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Decolorization of textile azo dyes by newly isolated halophilic and halotolerant bacteria

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Abstract

Studies were carried out on the decolorization of textile azo dyes by newly isolated halophilic and halotolerant bacteria. Among the 27 strains of halophilic and halotolerant bacteria isolated from effluents of textile industries, three showed remarkable ability in decolorizing the widely utilized azo dyes. Phenotypic characterization and phylogenetic analysis based on 16S rDNA sequence comparisons indicate that these strains belonged to the genus *Halomonas*. The three strains were able to decolorize azo dyes in a wide range of NaCl concentration (up to 20% w/v), temperature (25–40 °C), and pH (5–11) after 4 days of incubation in static culture. They could decolorize the mixture of dyes as well as pure dyes. These strains also readily grew in and decolorized the high concentrations of dye (5000 ppm) and could tolerate up to 10,000 ppm of the dye. UV–Vis analyses before and after decolorization and the colorless bacterial biomass after decolorization suggested that decolorization was due to biodegradation, rather than inactive surface adsorption. Analytical studies based on HPLC showed that the principal decolorization was reduction of the azo bond, followed by cleavage of the reduced bond.

Keywords: Biodegradation; Bioremediation; Halophilic bacteria; *Remazol Black B*; Textile effluents

1. Introduction

Since 1856, when the first synthetic dye, mauevin, was discovered, more than 100,000 new synthetic dyes have been generated. These dyes are used in different industries, including textiles, cosmetics, paper, leather, pharmaceutical, and food, with an annual consumption of about 0.7 million ton (Craliell et al., 1995; Chen et al., 2003). The textile industry accounts for two-thirds of the total dyestuff market. Azo dyes, the largest chemical class of dyes with the greatest variety of colors, have been used extensively for textile, dyeing, and paper painting (Craliell et al., 1995; Fang et al., 2004). These dyes cannot be easily degraded, while some are toxic to higher animals (Fu and Viraraghavan, 2001). During the dyeing process, approximately 10–15% of the used dye, depending on the structure, is released into the wastewaters, causing serious environmental and health hazards (Chen et al., 2003). Presence of the dyes in aqueous ecosystems diminishes the photosynthesis by impeding the light penetration into deeper layers thereby deteriorating the water quality and lowering the gas solubility. Furthermore the dyes and/or their degradation products may be toxic to flora and fauna (Talarposhti et al., 2001). Thus, decolorization of textile wastewaters has been a major environmental concern for a long time.

Chemical or physico-chemical treatment methods, such as coagulation/adsorption, complete destruction of dye molecules by electrolysis or ozonation, etc., are in general inefficient, costly and of limited applicability, while sometimes producing large amounts of toxic waste which is difficult to dispose of (Banat et al., 1996; Verma et al., 2003; Zhang et al., 2004). The conventional aerobic wastewater

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treatment process usually cannot efficiently decolorize the effluents contaminated with azo dyes, because of their strong electron-withdrawing group, which protects them against attack by the oxygenases (Nigam et al., 1996). Bioremediation is becoming important, because it is cost-effective and environmentally friendly, and produces less sludge (Robinson et al., 2001; Chen et al., 2003). Many microorganisms are capable of decolorizing the azo dyes, including Gram-positive and Gram-negative bacteria (Sani and Banerjee, 1999; Kodam et al., 2005; Moosvi et al., 2005) and fungi (Balan and Monteneiro, 2001; Verma and Madamwar, 2005).

Bioremediation in salty environments inevitably requires the application of halotolerant and halophilic microorganisms, which are able to grow under such harsh conditions. Since externally added bacteria may have some deleterious effects on the ecosystem, applying or activating the indigenous microflora is preferred if possible (Margesin and Schinner, 2001). In many cases, the textile effluents contain high salinity, thereby causing problems for conventional biological treatments (Mellado and Ventosa, 2003). Halotolerant and halophilic bacteria usually tolerate noticeable amounts of toxic metals in their environment. Thus they are utilized in bioremediation of oil (Delille et al., 1998; Margesin and Schinner, 1999) and oxyanion pollution (Nriagu and Payna, 1988; Kinkle et al., 1994), but their effectiveness in decolorization of textile effluents has not yet been reported. Thus, the aim of this work was to investigate the role of halotolerant and halophilic bacteria in decolorization of textile effluents, in particular in arid and salty environments in Iran.

