



# Decolorization of dye solution containing Remazol Black B by *Aspergillus niger* isolated from hypersaline environment

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## Abstract

In this study, a factorial design was planned for removal of Remazol Black B dye by the fungus called *A. niger* isolated from İzmir Çamaltı Saltpan in Turkey. Initial pH, molasses concentration, initial dye concentration, and the effect of salt on dye removal were examined at the batch scale level. The optimal dye concentration, pH, and molasses dosage were determined as 100 ppm, 3, and 10 g/L for the most effective decolorization rate, respectively. In this study, *A. niger* removed 98.97% of Remazol Black B (RBB) after 2 days of incubation at 100 ppm dye concentration. In conclusion, it is recommended that fungi isolated from hypersaline environments can be used as potential decolorization agents in the treatment of industrial wastewater containing dye and salt.

**Keywords** Fungi · Biodegradation · Dye · Factorial analysis

## Introduction

Dyes are the natural and xenobiotic substances that give color to the world. There are over 100.000 commercial dyes found in the world, and every year more than 10<sup>9</sup> kg of dyestuff is produced. Approximately 20–25% of these dyes are given to the natural environment as waste. The type of dyestuff, which constitutes more than half of the annual dye quantity produced, is the azo dyes characterized by having one or more azo bonds ( $-N=N-$ ) (Dos Santos et al. 2007). Azo dyestuffs create problems when they are mixed with

wastewater due to their color, biologically difficult degradation, persistence and potential toxic effects on organisms.

The increase in demand for textile products has increased the activity of the textile industry and also increased the amount of wastewater at the end of the process (Yönten et al. 2021). The textile industry effluents are one of the main sources of serious water pollution problems worldwide. Textile wastewater is classified as difficult treated wastewater due to the toxic chemicals, especially the dyes, content. Since the discharge of the dyestuffs used in the textile industry to the surface waters disrupts the ecological balance, the need for treatment before discharge into such wastewater has emerged. Studies in this direction are becoming increasingly important (Weisburger 2002; Ghobadi Nejad et al. 2019). Physical, chemical and biological methods are used for the treatment of colored wastewater containing azo dyes (Tahir et al. 2021). Physicochemical methods are listed as membrane filtration, coagulation, precipitation, flotation, adsorption, ion exchange and electrolysis (El Messaoudi et al. 2021). Biological methods are listed as bacterial and fungal biosorption, aerobic and anaerobic biodegradation (McMullan et al. 2001; Madhushika et al. 2021).

The selection of the usage of these techniques depends on the type of dye, the content of wastewater, the dosage and cost of the required chemicals, the energy and material cost and the environmental damage. Physical and chemical treatment methods have some disadvantages such as

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high cost, toxic by-product formation, excessive energy consumption and concentrated sludge formation (Derco et al. 2021). Therefore, biological treatment methods are generally preferred (Korenak, et al. 2018).

To date, most of the researches has been conducted on the utilization of different microorganisms in dye removal (Jadhav et al. 2016; Wei and Zhang 2018). In addition to toxic dyes, textile wastewater is in the category of wastewater that can be difficult to treat with its high salt content (Mirbolooki et al. 2017). The use of adapted microorganisms that can survive in these difficult conditions owned by textile wastewater can be solved for this problem. Hypersaline environments are environments with harsh environmental conditions such as high salt concentrations, usually alkaline pH, exposure to UV rays and variable temperatures. Also, these kinds of environments are under the stress of abiotic factors having low nutritional status and water activity and/or containing pollutants such as toxic metals and xenobiotics (Sachdev et al. 2021). Organisms that grow or survive in such extreme conditions have adapted to this type of environment. It is also expected that the biological treatment of complex and toxic wastewater such as textile wastewater can be achieved with organisms adapted to this kind of harsh condition.

