Progress in Biological Sciences
Vol. 4, Number 1, Winter / Spring 2014/33-42

Optimization for decolorization of azo dye *Remazol Black B* by a *Halomonas* strain using the Taguchi approach

Received: November 30, 2013; Accepted: March 15, 2014

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ABSTRACT

Halophilic and halotolerant microorganisms are good candidates for decolorization of azo dyes which are routinely used in the dyeing process in textile industries. In this paper, the optimization of biological decolorization of synthetic dye solutions containing *Remazol Black B* by the previously isolated halophilic bacterium *Halomonas sp.* D_2 is investigated. In a primary investigation using a one-factor–at-a-time method, temperature, initial pH of the solution, and concentrations of glucose, yeast extract, and sodium chloride were chosen for optimizing dye removal using the Taguchi method. Based on the statistical analysis of the results, the most significant parameter by far was the yeast extract concentration which accounted for 72.67% of the total effect, followed by pH (11.84%) and the NaCl concentration (8.90%). The optimized conditions for dye removal were predicted to be a temperature of 35°C, an initial pH of 10, glucose concentration of 1% (w/v), yeast extract concentration of 1% (w/v), and sodium chloride of 10% (w/v). Under these conditions, 95% decolorization was achieved in confirming experiments.

Keywords: azo dye, *Halophilic* bacteria, decolorization, media optimization, Taguchi method.

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Introduction

Presently, more than 100,000 new synthetic dyes are used in textile, cosmetic, paper, leather, pharmaceutical, food, and other industries, with a worldwide annual consumption of about 7×10^5 tons (1, 2). Azo dyes, the largest class of chemical dyes, have been used extensively for textile dyeing, and paper painting (1, 3). During the dyeing process, approximately 10–15% of the used dye, depending on the structure, does not bind to fibers and is released into sewage treatment systems or the environment, causing serious environmental and health hazards (2). The presence of dyes in aqueous environments deteriorates water quality, because it reduces photosynthesis by impeding light penetration into deeper layers. Furthermore, the dyes and/or their degradation products could be toxic, mutagenic, and/or carcinogenic (4, 5). Because of these issues, the decolorization of textile wastewaters has been a major environmental concern for a long time.

Chemical or physical-chemical methods such as coagulation/adsorption, electrolyses, and ozonation can be used to treat contaminated effluents. These methods, however, have certain disadvantages, such as low efficiency, high cost, and the generation of byproducts that are even more toxic than the original pollutants (6-8). Biological methods have attracted intensive research interest as they are cost-effective, environmentally friendly, and produce less sludge (2, 9).

The importance of halophilic and halotolerant microorganisms in bioremediation of different pollutants such as heavy metals, oil, and oxyanion pollutants have been reported (10-13). Their ability to decolorize azo dyes was also studied and reported for the first time by the same researchers (14).

In this study, the biological decolorization of *Remazol Black B* by one such organism was investigated. *Halomonas sp.* D_2 strain, previously isolated from textile effluents, was selected because it was shown to have a remarkable ability to decolorize different azo dyes. It could readily decolorize *Remazol Black B* as the representative azo dye in increased amounts of salinity and pH which is characteristic of textile effluents. The decolorization mechanism of the D_2 strain was also proposed to be degradation rather than adsorption; therefore, it was selected for further study (14). The purpose of this study was to systematically investigate the effects of different cultivation parameters in order to identify optimum decolorization conditions for the halophilic strain known as *Halomonas* sp. D_2. Based on the results obtained in one-factor-at-a-time experiments, the main operational parameters and their levels were selected for further optimization of the process. Since traditional approaches would be time-consuming and costly because of the high number of testing parameters, the Taguchi method, an efficient experimental design technique, was employed in this study. By developing a set of standard Orthogonal Arrays (OA) and a methodology for the analysis of results, this approach (15, 16) can extract required information such as main effects and interaction effects of design parameters from results of a minimum number of tests even when quite a large number of parameters are being investigated (17, 18).

Materials and Methods

Chemicals

All reagents used were of analytical grade. The culture media, yeast extract, glucose, sodium chloride, and all other chemicals were obtained from MERCK (E. Merck,

Darmstadt, Germany). *Remazol Black B* was purchased from Ciba Geigy GmbH (CIBA, Iran).