2. Methods

2.1. Chemicals

Seven commercially used textile azo dyes including *Remazol Black B*, *Maxilon Blue*, *Sulphonyl Scarlet BNLE*, *Sulphonyl Blue TLE*, *Sulphonyl Green BLE*, *Remazol Black N*, and *Entrazol Blue IBC* were obtained from the Ciba Geigy GmbH representative in Iran (CIBA). Stock solutions were prepared in distilled water and autoclaved at 121 °C and 1 atm for 15 min. These seven dyes were used only for screening of the effective strains, and *Remazol Black B* was used as a model dye for further experiments. All the culture media, organic and inorganic compounds, and reagents used in HPLC and TLC analysis were obtained from MERCK (E. Merck, Darmstadt, Germany). For DNA extraction, the SIGMA GENELUTE DNA kit (St. Louis, Missouri. USA) was used.

2.2. Strains and culture conditions

The textile effluents were collected in sterile collection tubes from the sludge and wastewater of the ditches at industrial sites located in the cities of Qom and Kashan in centre of Iran (1.5% and 3% (w/v) salinity, respectively). Then 5 ml of the textile wastewater were added to each of the 250 ml conical flasks containing 50 ml of the screening medium and the pH was adjusted to 7.2 ± 0.1 with 1 M KOH. Inoculated flasks were incubated in an orbital shaking incubator (Orbital Incubator, SI 50, Stuart Scientific) at 34 °C and 150 rpm for 48 h. The screening medium was composed of tryptic soy broth (TSB) medium with 5% (w/v) salt containing 4.8 g of $MgSO_4$, 3.5 g of $MgCl_2$, 1 g of KCl, and 40.5 g of NaCl in one liter of distilled water. Isolated strains were purified by plate streaking technique on tryptic soy agar supplemented with 5% (w/v) of the mentioned salts. The pure isolates were maintained in a complex medium of the following composition ($g\,l^{-1}$): proteose peptone 3, yeast extract, 10, glucose 1, agar 20, supplemented with 5% (w/v) of the same salts used in the screening medium.

Salt tolerance experiments were performed on the tryptic soy broth plus various concentrations of NaCl (0–25% w/v) at 34 °C. The growth at various temperatures (5, 10, 15, 20, 25, 30, 35, 40, 45, 50, and 55 °C) was determined. The pH range suitable for growth was deduced, and the final pH was adjusted between 5 and 12. For the pH higher than 6, 0.1 M Tris–HCl and for lower than pH 6, 0.1 M sodium acetate buffers were used. Bacterial growth was measured at 620 nm by a spectrophotometer (Shimadzu model UV-160 A).

Then 10 ml of the decolorizing medium (10 g of glucose, 5 g of yeast extract, 50 g of NaCl, and 0.05 g of azo dye in one liter of distilled water) was sterilized in culture tubes, inoculated with 1% of 1.5×10^8 cfu ml^{-1} of the bacterial suspensions in distilled water plus 3% (w/v) NaCl, and incubated at 34 °C. The pH was adjusted to 7.2 with 1 N KOH before sterilization. Decolorization of the seven commercial azo dyes mentioned above was investigated.

In order to select the effective strains, the following additional tests were performed: (1) The ability to decolorize mixtures of dyes at a total concentration of 50 ppm; (2) Decolorization assay in the pH range from 5 to 11. pH adjustment was done with 2N KOH or 2N HCl; (3) Decolorization ability at 600, 800, 1000 and 5000 ppm of the *Remazol Black B*; (4) The ability to utilize *Remazol Black B* as the sole carbon source. Yeast extract and glucose in the decolorizing medium were replaced with 1 $g\,l^{-1}$ ammonium nitrate and 1 $g\,l^{-1}$ *Remazol Black B*, respectively.