Halophilic/halotolerant fungi can be tolerated harsh conditions such as high alkali and saline environments due to their adaptation mechanisms (Wei and Zhang 2018). It is assumed that the fungal species isolated from hypersaline environments can be the best candidates for the treatment of wastewaters having harsh conditions. Most of the fungal strains were reported with their effective dye removal performances from aqueous solutions in the literature (Nerud et al. 2004; Yang et al. 2011; Gül 2013). However, there is limited information about the usage of fungal strains isolated from hypersaline environments in wastewater treatment technologies. This study aims to determine the dye removal performance of the fungal strain isolated from the hypersaline environment from dye-contaminated solutions.

## Materials and methods

### Microorganisms and dyes

In this study, *Aspergillus niger* previously isolated from İzmir Çamaltı Saltpan, Turkey has been used. The fungal isolate has been identified based on their morphological characteristics and the identification was supported by an internal transcribed spacer (ITS)-based phylogenetic analysis. The sequences were submitted to GenBank with the accession numbers KU958178 (İrdem and İlhan 2021).

### Preparation of dye solutions

The synthetic dye called Remazol Black B (RBB) was obtained from Sibel Textile, Bilecik/Turkey. The dye stock solution was prepared by dissolving the powdered dyestuff in distilled water to a final concentration of 20,000 ppm. Desired volumes of the stock solutions were diluted and added to the experimental solutions.

### Decolorization studies

The effect of dye concentration, pH, and molasses dosage was determined in the liquid medium composed of beet molasses solution, 1.0 g/L  $(\text{NH}_4)_2\text{SO}_4$ , and 0.5 g/L  $\text{KH}_2\text{PO}_4$ . The dye-containing medium without fungal inoculation was used as the control group. The dye degradation capabilities of fungi were evaluated according to the transparent zone formed around the colony on the petri dish contained dyeing-agar (Verma et al. 2015). The experimental design was planned to determine the optimum conditions for the maximum decolorization performance by fungus.

### Analytical methods

For dye analysis, 2 mL of samples were taken at definite times from the working solution containing fungi and dye. The samples were centrifuged at 10,000 rpm for 2 min, and the supernatant was used for dye analysis after appropriate dilutions. The concentration of Remazol Black B was examined by measuring the absorbance at 600 nm. Distilled water was used as a blank (Caner et al. 2009).

Absorbance measurements were performed using a Selecta UV visible spectrophotometer. The percentage decolorization of dye was calculated from Eq. (1):

$$\text{Decolorization (\%)} = (C_o - C_f) / C_o \times 100 \quad (1)$$

In the equation,  $C_o$  is the initial dye concentration (ppm) and  $C_f$  is the final dye concentration (ppm).

### Factorial experimental design

When there is more than one factor in the experimental design, factorial design should be used to avoid misleading results due to the interaction effect. Factorial designs allow the effects of one factor to be estimated at different levels of other factors. Many new experimental design methods have emerged due to the existence of constraints such as time, cost and labor in a factorial design. However, if these constraints are overcome in the study, the efficiency of the results to be obtained from the factorial design is much

higher than these methods. For this reason, if there is no restriction in the application studies, the application of the factorial design, even if it is traditional, is very important in terms of the reliability of the results to be obtained (Montgomery 2009).

Factorial designs proposed by Fisher (1935) and Yates (1937) are very popular designs used to investigate the main effects and interaction effects (interaction) of two or more factors at the same time (Fisher 1935; Yates 1937). Factorial designs are much more effective than traditional designs that investigate the effect of one factor at a time, as it saves time and money. Besides, factorial designs determine the interactions between factors. Optimization of environmental conditions provides great advantages in terms of increasing efficiency and reducing cost. The process is easy to control with this method. In application,  $2^k$ ,  $3^k$  factorial designs are widely used. However, may not all factors have an equal number of levels. In this study, 2 levels of molasses, 3 levels of pH, 2 levels of NaCl, and 3 levels of dye concentration were investigated. In this case, the general factorial design is defined as follows (Montgomery 2009).

