (b) 4hr and (c) 6hr.

exhibited optimal growth at pH 7, 8 and 9 (data not shown), with maximum decolorization rate of about 90% at pH 9 within 6 hrs in screening medium under static conditions. Decolorization rates of about 44, 58 and 50% were observed at pH 7, 8 and 10, respectively. Least decolorization rate of 15% was seen at pH 6 and the strain failed to grow at pH 5. Fig. 2b shows the result temperature effect on AB-24 decolorization. The optimal temperature for AB-24 decolorization by *B. halodurans* was 37°C. Decolorization rate gradually decreased with increase in temperature ranging from 40–50°C. This could be due to loss in cell metabolism and viability, which was also observed with decolorization of azo dyes by *Shewanella putrefaciens* strain AS96 (Khalid *et al*., 2008). Percent decolorization rates of about 71, 52 and 33% were noticed at 40°C, 45°C and 50°C, respectively. Lowest rate of decolorization (8%) was observed at 55°C. Apart from high temperature and alkaline conditions, high level of salts were found in the industrial effluents containing azo dyes (Aksu, 2003). Hence, the potential of *B. halodurans* to decolorize azo dye AB-24 at various concentrations of salt was assessed and the results are shown in Fig. 2c. AB-24 was reduced by 50% with 2% NaCl. At 5% salt, *B. halodurans* reduced 90% of AB-24 (1000 mgl^{-1}) in 6 hrs under static conditions. Further, increase in NaCl concentration resulted in significant decrease in decolorization rate. Decreasing decolorization rates by 77, 44 and 10% was observed at 7, 10 and 12% NaCl concentration, respectively. High salt concentrations cause inhibitory effects in microorganisms leading to reduction in biological activity (Kargi, 2002). Slightly similar results were obtained with AR-27 dye decolorization by *Shewanella aquimarina* with 5% NaCl exhibiting 98% dye reduction under static anaerobic conditions (Meng *et al*., 2012). The strain exhibited negligible growth in the absence of NaCl and did not grow above 12% salt concentration.