2.3. Identification of the isolates

Morphological and physiological characterizations were performed in basal culture media containing 5% (w/v) NaCl and were tested using methods described by Smibert and Krieg (1994). The genomic DNA of the three selected strains were extracted with the Genelute DNA extraction kit, following the manufacturer's recommended procedure. The 16S rDNA genes were amplified using 8F (5′-AGAGTTTGATYMTGGCTCAG-3′) and 1541R (5′-AAGGAGGTGATCCAGCCGCA-3′) universal primers, for two of the selected isolates (namely strain D2 and strain

A3). For the third selected isolate (strain Gb), 8F and 1492R (5′-GGTTACCTTGTTASGACTTC-3′) universal primers were used. The PCR reaction conditions included initial denaturation at 94 °C for 5 min, 35 cycles including 94 °C for 30 s, 57 °C for 1 min, 72 °C for 100 s and final extension in 71 °C for 15 min. The purified PCR products were sequenced in both directions using an automated sequencer by SeqLab Laboratory (Germany).

2.4. Decolorization under different culture conditions

Decolorization under different culture conditions was done by changing, one at a time, the factors with the basic conditions of temperature 34 °C, pH 7.2, 5% (w/v) NaCl, and 50 ppm *Remazol Black B*. Different decolorization efficiencies were obtained at different pHs, salt sources, salt concentrations, nitrogen sources, aeration conditions and incubation temperatures. The effect of the aeration condition was examined under three culture conditions, namely, static (no shaking), agitated (aerobic), and anaerobic. In the case of the aerobic tests, the culture flasks were shaken on a rotary shaker running at 150 rpm. In the anaerobic condition, tubes containing decolorizing medium were sealed with rubber septa and incubated in anaerobic jars, and in the static condition tubes were placed in the incubator directly. All assays were performed in triplicate with the uninoculated culture as the control.

2.5. Decolorization and growth assay

A standard graph for absorbance versus dye concentration for each dye was obtained by plotting the corresponding maximum absorbance in the UV–Vis spectra at different dye concentrations prepared by dissolving the dye in distilled water. To measure decolorization, sampling was done at different time intervals from the inoculated decolorizing media and clarified (centrifuged at 7500*g* for 4 min) in order to prevent absorbance interference from the cellular or other suspended debris. Uninoculated culture media with and without added dyes were used as negative controls. The decolorization efficiency of different isolates was expressed as

$$\text{Decolorization } (\%) = (A_0 - A)/A_0 \times 100,$$

where A_0 is the initial absorbance and A is the absorbance of medium after decolorization at the λ_{max} (nm) of each dye. Turbidity (a measure of the bacterial growth) was calculated by determining the difference between the absorbance of culture samples before and after centrifugation at 600 nm (Dong et al., 2003).

$$\text{Turbidity} = \text{OD}_{(\text{before centrifugation})} - \text{OD}_{(\text{after centrifugation})}$$

The clarified samples from the decolorization media were used for determining the possible changes in the absorption spectra of the dye in the UV–Vis range against a baseline defined by the absorbance of clarified samples from dye-free media.

2.6. Comparing the active versus inactive cells

Fresh culture media of each strain were prepared, half of them autoclaved. Both the autoclaved (inactive) and living cells were centrifuged at 7500*g* for 4 min. To determine if extracellular bioproducts or bacterial cells are involved in decolorization, the supernatant and pellets of the living and nonliving cells were incubated with the dye and their UV–Vis absorption was used as a measure of their decolorization activity (Khehra et al., 2005).

2.7. Biodegradation assay via TLC

After complete decolorization by the D2 strain, the decolorized medium was centrifuged at 7500*g* for 4 min, and the supernatant extracted with chloroform after alkalization to pH 8 to extract the biotransformed products (Supaka et al., 2004). The extracted and the aqueous phases were separately evaporated in a rotary evaporator. The concentrated extract and the aqueous phases were dissolved in 1 ml chloroform and 1 ml methanol, respectively, and used for thin layer chromatography (TLC). The mobile phase for both the organic and the aqueous extracts was petroleum ether: chloroform: methanol (4:1:1). The bands of aromatic compounds were observed under UV light (365 nm). TLC and HPLC analyses were done for the D2 strain (the best decolorizer among the three selected strains).

