Decolorization of Cibacron Black w-55 under Alkaline Conditions by New Strain of *Halomonas* sp. Isolated from Textile Effluent

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ABSTRACT: *Among the 30 strains of moderately halophilic bacteria isolated from the salty effluents of textile industries around Qom city in central Iran, a Gram-negative rod shape bacterium designated as strain IP_8 showed a remarkable ability in decolorizing of azo dyes over wide ranges of pH (7-11) and temperature (25-45 ºC), in presence of NaCl and Na_2SO_4 (0.5-1.5 M) under anaerobic and aerobic conditions. Phenotypic characterization and phylogenetic analysis based on 16S rDNA sequence comparisons indicated that this strain was a member of the genus Halomonas with the greatest similarity to Halomonas axialensis. UV-Vis analysis before and after decolorization and the colorless bacterial biomass after treatment suggested that decolorization was due to biodegradation. HPLC analysis of dye treatment confirmed that the principal biodegradation was occurred after 48h of incubation in aeration culture. The effect of metal salts on decolorization showed that $AgNO_3$ and $NaAsO_4$ were of higher and lower effect on decolorization, respectively.*

KEY WORDS: *Decolorization, Cibacron Black w-55, Halophilic bacteria, Textile effluents.*

INTRODUCTION

It is estimated that 10%-15% of the dyes used in textile industries end up in the effluent during the dyeing processes [1]. A wide range of physicochemical methods has been developed for removal of synthetic dyes from dye-containing effluents. Although in recent years Zero-Valent Iron (ZVI) has been used as a reagent for removal of pollutants from the environment (2) but there is a problem caused by the accumulation of iron in the sludge leads to environmental problems. However, biological methods because of availability, being indigenous,

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non toxic, low cost and complete mineralization, successfully are used for remove dyes in extreme effluents [3-5].

Over the past decades, many microorganisms including bacteria [6,7] fungi [8-11] yeast [12] actinomycetes [13] and algae [14] have been known to be capable of decolorizing wastewater containing azo dyes.

A high salt concentration is another characteristic of textile effluents leading to moderate inhibition of most microbial activities. Nitrate, sulfate, chloride and carbonate salts can all be used during reactive dyeing [15]. On the other hand, due to the use of soda ash in the process of dying, pH of effluent is alkaline. Soda ash changes the pH of the fiber-reactive dye and cellulose fiber so that the dye reacts with the fiber, making permanent connection that holds the dye to the fiber. Therefore after dying, bioremediation of textile effluents inevitably requires the application of alkalophilic and halophilic microorganism, which are able to grow under such harsh conditions [16]. Thus, the purpose of this study was to achieve a halophilic bacterium for decolorization of reactive dye under alkaline conditions.

EXPERIMENTAL SECTION

Chemicals

Remazol Black B, Remazol Black GF and Cibacron Red 6B were provided by Iran Merinos Textile Industry and the reactive dye (Cibacron Black w-55) was donated by the local representative and technical office of the CIBA GMBH, Switzerland in Tehran. The first three dyes above were only used for screening of strains, and Cibacron Black w-55 dye was used as a model for further experiments. All culture media, organic and inorganic compounds and reagents used in HPLC analysis were purchased from MERCK (E. Merck, Darmstadt, Germany).

Bacterial strains and culture conditions

The samples of textile effluents were collected in sterile collection tubes from textile industries around Qom city in the centre of Iran (0.5% (w/v) salinity). Volumes of 5 mL of the effluent samples were added to 250 mL conical flasks containing 50 mL of the screening medium [17] with the pH adjusted to 7.2±0.1 with 1 M KOH. Inoculated flasks were incubated at 34 ºC in an orbital shaker (Orbital Incubator, SI 50, Stuart Scientific) at 34 °C and 150 rpm for 48 h. Isolated strains were purified by plate streaking technique on SW-7.5 agar supplemented with 7.5% (w/v) of the mentioned salts. Salt tolerance experiments were performed on the SW medium with different NaCl concentrations (0-25% w/v). The bacterial growth was monitored over wide temperature (5, 10, 15, 20, 25, 30, 35, 40, 45, 50, and 55 °C) and pH (5-12) ranges. pH higher than 6 and lower than pH 6 were adjusted with 0.1 M Tris-HCl and 0.1 M sodium acetate buffers, respectively. Bacterial growth was measured at 620 nm by a spectrophotometer (Shimadzu model UV-Vis -160A).