Culture conditions

The experiments were conducted in culture tubes containing 10 mL of basic decolorization medium (glucose, 10 g; yeast extract, 5 g; NaCl, 50 g; and *Remazol Black B*, 0.05 g in one liter of distilled water). The pH was adjusted to 7.2 with 1 N KOH before sterilization. *Remazol Black B* and glucose stock solutions were prepared and autoclaved separately at 121°C and 1 atm for 15 min. The sterilized medium in culture tubes was inoculated with 1% its volume of bacterial suspensions containing 1.5×10^8 CFU ml^{-1} and incubated at 34°C without shaking.

Experimental design

One-factor-at-a-time experiments

A set of different culture conditions was chosen to examine their effects on decolorization. Urea, ammonium sulfate, yeast extract, pepton, trypton, and soybean were used as organic and inorganic nitrogen sources at fixed concentrations of 0.5% (w/v). Glucose, sucrose, fructose, lactose, and maltose were used at 1% (w/v) as carbon sources. Sodium chloride, potassium chloride, sodium sulfate, potassium nitrite, sodium nitrate, and magnesium sulfate, which are known to be the most effective salts for the growth of halophilic microorganisms, were used as salt sources at 5% (w/v). The effects of pH and incubation temperature were also studied.

Taguchi design

Results obtained using the one-factor-at-a-time method revealed the prominent effect of five factors (glucose, yeast extract, NaCl, pH, and incubating temperature) on decolorization efficiency, and the effects of each of these were studied at four levels (Table 1). A standard orthogonal array of 16 experiments was designed using Qualitek-4 software (L16). The experiments were conducted in triplicate under the conditions defined in (Table 2). A medium containing the dye without bacterial inoculation was used as the abiotic control to calculate the extent of biological decolorization.

Table 1. The parameters selected for optimization of microbial decolorization process, each at four levels

No.	Factor	Level 1	Level 2	Level 3	Level 4
1	**Glucose (% w/v)**	0.1	0.3	0.5	1
2	**Yeast Extract (% w/v)**	0.05	0.1	0.5	1
3	**NaCl (% w/v)**	1	5	10	15
4	**pH**	7	8	9	10
5	**Temperature (°C)**	25	30	35	40

Table 2. Experimental conditions determined based on the standard Taguchi L16 orthogonal array and the results obtained in the decolorization assay.

Run No.	Factor					Decolorization extent (%)	Standard deviation	S/N ratio
	Glucose (% w/v)	Yeast extract (% w/v)	NaCl (% w/v)	pH	Temperature (°C)			
1	0.1	0.05	1	7	25	11.23	1.33	20.876
2	0.1	0.1	5	8	30	16.33	2.01	24.123
3	0.1	0.5	10	9	35	58.8	4.01	35.347
4	0.1	1	15	10	40	79.4	2.95	37.984
5	0.3	0.05	5	9	40	26.63	3.79	28.349
6	0.3	0.1	1	10	35	30.56	8.72	28.796
7	0.3	0.5	15	7	30	51.09	3.8	34.12
8	0.3	1	10	8	25	60.4	4.78	35.569
9	0.5	0.05	10	10	30	35.93	3.43	31.026
10	0.5	0.1	15	9	25	24.1	2.25	27.564
11	0.5	0.5	1	8	40	21.5	0.1	26.648
12	0.5	1	5	7	35	73.2	0.55	37.289
13	1	0.05	15	8	35	28.1	0.86	28.996
14	1	0.1	10	7	40	34.66	1.65	30.778
15	1	0.5	5	10	25	62.5	2.09	35.907
16	1	1	1	9	30	62.76	2.05	35.945

Decolorization assay

To measure microbial decolorization, samples were collected after 4 days of incubation. In order to prevent absorbance interference from cellular or other suspended debris, media were clarified by centrifugation at 7500 g for 4 min. Based on the absorbance spectrum of the *Remazol Black B*, 600 nm (λ_{max} of dye) was used to measure the remaining dye after the decolorization study. Sterile culture media (with and without added dye) were used as controls to determine the extent of decolorization. Decolorization efficiency was expressed as

$$\text{Decolorization\%} = (A_0\text{-}A_1)/A_0 \times 100,$$

where A_0 is the absorbance of abiotic control and A_1 is the absorbance of medium after decolorization at $\underline{\lambda_{max}}$ of the dye.

Data Analysis

The main effects of the tested parameters were calculated by averaging the experiment results achieved at each level for each parameter. In the L16 orthogonal array design, at each level of a specified parameter, all levels of other parameters appeared once in the experiments. Taguchi designs are balanced in a way that no factor is weighted more or less in an

experiment, thus allowing factors to be analyzed independently of each other. In other words, since the influences from different levels of other parameters are counterbalanced, the effect of each level of every parameter can be calculated independently.