2.8. HPLC analysis

HPLC analysis was carried out on a Ceccil model Adept CE 4900 chromatograph equipped with a Cecil model CE 4200 UV detector, an oven column model CE 4601, and a lichrosorb C18 column with a 4.6 mm inside diameter and 25 cm height. A mobile phase composed of 50% methanol, 0.3% H_3PO_4, and 49.7% water was used at a flow rate of 0.5 ml min^{-1}. The elutes were monitored by the UV absorption at 300 nm. To determine the dye fragments produced upon decolorization, the treated samples were used directly for HPLC analysis. As stated earlier, the peak at 300 nm in the UV–Vis spectrum of the culture media before and after decolorization does not change. We determined the chemical constitution of the species producing the peak. Since during the HPLC analysis, pH reaches 2–3, it was necessary to determine the effect of high acidity on the 300 nm peak. It was found that the peak was not affected. Therefore, *Remazol Black B* decolorized media with the D2 strain were centrifuged (7500*g* for 4 min) clarified by 0.22 μ filters, and analyzed with HPLC every day during the incubation period until complete decolorization occurred.

3. Results and discussion

3.1. Isolation and identification

After one week of incubation, 27 halophilic and halotolerant strains were isolated. Primary biochemical and

physiological tests were performed to differentiate the isolated strains. All strains were assayed to decolorize seven commercial azo dyes. Based on further decolorization tests, including those performed at higher dye concentrations up to 5000 ppm, decolorization of mixtures of the dyes, at different pHs, and using the dye as the sole carbon source, three strains, namely, D2, A3, and Gb strains were selected for further study.

1411 bp of 16S rDNA gene of the D2 strain (GenBank accession no. *DQ372908*), 1182 bp of the A3 16S rDNA gene (GenBank accession no. *DQ372909*), and 1451 bp of Gb 16S rDNA gene (GenBank accession no. *DQ489548*) were determined. The D2 16S rDNA gene had 359 A, 266 T, 341 C, and 445 G bases, the A3 16S rDNA gene had 301 A, 222 T, 283 C, and 376 G bases, and the Gb 16S rDNA gene had 357 A, 280 T, 344 C and 465 G bases. The nucleotide alignment of the three strains showed the most phylogenetic similarity to *Halomonas* genus. The D2 strain was 99% similar to *Halomonas aquamarina*, the A3 strain was 99% similar to *Halomonas meridiana*, and the Gb strain was 98% similar to *Halomonas salina*.

The isolates shared the main phenotypic features of the genus *Halomonas*; however, some characteristics, such as motility, temperature, and the NaCl concentration range for growth, nitrate reduction, and urease activity, were sufficiently different from the similar species of *Halomonas*. On the other hand, the 16S rDNA analysis indicated that the isolates were phylogentically related to a low degree when compared with the other known halophilic Gram-negative bacteria such as *Vibrio* and *Salinivibrio* (data not shown). However, further studies should take more characteristics into account, especially DNA–DNA hybridization data.

3.2. Decolorization under different culture conditions

The selected strains grew in the medium having 10,000 ppm of *Remazol Black B* (the decolorization rate was not checked at this concentration). Reduction in cell growth might result from the toxicity of dyes through the inhibition of metabolic activities. Azo dyes generally contain one, or more sulphonic-acid groups on the aromatic rings, which might act as detergents, thereby inhibiting the growth of the microorganisms. Such dyes may affect DNA synthesis since it has also been reported that dyes are inhibitors of the nucleic acid syntheses, or cell growth (Wuhrmann et al., 1980; Chen et al., 2003).