Cultured tubes containing 10 mL of the decolorizing medium (SW-7.5 and 0.05 g of the dye in one liter of distilled water) were inoculated with 1% of 1.5×10^8 CFU mL^{-1} of the bacterial suspensions and incubated at 34°C. The pH was adjusted to 7.2 with 1M KOH before sterilization. Decolorization assay of the dye was carried out at different pH (7.0-11) and temperatures (25-45ºC), different salt sources (NaCl, Na_2SO_4), and salt concentrations (2.5-15%) and metal salts (Zn, Cu, Ag, Cr, Te, As, Ni), under different aeration conditions. The aerobic tests were performed with culture flasks either on a rotary shaker running at 100, 150 and 200 rpm or in a static. The anaerobic tests were performed with tubes containing decolorizing medium sealed with rubber septa incubated in anaerobic jars. All assays were performed in triplicate with the non-inoculated culture as the control.

Identification of the Strain

Morphological and physiological characteristics of the isolate were studied either on SW-7.5% agar and broth as described previously [17, 18]. Nutritional assays were performed on modified Koser medium [17]. Genomic DNA extraction was done according to *Redburn & Patel* (1993) and the 16S rDNA gene of isolate was amplified using the universal bacterial primers 8F (5'-AGAGTTTGATCCTGGCTCAG) and 1541R (5'-AAGGAGGTGATCCAGCCGCA-3'). The purified PCR product was sequenced in both directions using an automated sequence by SeqLab laboratory (Germany) and then deposited in GenBank.

Decolorization assay

A standard graph for absorbance versus dye concentration for Cibacron Black w-55 was obtained by plotting the corresponding maximum absorbance in the UV-Vis spectra (Shimadzu- UV-vis160A) at different

dye concentrations prepared by dissolving the dye in distilled water. To measure decolorization percentage, sampling was performed at regular intervals over 24 h. An aqueous sample of 1.5 mL was taken from each flask, centrifuged at 4000 rpm for 20 min and supernatants were decanted and monitored for UV-Vis spectrum [7]. Non-inoculated culture medium with and without the dyes were used as negative controls.

Comparing the active versus inactive cells

Fresh culture media of strain IP_8 was prepared, half of them autoclaved. Both the autoclaved (inactive) and living cells were centrifuged at 4000 rpm for 20 min. To determine if extracellular bioproducts, 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 [17].

Analysis of decolorization product

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% H3PO4, and 49.7% water was used at a flow rate of 0.5 mL/min and then 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. Cibacron Black w-55 decolorized media with the IP_8 strain were centrifuged (7500g for 4 min) clarified by 0.22nm filters, and analyzed with HPLC every day during the incubation period until complete decolorization was reached.

RESULTS AND DISCUSSION

Bacterial Characteristics

Among several strains of moderately halophilic bacteria that were able to decolorize some important textile dyes, one strain exhibited decolorization at pH higher than 7 and higher concentration of dye on SW-7.5. This strain was selected for further identification and decolorization studies. According to the Bergey's Manual of Systematic Bacteriology, the strain was tentatively named as "*Halomonas* sp. strain IP_8 (GenBank accession no DQ767689).

The strain grew well at NaCl concentration range of 1-20% (w/v) with the optimum growth at 3-5 % (w/v), while no growth was seen in the absence of NaCl. The strain IP_8 grew over the temperature range of 10 to 50 ^{0}C and pH range of 6 to 12 with the optimum growth at 35-37 °C and pH 8.0-8.5, respectively.

Decolorization and growth assay

Kinetics of bacterial growth and dye decolorization were investigated in the decolorizing medium (Fig. 1). No decolorization activity was shown during the early- and mid-exponential growth phase. However, the decolorization activity started by the end of the exponential phase and continued in the stationary phase. The dye concentration reduced largely from 50 mg/L to ~20 mg/L within 16-24 h.

Effect of temperature and pH on decolorization

Decolorization of the reactive dye, Cibacron Black w-55 by the IP_8 strain was observed at various temperatures ranging from 25 to 45 ºC with the optimum decolorization temperature at 35 ºC (Table 1). At two extreme temperature values (25 and 45 $^{\circ}$C), the rate of decolorization decreased dramatically to ~61% and ~30% respectively. (Decolorization assay were done at the 2th day of incubation in the SW-7.5)

The effect of pH on decolorization of the IP_8 strain is shown in Fig. 2. No decolorization was seen when the initial pH was below 6.0, but its rate increased as pH increased from 7 to 11. These results, is in contrast with all the known decolorizing bacteria that have a narrow pH range [3, 18] and in accordance with those of *Asad et al.* [5] results. This could be due to improved bacterial growth at evaluated 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 [19]. It should be mentioned that the wastewater samples used for isolation of the halophilic strains were also alkaline, pH 8-9.