Analysis of variance (ANOVA) was used to quantitatively estimate the significance and relative contribution of each parameter to the overall decolorization. The *F*-ratio in an ANOVA shows the variation due to the applied treatment relative to the variation caused by experimental error. A statistically significant *F*-ratio signifies that the null hypothesis should be rejected, and that means the treatment is effective in dye removal.

Results

We have previously shown that among the 27 strains of the halophilic and halotolerant bacteria isolated from saline textile effluents, *Halomonas* sp. D_2, can decolorize azo dyes under a wider range of NaCl concentrations (up to 20% w/v), temperatures (25–40°C), and pH values (5–11) after 4 days of incubation in static culture (Fig. 1) (14). It can grow well in culture media with high concentrations of dye up to 10,000 ppm which are toxic to many other reported bacteria.

To find the main operational parameters and their optimal levels, effects of different media components and culture conditions were investigated using the one-factor-at-a-time approach. Maximum decolorization was formerly observed when yeast extract was used as a nitrogen source (14). Here it was shown that, while the D_2 strain retained its decolorization ability in the presence of any of the six mentioned salts, maximum decolorization was observed with sodium chloride (Fig. 2a). Among different carbon sources, glucose had the strongest effect on decolorization (Fig. 2b). Moreover, pH and incubation temperature were shown to affect the efficacy of decolorization by the bacterium (data not shown). Since the conventional method of optimization is laborious, the Taguchi method was applied in L16 array to evaluate the influence of the five mentioned, effective variables at four levels, as shown in Table 1.

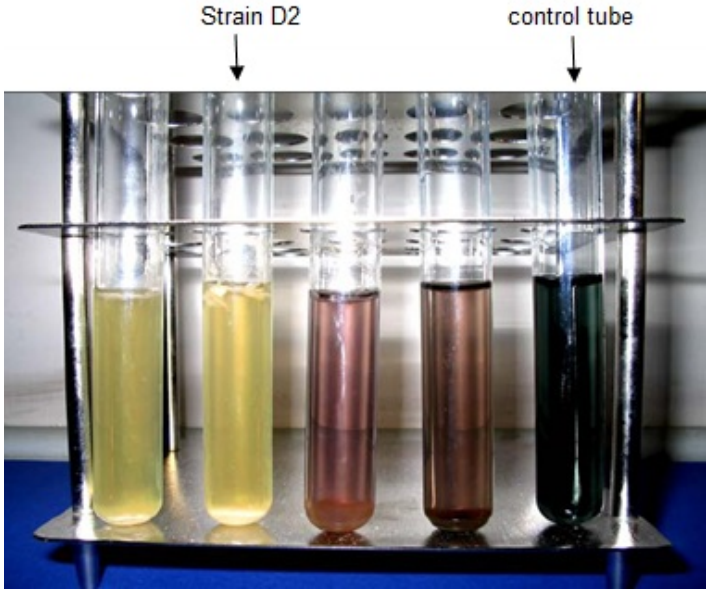

Figure 1. Decolorization of azo dye by strain D_2 in comparison with three other strains also capable of azo dye decolorization. Decolorization medium was composed of glucose, 1% w/v; yeast extract, 0.5% w/v; NaCl, 5% w/v; and *Remazol Black B*, 50 ppm at pH 7.2.