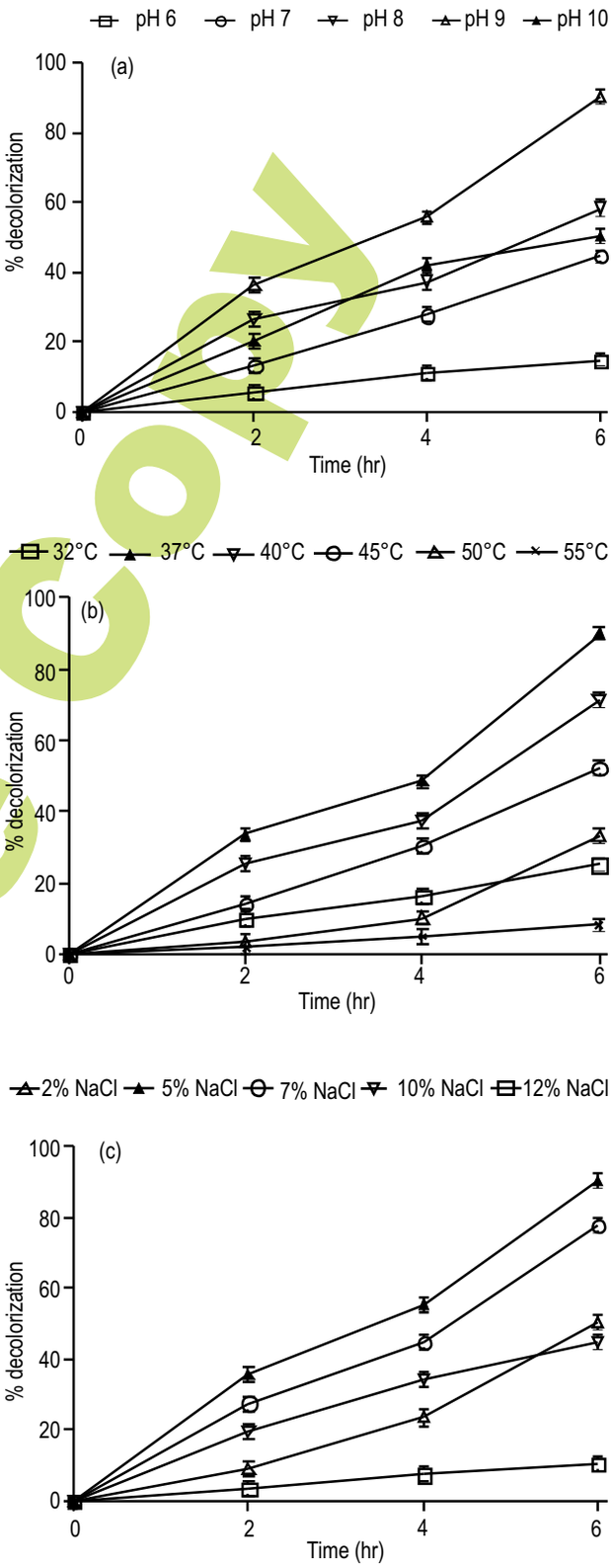

Fig. 2 : Effect of (a) pH, (b) temperature and (c) salinity on decolorization of Acid Black-24 by *Bacillus halodurans*

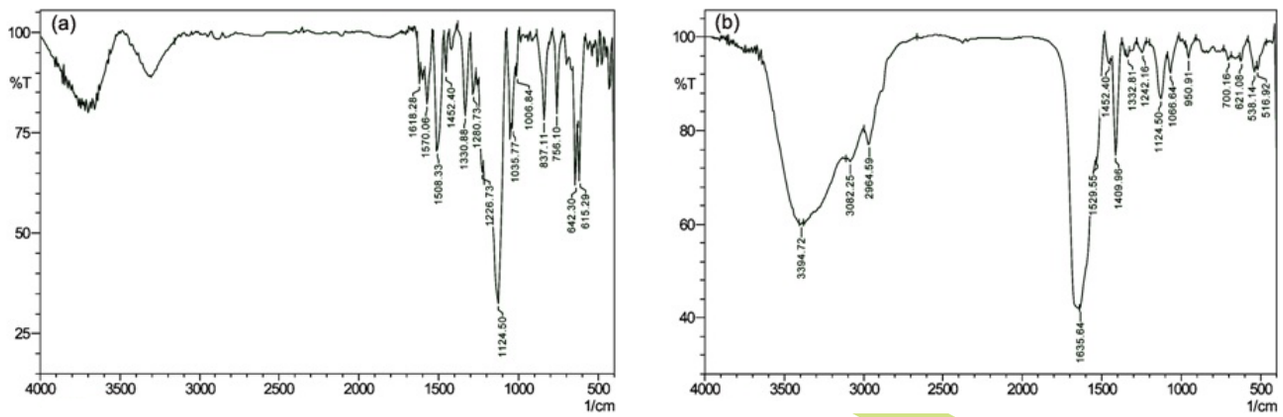

Fig. 3 : (a) FTIR spectra of azo dye AB-24 and (b) FTIR spectra of degraded sample