Effect of aeration

Decolorization under anaerobic condition was lower than both shaking and static conditions (Table 2). This is because bacterial growth under static condition was higher than anaerobic condition (Data not shown). Increasing rotation from 100 to 200 rpm resulted to lower

Table 1: Effects of temperature on decolorization of Cibacron Black w-55(50mg/l) by strain IP_8.

Temperature(ºC)	Decolorization(%)
25	61.58±5.21
30	79.53±3.8
35	89.99±3.74
40	86.84±3.41
45	30.52±3.93

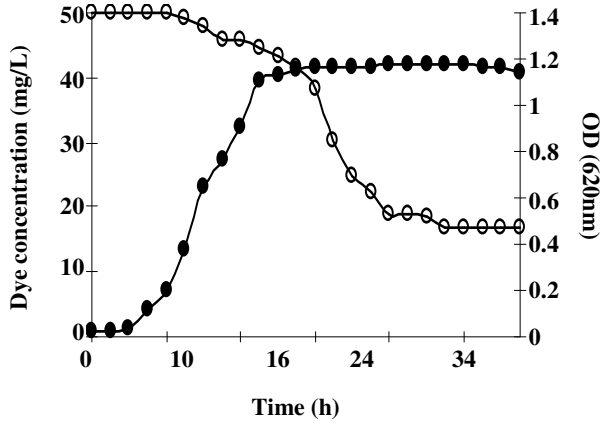

Fig. 1: Decolorization by growing cells. (–○–) Decolorization curves of Cibacron Black w-55; (–●–) growth curve of IP_8 during 40h in SW-7.5.

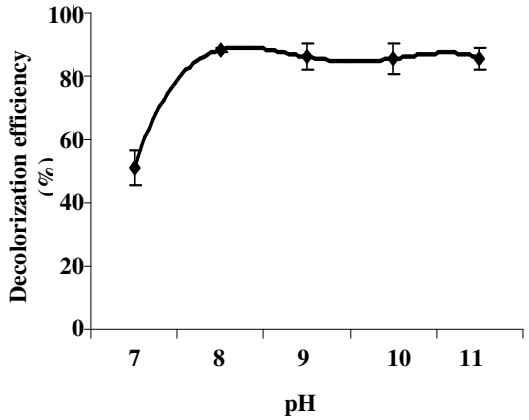

Fig. 2: Effects of pH on decolorization of IP_8 strain. Results represent the means of three experiments, and bars indicated ±deviation. Absence of bars indicates that errors were smaller than symbols. Decolorization assay were done at the 2 th day of incubation in the SW-7.5

decolorization, demonstrating the inhibition effect of oxygen on decolorization that is corresponding to *Chen et al.* results [3].

Effect of salts

Decolorization of Cibacron Black W-55 by the strain IP_8 was greatly affected by addition of various salt sources. No decolorization was seen in the presence of 0.5, 1 and 1.5 M $NaNO_3$, $NaNO_2$ and KCl. Maximum decolorization was detected in the presence of 1-1.5M NaCl. The dye was also decolorized in the presence of Na_2SO_4 (Table 3). The decolorization of the reactive dye increased to 98% when the concentration of NaCl increased from 0.5 M to 1.5 M, while increase of Na_2SO_4 from 0.5 M to 1.5 M had an inhibitory effect on decolorization activity of the strain. The maximum decolorization was observed at 0.5 M Na_2SO_4 (85%). Studies by *Ola et al*. (2006) with cibacron black PSG under aerobic and anaerobic conditions using Bacillus cereus led to 67.33% color removal after 5 days of incubation when NH_4NO_3/glucose was incorporated in to the fermentation medium. Therefore the rate of decolorization of IP8 strain compared to previous results was marked. The effect of salts (nitrate and sulfate) on the decomposition of the azo dye Reactive Red 141 under anaerobic conditions showed that nitrate delays the onset of decomposition while sulfate had no effect the biodegradation process [4, 15].

Effect of metal salts

The $K2TeO_3$ and $AgNO_3$ in 0.1mmol and $CuSO_4$, $K2CrO_4$ in 0.5 mmol indicated an inhibitory effect on decolorization of Cibacron Black W- 55. Addition $CAsO_4$, $CaCl_2$ and $NiSO_4$ at low concentration (< 0.1 mmol) cause increase the efficiency of decolorization and higher than 0.5 mmol/L indicated decrease (Table 4).