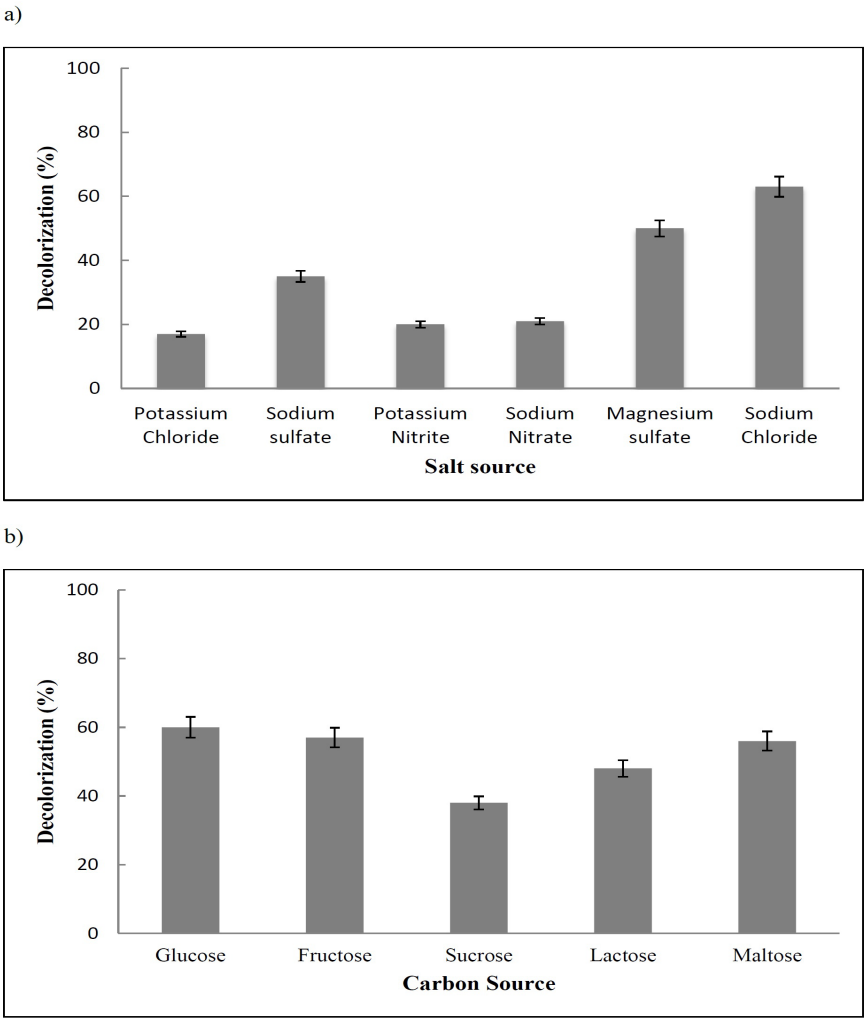

Figure 2. Effects of different (a) salts and (b) carbon sources on decolorization of *Remazol Black B* by *Halomonas sp.* D_2. Decolorization medium was composed of carbon source (glucose in part a), 1% w/v; yeast extract, 0.5% w/v; salt (NaCl in part b), 5% w/v; and *Remazol Black B*, 50 ppm at pH 7.2.

The experimental results for the microbial decolorization under different conditions can be seen in Table 2. It was observed that the various conditions tested resulted in notably different levels of decolorization, ranging from 11.23% to 79.4%. The *F*-ratio analysis of the experimental results also suggested that the selected variables strongly affect the decolorization process.

The analysis of variance (ANOVA) showed that by far the most significant parameter was yeast extract concentration, which accounted for 72.67% of the total effect, followed by pH (11.84%) and NaCl concentration (8.90%). Generally, the decolorization rate improved as the yeast extract concentration, salinity, and pH increased. Temperature and glucose concentration, respectively, only accounted for 2.10% and 1.88% of decolorization efficiency; these parameters could be pooled out of the statistical analysis (Table 3, Fig. 3). It should be noted that the percentage of contribution reported for each parameter is only valid within the studied ranges of parameters.

Table 3. Analysis of variance (ANOVA) of the obtained results

No.	Factor	DOF	Sum of squares	Variance	*F*-ratio	Pure sum	Percent
1	Glucose (% w/v)	3	431.978	143.992	12.277	396.795	1.876
2	Yeast Extract (% w/v)	3	15399.201	5133.067	437.684	15364.018	72.670
3	NaCl (% w/v)	3	1917.500	639.166	54.500	1882.316	8.903
4	pH	3	2538.564	846.188	72.152	2503.38	11.840
5	Temperature (°C)	3	479.583	159.861	13.630	444.400	2.101
	Error	32	375.288	11.727	-	-	2.610
	Total	47	21142.116	-	-	-	100.00%

DOF: degree of freedom.

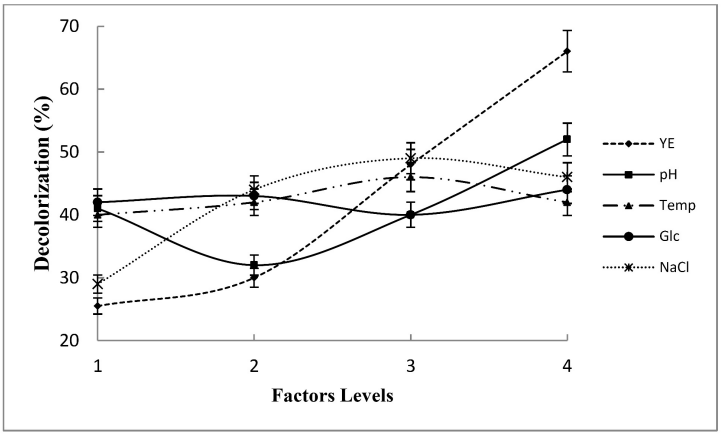

Figure 3. Main effect analysis of the studied factors (yeast extract, glucose, NaCl concentrations, pH, and Temperature) at 4 levels as described in Table 1.