The anaerobic culture condition was best for decolorization but was not very different from the static culture tubes, probably due to oxygen depletion after growth under static conditions (Fig. 1). Similar to most of the bacterial decolorizations, none of the strains was able to decolorize the dye under aerobic (shaking) conditions (Chen et al., 2003; Supaka et al., 2004; Khehra et al., 2005; Moosvi et al., 2005). Bacterial degradation of azo dyes under anaerobic cultures often occurs via an enzymatic reaction. This is due to the strong electron-withdrawing tendency of the azo group compared with oxygen from the reduced electron carrier, i.e., NADH (Wuhrmann et al., 1980; Zimmermann et al., 1982; Banat et al., 1996; Chen et al., 2003). In both anaerobic and static culture conditions, the strain D2 had the largest decolorization rate while A3 had the lowest.

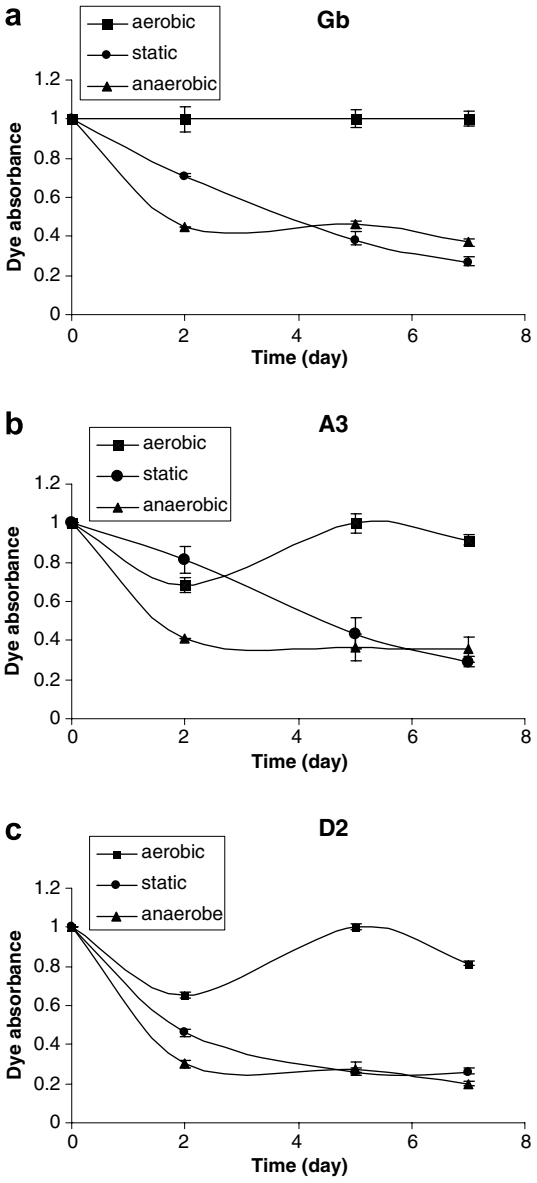

Fig. 1. Effect of different culture conditions including anaerobic, static and aerobic condition on decolorization of *Remazol Black B* (50 ppm) at 600 nm with three selected strains. (a) Gb strain, (b) A3 strain, (c) D2 strain. Results represent the means of three separate experiments, and deviation bars indicated.

The effect of pH and temperature on decolorization of the strains is shown in Fig. 2. It could be inferred that the decolorization rate increased as the pH increased. All three strains decolorized the azo dyes in a wide range of pH, a desirable characteristic, i.e., in contrast with common decolorizing bacteria that have a narrow pH range (Chen et al., 2003; Dong et al., 2003; Kodam et al., 2005; Moosvi et al., 2005). This could be due to improved