The biodegradation of organic component can be reduced by metal toxicity in aerobic and anaerobic systems. Metals including copper, zinc, cadmium, chromium (III and VI), nickel, mercury, and lead are reported to inhibit biodegradation process [19, 20]. Also KN-R decolorization by *Rhodocyclus gelatinosus* were reduced to less than 3% in presence of 1 mmol $HgCl_2$, $AgNO_3$, $CuSO_4$, $ZnSO_4$ [19] .

HPLC and UV-Vis analysis

UV-Vis absorption spectrum over the range of 200-800 nm during 48 hours of decolorization process

Table 2: Effect of different culture conditions including anaerobic, static and shaking on decolorization of Cibacron Black w-55(50mg/l) by strain IP_8.

Culture conditions	Decolorization (%)
Shaking (rpm):	
100	79.5
150	34.7
200	22.1
Static condition	88.6
Anaerobic condition	43.18

Table 3: The Effect of NaCl and Na2SO4 concentrations on decolorization by IP8. Decolorization assay were done at the 2th day of incubation in the SW-7.5.

	Decolorization (%)	
Salt(M)	Na_2SO_4	NaCl
0.5	83.5±12.02	17.8±9.4
1	3.8±2.5	92.7
1.5	0	95.83±1.47

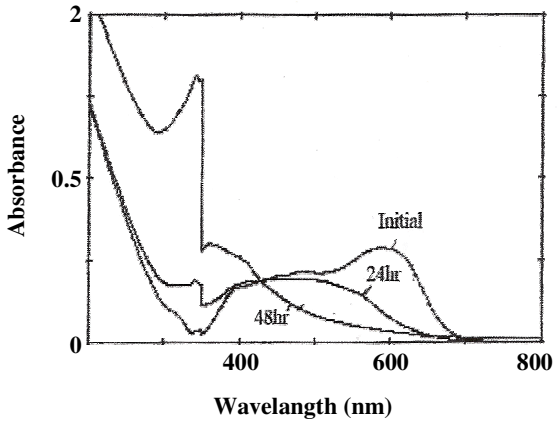

Fig. 3: The variation in UV-Vis spectra of Cibacron Black w-55 before and after decolorization of IP_8 strain. The decolorized media of strain was centrifuged before drawing the UV-Vis spectra to delete the interference of cellular absorbance.

indicated that the maximum absorption was in 600 nm there was a decline in the 600 nm peak after 24 h and new peaks were observed in wavelength of about 350 nm during 48 h, which may be related to the new products resulting from decomposition of dye compounds (Fig. 3). Inspecting the cell mats also showed that microorganisms retained their natural color after decolorization of Cibacron Black w-55. According to the literature [16,18], 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 [16,1].

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. 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.

These observations were confirmed by HPLC results. Fig. 4-a is related to the control sample which contains the medium SW-7.5 and the dye Cibacron Black W-55. The first and second peaks are probably related to peptone and yeast extract of the medium and the third to the Cibacron dye. According to the retention time there is a significant difference between control and the samples treated with IP_8 strain within 48h. The peak related to the dye, seemed to disappear completely (Fig. 4b). On the other hand, the effect of aeration after decolorization is an evidence for decomposition and elimination of the dye (Fig.4 c). Some toxic compounds with complex aromatic molecular structure could not be biodegraded directly by microorganisms [21]. Thus the addition of a special substrate such as peptone, which is ready to be utilized by microorganism, can facilitate or stimulate the biodegradation of the toxic chemical, by stimulating the bacteria metabolism [21].

CONCLUSIONS

Textile dye wastewater is well known to contain strong color, high pH, COD, temperature and low

Table 4: Effect of metal salts (0.1, 0.5 and 1 mmol/l) on Cibacron Black w-55 decolorization by strain IP_8 (in 48h)*.

Metal compounds		Decolorization efficiency (%) (OD_{600})	Growth (OD_{660})	(OD_{600} / OD_{660}))
Contrast		85.78	1.28	67.01
$NaAsO_4$	0.1	92.96	1.16	80.13
	0.5	88.44	1.75	50.53
	1	80.89	0.88	91.92
$NiCl_2$	0.1	90.4	1.05	86.09
	0.5	93.59	1.32	70.90
	1	74.72	1.22	61.24
$ZnSO_4$	0.1	65.31	0.93	70.22
	0.5	41.69	0.73	57.10
	1	17.89	0.56	31.94
K_2CrO_4	0.1	34.45	1.01	34.11
	0.5	-	0.14	-
	1	-	0.36	-
$CuSO_4$	0.1	17.52	1.73	10.13
	0.5	-	1.4	-
	1	-	1.15	-
K_2TeO_3	0.1	-	0.13	-
	0.5	-	-	-
	1	-	-	-
$AgNO_3$	0.1	-	-	-
	0.5	-	-	-
	1	-	-	-