$$\begin{aligned}
 y_{ijklm} = & \mu + \tau_i + \gamma_j + \delta_k + \vartheta_l + \tau\gamma_{ij} \\
 & + \tau\delta_{ik} + \tau\vartheta_{il} + \gamma\delta_{jk} + \gamma\vartheta_{jl} + \delta\vartheta_{kl} \\
 & + \tau\gamma\delta_{ijk} + \tau\gamma\vartheta_{ijl} + \tau\delta\vartheta_{ikl} + \tau\gamma\delta\vartheta_{ijkl} + \epsilon_{ijklm}, \\
 & i = 1, 2; \quad j = 1, 2, 3; \quad k = 1, 2; \\
 & l = 1, 2, 3; \quad m = 1, 2.
 \end{aligned}$$

Here,  $y_{ijklm}$  is the  $m$ th observation value in level  $i$  of factor A, level  $j$  of factor B, level  $k$  of factor C, level  $l$  of factor D;  $\mu$  is the overall average;  $\tau_i$  is the effect of the  $i$ th level of factor A;

$\gamma_j$  is the effect of the  $j$ th level of factor B;  $\delta_k$  is the effect of the  $k$ th level of factor C;  $\vartheta_l$  is the effect of the  $l$ th level of factor D;  $\tau\delta_{ik}$  is the interaction effect of factors A and B;  $\tau\gamma\delta_{ijk}$  is the interaction effect of factors A, B and C factors;

**Table 1** The level of factors in the factorial design

		Value label	<i>N</i>
Molasses	-1	5 g/L	36
	1	10 g/L	36
pH	-1	3	24
	0	5	24
	1	7	24
NaCl	-1	0	36
	1	5%	36
Dye concentration	-1	50 ppm	24
	0	100 ppm	24
	1	150 ppm	24

**Table 2** The factorial experimental design for optimization parameters of RBB decolorization

RUN	Molasses	pH	NaCl	Dye concentration
1	-1	-1	-1	-1
2	-1	-1	-1	0
3	-1	-1	-1	1
4	-1	-1	1	-1
5	-1	-1	1	0
6	-1	-1	1	1
7	-1	0	-1	-1
8	-1	0	-1	0
9	-1	0	-1	1
10	-1	0	1	-1
11	-1	0	1	0
12	-1	0	1	1
13	-1	1	-1	-1
14	-1	1	-1	0
15	-1	1	-1	1
16	-1	1	1	-1
17	-1	1	1	0
18	-1	1	1	1
19	1	-1	-1	-1
20	1	-1	-1	0
21	1	-1	-1	1
22	1	-1	1	-1
23	1	-1	1	0
24	1	-1	1	1
25	1	0	-1	-1
26	1	0	-1	0
27	1	0	-1	1
28	1	0	1	-1
29	1	0	1	0
30	1	0	1	1
31	1	1	-1	-1
32	1	1	-1	0
33	1	1	-1	1
34	1	1	1	-1
35	1	1	1	0
36	1	1	1	1

$\tau\gamma\delta\vartheta_{ijkl}$  is the interaction effect of factors A, B, C, and D factors and  $\epsilon_{ijklm}$  is the random error.

The level of factors in the factorial design is given in Table 1. The 36 different experimental patterns for different levels of molasses, pH, NaCl and dye concentration are given in Table 2. The studies were carried out in 250 ml of flasks with a volume of 100 ml. The mixture was allowed to incubate for 7 days with agitation at 150 rpm at 27 °C.

After the incubation period was completed, the samples were taken from each flask and filtered through the filter

**Table 3** Variance analysis for decolorization

Source	Type II sum of squares	df	Mean square	F	Sig
Corrected model	19,325.018 <sup>a</sup>	35	552.143	293.797	0.000
Intercept	464,874.356	1	464,874.356	247,360.605	0.000
Molasses	1297.110	1	1297.110	690.195	0.000
pH	3198.084	2	1599.042	850.854	0.000
NaCl	7958.860	1	7958.860	4234.926	0.000
Dye concentration	656.342	2	328.171	174.621	0.000
Molasses *pH	480.454	2	240.227	127.825	0.000
Molasses* NaCl	273.559	1	273.559	145.561	0.000
Molasses* Dye Con	144.397	2	72.198	38.417	0.000
pH* NaCl	2273.524	2	1136.762	604.874	0.000
pH* Dye Con	536.015	4	134.004	71.304	0.000
NaCl*Dye Con	111.316	2	55.658	29.616	0.000
Molasses*pH*NaCl	707.371	2	353.685	188.197	0.000
Molasses*pH*Dye Con	450.116	4	112.529	59.877	0.000
Molasses*NaCl*Dye Con	65.583	2	32.792	17.449	0.000
pH*NaCl*Dye Con	866.567	4	216.642	115.276	0.000
Molasses*pH*NaCl*Dye Con	305.719	4	76.430	40.668	0.000
Error	67.656	36	1.879		
Total	484,267.030	72			
Corrected total	19,392.674	71			