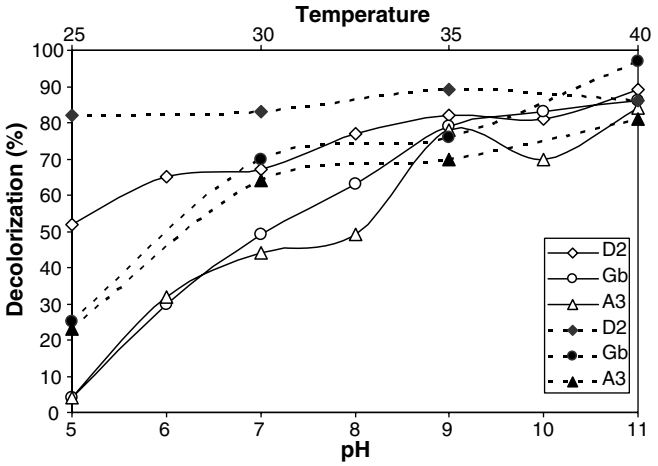

Fig. 2. Effect of pH (—) and temperature (...) on Decolorization of A3, Gb and D2 strains. For investigating pH effect, similar decolorization media with different pHs were made and inoculated with three strains. 25, 30, 35 and 40 °C were used for analyzing decolorization of selected strains in different incubation temperatures. Decolorization amount were analyzed at 2nd, 4th and 6th day of incubation. The 4th day was the best day for comparison of different media. Results represent the means of three separate experiments.

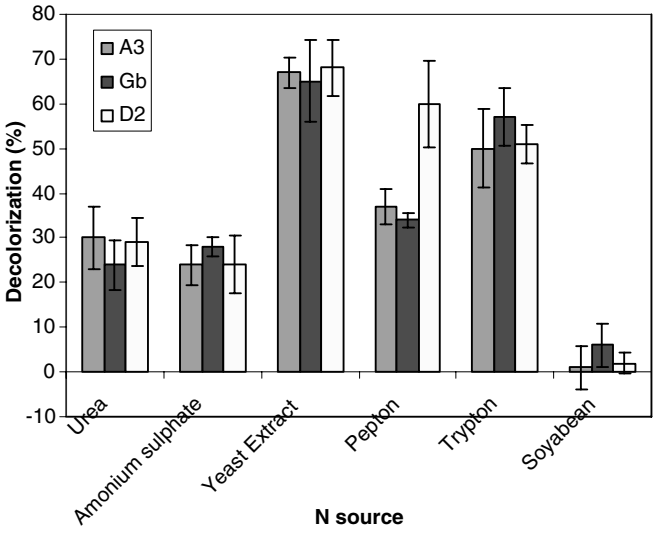

Fig. 3. Effect of various nitrogen sources on decolorization of three strains. Different nitrogen sources were used at 5 g l^{-1} in decolorization medium. Decolorization assay were done at the 4th day of incubation. Results represent the means of three separate experiments, and deviation bars indicated.

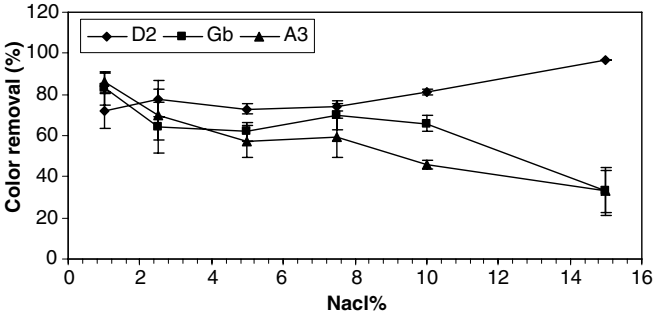

Fig. 4. Effect of salinity on decolorization of A3, Gb and D2 strains. Similar decolorization media with various salt concentrations were prepared and assayed every day after incubation up to one week but the result of the 4th day is illustrated in the figure. Results represent the means of three separate experiments, and deviation bars indicated.

bacterial growth at elevated pHs. The pH tolerance is quite important because reactive azo dyes bind to cotton fibers by addition or substitution mechanisms under alkaline conditions and high temperatures (Aksu, 2003). It should be mentioned that the wastewater samples used for isolation of the halophilic and halotolerant bacteria were also alkaline, pH 8–9. Therefore, they are suitable for practical bioprocesses in treating dyeing mill effluents. The culture temperature did not affect the decolorization rate for the D2 strain, but sharply increased decolorization rate for the other two strains in the range of 25–30 °C and more slowly in the range of 30–40 °C. These observations could be attributed to the increase in enzyme activity and growth increase with the temperature.