Comparison of FTIR of control dye (Fig. 3a) with extracted metabolites (Fig. 3b) revealed significant changes in the spectrum. Peaks in the FTIR spectrum of control dye represented –N=N– stretching and C–C stretching frequencies of azo groups and aromatic rings at 1618.28 cm^{-1} and 1570.06, 1508.33, 1452.40 cm^{-1}, respectively. Whereas peaks at 1330.88 cm^{-1} and 1280.73 cm^{-1} indicated C–N stretching frequencies of aromatic carbons attached with nitrogen functionality. The peaks at 1124.50 cm^{-1}, 642.30 and 615.29 cm^{-1} represented S=O stretching frequency of SO_3Na group on aromatic ring. After decolorization of dye, the FTIR spectrum of extracted metabolites showed considerable change in the positions of peaks as compared to spectrum of control dye. A peak at 3394.72 cm^{-1} represented N–H stretching of amino group in the extracted metabolites FTIR spectrum, whereas two newly formed peaks at 1529.55 cm^{-1} and 1452.40 cm^{-1} represented C=C stretching frequencies of aromatic ring. The peaks at 3082.25 cm^{-1} and 1635.64 cm^{-1} indicated C–H stretching of aromatic ring and –N=N– stretching of azo group, respectively. Similarly, peaks at 1124.50 cm^{-1}, 621.08, 538.14, 516.92 cm^{-1} and 1332.81 cm^{-1}, 1242.16 cm^{-1} represented S=O stretching frequencies of SO_3Na and C–N stretching frequencies of aromatic carbon attached with nitrogen in the extracted metabolites. The peaks at 950.91 cm^{-1} and 700.16 cm^{-1} were indicative of C–H deformation of aromatic ring protons. Appearance of a peak at 3394.72 cm^{-1}, which represented formation of $–NH_2$ group in the FTIR spectrum of extracted metabolites after decolorization of dye led to confirmation for degradation of Acid Black- 24. HPLC analysis