With respect to yeast extract, 40.5% of decolorization enhancement was observed with its increase from level 1 to 4 (Fig. 3). Considering the halophilic nature of strain D_2, it was expected that salinity would have an enhancing effect on decolorization activity. It was observed in the main effect analysis that an increase in NaCl concentrations from 1% to 5% caused a 13.15% enhancement in the extent of decolorization. This enhancing effect of NaCl was also obvious at 10% but diminished at 15%. This feature could be of great value for application in saline wastewater treatments of textile effluents. Among the pH values tested, the lowest decolorization was obtained at pH 8. A 20.52% increase in decolorization was observed by increasing pH from 8 to 10. Although incubation temperature did not noticeably affect the activity of strain D_2, main effect analysis showed that the highest decolorization was achieved at 35°C. Glucose concentration in the media showed the least contribution to overall decolorization. Considering the evaluation results of various sugars (Fig. 2), it could be concluded that a minimal concentration of glucose could be adequate for supporting the growth and decolorization activity of strain D_2.

From the average response analysis, the optimum decolorization parameter combination was predicted to be 1% yeast extract concentration, pH 10, 10% (w/v) NaCl, 35°C temperature, and 1% (w/v) glucose. Expected decolorization under these conditions was predicted to be 93.85% ±3.12% at the 90% confidence level. Verification experiments performed under the predicted conditions

indicated the validity and adequacy of the predicted models. The observed result of decolorization (95%) was well accorded with the predicted results.

Considering the high cost of temperature control and glucose amendment in wastewater treatments and the prediction that these parameters do have profound effects on decolorization efficiency, the ANOVA analysis was repeated on the pooled data without considering temperature and glucose effects. The optimum prediction result showed that 83.83±3% azo dye decolorization could be achieved even while ignoring these two parameters. This finding could have great economic implications for industrial applications.

Discussion

High salt concentrations in textile effluents is one of the major limiting factors for decolorization. Halophilic and halotolerant bacteria, which thrive under high salt conditions, are appropriate candidates for this process. There are few reports about bacteria capable of azo dye decolorization in increased amounts of salinity. Khalid et al. reported two bacterial strains capable of azo dye decolorization in the presence of up to 10% (w/v) NaCl (19). In another report, *Halomonas sp.* was reported to decolorize up to 90% of azo dyes in the presence of NaCl in a narrow pH range of 6.5- 8.5 (20). Considering the potential advantages of biological agents as opposed to chemical protocols for decolorization, the application of halophilic and halotolerant microorganisms could be promising for the treatment of textile saline wastewater.

In the current study, the Taguchi method was successfully applied to optimize decolorization of the textile azo dye *Remazol Black B* with the halophilic bacterial strain *Halomonas* sp. D_2. Within the level ranges tested, the most significant parameter was shown to be yeast extract concentration, which accounted for 72.67% of the total effect. This phenomenon may be associated with the essential metabolic role of yeast extract in the regeneration of NADH, which is the electron donor in azo bond reduction (1); this result is in accordance with other reports (21, 22).

The next significant parameter was pH (11.84%). The increasing effect of pH could be related to the fact that optimum growth pH for D_2 strain was determined to be between the values of 8-10 (data not shown) and to the cooperative relationship that exists between growth and decolorization (2, 14). It is worth mentioning that tolerance to high pH values is important, because reactive azo dyes bind to cotton fibers through chemical reactions under alkaline conditions and high temperatures (23). It should also be mentioned that the textile effluent sample used to isolate the decolorizing halophilic bacteria was alkaline with pH 8-9 (14). The third significant factor was shown to be NaCl concentration (8.90%). As the D_2 strain is a halophilic bacterium, increased amounts of NaCl, as long as the amount is in accordance with the bacterium's optimum salt range, could enhance its decolorization ability via improving its growth requirements.

Verification experiments performed under the predicted conditions indicated the validity and adequacy of the predicted models. The 95% extent of dye removal achieved was much higher than the 65% level achieved under basic conditions. The conditions are expected to be very useful in practical applications. Furthermore, the Taguchi approach was shown to be quite satisfactory.

Acknowledgements

The authors would like to thank the research council of the University of Tehran for financially supporting this study.

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