Fig. 3 shows the effect of various organic and inorganic nitrogen sources on the decolorization of *Remazol Black B* by the three selected strains. In accordance with other reports, the best decolorization was achieved with the yeast extract (Chen et al., 2003; Dong et al., 2003; Kodam et al., 2005; Moosvi et al., 2005). The results clearly indicated that decolorization was greatly affected by addition of various nitrogen sources. Metabolism of the yeast extract is considered essential for regeneration of NADH, which is the electron donor for azo bond reduction (Craliell et al., 1995). It was also found that decolorization efficiency increased with increasing the yeast extract concentration (from 1 to 5 g l^{-1}), but only slightly in the range of 5–10 g l^{-1}.

The effect of salt concentration on decolorization by the three strains in the range 10–150 g l^{-1} showed that the D2 strain decolorization was slightly dependent on salt concentration. The strains A3 and Gb showed a slight decrease in decolorization upon an increase in salt concentration (Fig. 4). The ability to decolorize in up to 20% (w/v) salinity in the media could make these two strains potential strains for decolorization of textile azo effluents in saline ecosystems. It could be inferred from Fig. 5 that under these basic conditions decolorization increased with increasing the bacterial growth, which agreed with others researchers' findings (Chen et al., 2003).

3.3. UV–Vis analysis

Experiments revealed that *Remazol Black B* was decolorized by all three strains in such a way that its absorption peaks (peaks A and C in Fig. 6) in the visible range disappeared while the absorption peak in the UV range (peak B in Fig. 6) did not diminish. Inspecting the cell mats also showed that microorganisms retained their natural color after decolorization of *Remazol Black B*. According to the literature (Knapp and Newby, 1995; Sani and Banerjee,

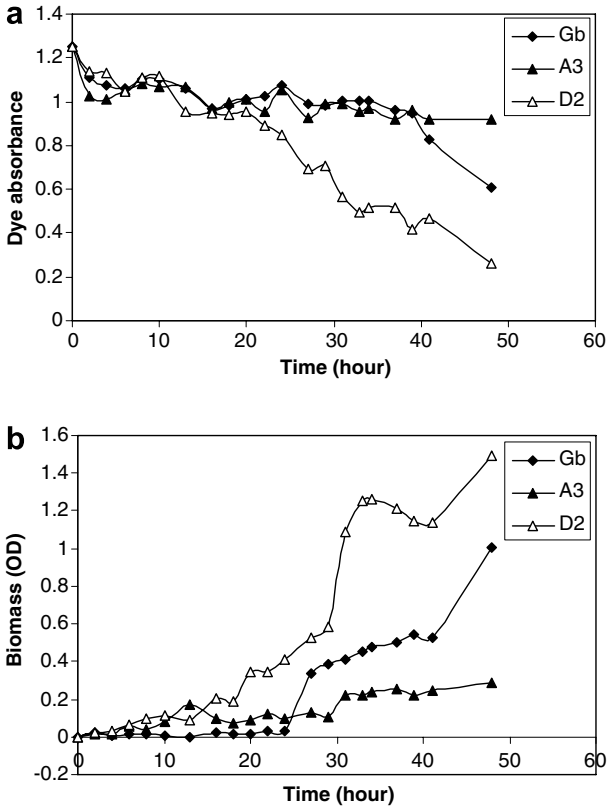

Fig. 5. Growth and decolorization of three strains within 48 h of incubation at 34 °C, and 50 ppm of dye. (a) color removal, (b) biomass optical density.

1999), decolorization of dyes by bacteria could be due to adsorption by microbial cells, or to biodegradation. In the case of adsorption, the UV–Vis absorption peaks decrease approximately in proportion to each other, whereas in biodegradation, either the major visible light absorbance peak disappears completely, or a new peak appears. Dye adsorption can also be clearly judged by inspecting the cell mats. Cell mats become deeply colored because of the adsorbed dyes, whereas those retaining their original colors occur when biodegradation takes place (Chen et al., 2003).