**pH of the medium was adjusted to 8. Values are averages of three independent experiments.*

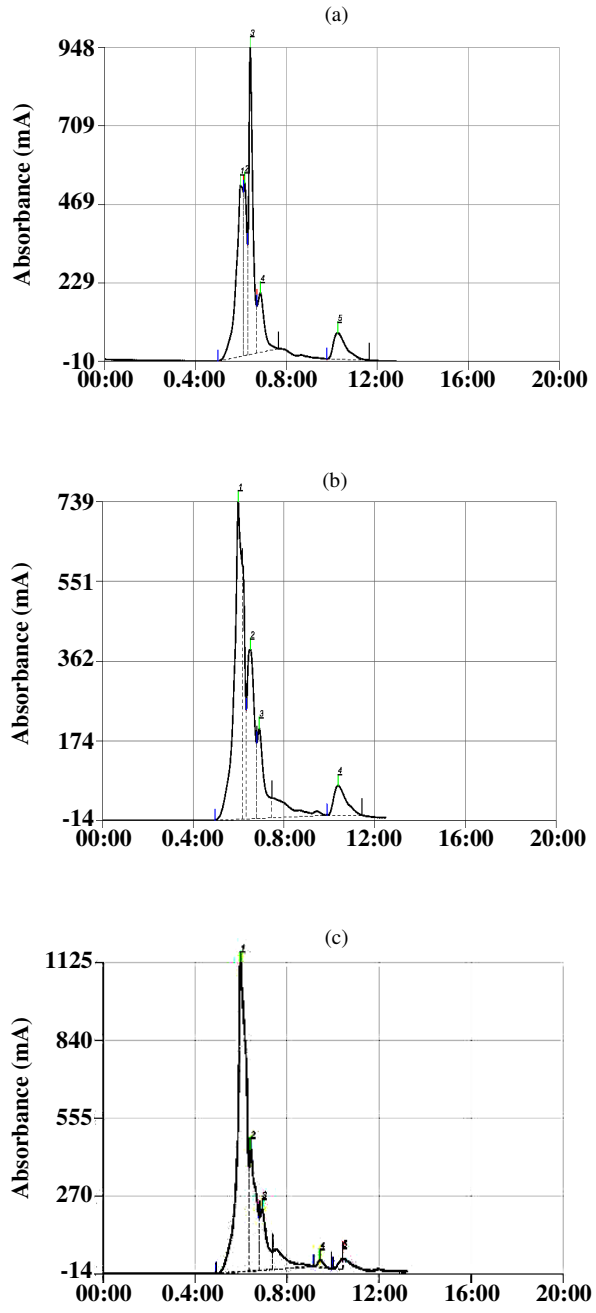

Fig. 4: a) HPLC chromatogram related to decolorization medium, SW-7.5, before bacterial treatment. The dye concentration, Cibacron Black w-55, was 50 mg/l at initiation. b) HPLC chromatogram related to decolorization medium, SW-7.5, after 48h treatment by strain IP8 under static condition. The dye concentration, Cibacron Black w-55, was 50 mg/L at initiation. c) HPLC chromatogram related to decolorization medium, SW-7.5, after 48h treatment by strain IP8 under aeration at 150 rpm. The dye concentration, Cibacron Black w-55, was 50 mg/l at initiation

biodegradability. High concentrations (40 to 100 g/L) of salts are used in a dye bath to ensure maximum fixation of dye to the cellulosic fiber. Typical salts used include sodium nitrate, sodium sulfate and sodium chloride. Moreover, it has been reported that bacterial cultures generally exhibit maximum decolorization at pH values near 7, whereas the rate of decolorization for strain IP8 was optimum in the pH range from 7 to 11 and Maximum decolorization was detected in the presence of 1-1.5M NaCl. In comparison with decolorization of Cibacron black by Bacillus cereus (67.33% after 5day in concentration 0.05g/L^{-1}) [22] and Trametes villosa (85% after 7day in concentration 0.002g/L^{-1}) [23], IP_8 strain showed decolorization, more than previous results (98% after 48h in concentration 0.05g/ L^{-1}). However it could be concluded that *Halomonas sp*. strain Ip_8 is a good candidate for treatment of alkaline textile effluents especially the effluent containing reactive dyes from the dyeing process.

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