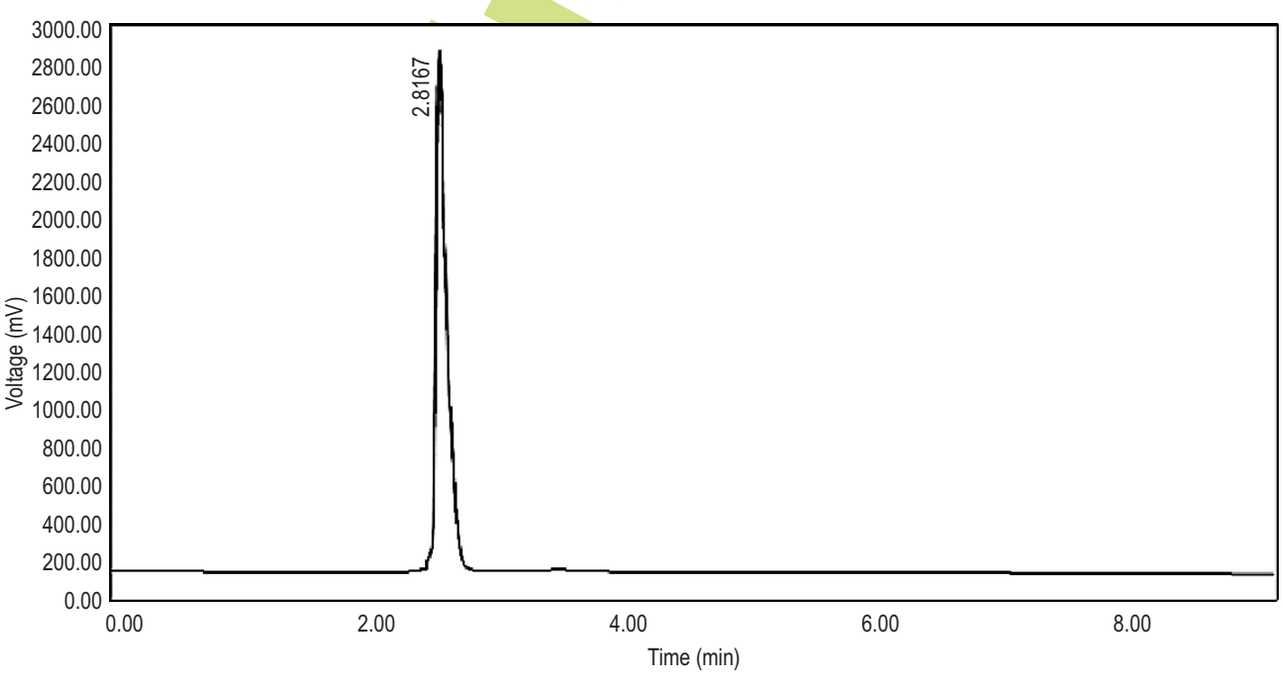

Fig. 4 : HPLC chromatogram of Acid Black-24 extracted at 0hr

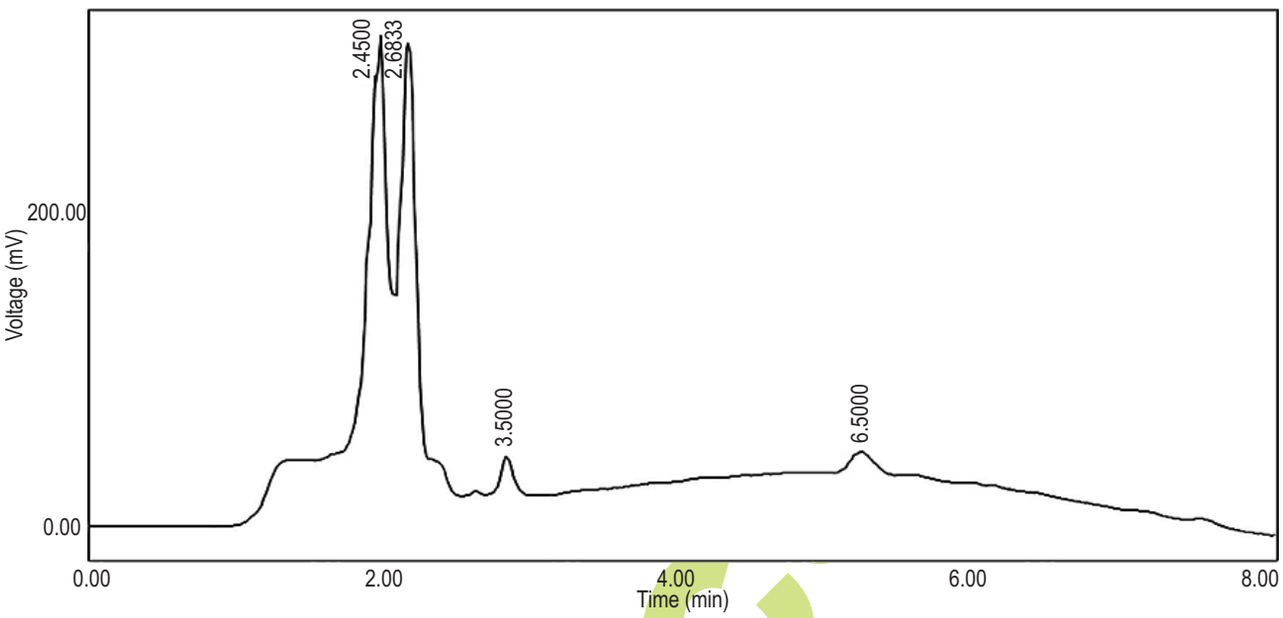

Fig. 5 : HPLC elution profile of degraded metabolites extracted at 6hr

report of dye sample withdrawn at beginning of static incubation (0 hrs) is shown in Fig. 4. A single peak of Acid Black-24 appeared at a retention time of 2.81 min. After a period of 6 hrs, as the degradation proceeded, HPLC chromatogram of degraded sample (Fig. 5) showed the presence of two major new peaks at 2.45 and 2.68 min, respectively and two minor peaks at 3.50 and 6.50 min. Absence of major dye peak and appearance of multiple peaks at different retention times is indicative of biodegradation of Acid Black-24. Similar degradation results with respect to UV-Vis, FTIR and HPLC were noticed in previous studies with *Aeromonas hydrophila* (Chen *et al.,* 2003) and *Pseudomonas aeruginosa* (Sarayu and Sandhya, 2010).

In conclusion, Acid Black-24 was efficiently decolorized and degraded by *Bacillus halodurans* under alkaline conditions within a short time of 6 hrs. The results indicate that *Bacillus halodurans* could be used as an effective strain in degrading various textile dyes and in the treatment of alkaline dyeing effluents.

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