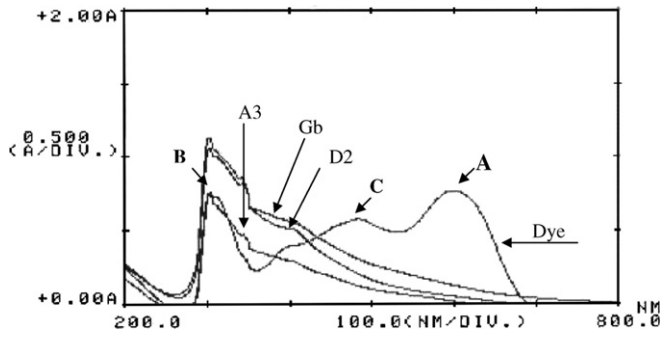

Fig. 6. The variation in UV–Vis spectra of *Remazol Black B* before and after decolorization of A3, Gb and D2 strains. The decolorized media of three strains were centrifuged before drawing the UV–Vis spectra to delete the interference of cellular OD.

Studying live versus inactivated cells proved that only live bacterial cells were able to decolorize the dye whereas inactivated cells were unable to do so. This proved that decolorization was not due to physical adsorption by inactivated cells. On the other hand, no decolorization activity was detected in the supernatant of culture media after the removal of cells. This implied that no secreted enzyme or any other bioproduct might be involved in decolorization.

3.4. Thin layer chromatography

The comparison of TLC chromatograms of the media extracted by the organic phase before and after decolorization by the D2 strain under UV light showed that the decolorized sample had three additional bands (Lanes 1, 2, 3 in Fig. 7a, column 2), which might have originated from the dye metabolites. Aromatic amines are the usual decolorization products of azo dyes that appear in the organic phase extract. Comparison of chromatograms of the aqueous phase extracts of the samples before and after treatment also shows the disappearance of the dye band in decolorized media (Lane a in Fig. 7b, column 1) indicating complete decolorization.

3.5. HPLC analysis

HPLC chromatograms of decolorized media by the D2 strain were obtained before and every day after incubation until complete decolorization occurred. Another medium was prepared with the same composition except for lack of the dye and inoculated with the D2 strain to remove the effect of growth metabolites. The UV–Vis analysis of the mentioned media was done every day until the complete disappearance of the visible peak at 600 nm. The chromatogram of the basic decolorization media before and after decolorization showed strong absorbance peaks

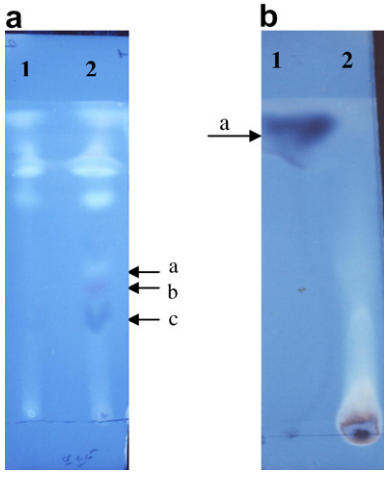

Fig. 7. comparison of TLC chromatograms of (a) organic phase extract and (b) aqueous phase extract of decolorization media before (column 1 in each figure) and after (column 2 in each figure) decolorization with D2 strain.

at retention times 6 min and 4 s and 6 min and 11 s, presumably related to co-elution of the dye molecule with other metabolites, which sometimes appears as two peaks. The HPLC chromatograms of the media samples during the first few days did not show any noticeable change whereas the UV–Vis spectra of the same samples showed the complete disappearance of the visible peaks, leaving only the 300 nm peak. During the bacterial decolorization the azo bond (chromophore) is reduced (O'Neill et al., 2000). Aromatic amines of these dyes have no absorption in the visible range but do in the UV range. Thus the intact peak at 300 nm in the decolorized media indicates the persistence of these structures. These indicate that after reduction of the azo bond, a chemical species is produced that has the same size and molecular structure as the parent dye but does not absorb in the visible range and exits the HPLC column with nearly the same retention time. Chromatograms after two days of incubation showed the appearance of two small peaks, one at the retention time of 4 min and 15 s and the other at 2 min and 15 s that do not exist in the media lacking the dye. These two peaks may belong to aromatic amines produced by metabolites of the dye degradation. These observations unanimously testify that decolorization proceeds by reduction of the azo bond followed by cleavage of the reduced bond (Nigam et al., 1996; O'Neill et al., 2000).

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