$R$  squared = 0.997 (Adjusted  $R$  Squared = 0.993)

paper, then the absorbance was measured at the wavelength of 600 nm in order to calculate the dye removal percentage.

The levels of factors such as molasses concentration, initial pH, salt, and initial dye concentration were given in Table 3. Accordingly, 2 levels were considered for molasses concentration (5 g and 10 g/L). The given experimental setups (Table 2) for each level were carried out in duplicates. As a result, the case of molasses concentration in the experimental design was carried out 36 times for both 5 and 10 g/L molasses concentrations.

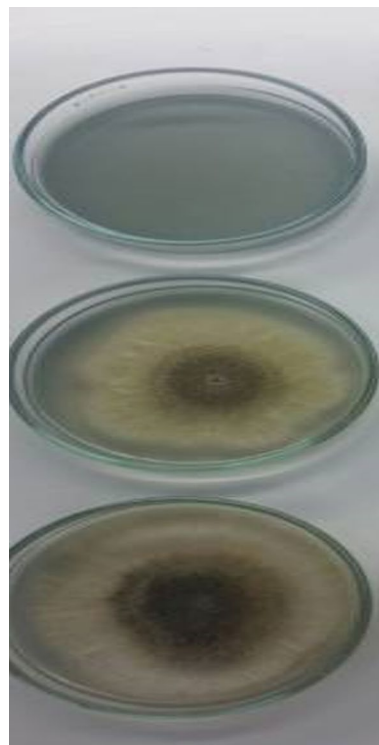
## Data analysis

Experimental results were analyzed using SPSS package software. The obtained data from Factorial Design were subjected to statistical analysis.

## Results

### Decolorization studies

It was observed that while fungal culture was growing in the malt extract agar (MEA) containing 20 ppm dye, also the transparent zones were occurred and indicated that dye was removed by living fungal strain. The photos related to the dye removal experiments carried out with Remazol Black B dye and *Aspergillus niger* are given in Fig. 1.



**Fig. 1** Selected fungal strain for decolorization experiments

### Factorial experimental design

As a result of a statistical-based study, the variance analysis obtained in terms of dye removal is given in Table 3. As shown in Table 3, the calculated value for the model was less than the significance value of 0.05. In this case, it was decided that the model established at 0.95 probability was statistically significant. Then, the significance tests for model coefficients were examined. The effects of the factors (single, double, triple, and quadruple) interaction included in the experiment were statistically significant at a 0.05 significance level (Table 3). Furthermore, it was determined that the established model explained the decolorization rate as 0.993.

When Table 3 is examined, the *p*-value calculated for the main effect of the Molasses factor is less than the significance level of 0.05. This indicates that the main effect of the Molasses factor is statistically significant. In other words, the molasses factor alone is effective in decolorization. Similarly, the main effects of factors such as pH, NaCl, and dye concentration were found statistically significant. Then, whether bilateral interactions in the factorial model are meaningful or not was investigated. Firstly, the effect of different level combinations of molasses and pH factors has been discussed. Table 3 shows that the *p*-value calculated for this interaction is less than 0.05. Combinations of different levels of molasses and pH factors are effective in decolorization.

In the experiments, the three different levels of pH and dye concentration were examined and the effects were found as statistically significant. Multiple comparisons should be made to determine a significant difference between the level of factors with a level number of 3 and above. The results obtained for multiple comparisons are given in Table 4.

### The effect of pH on dye removal

The relationship between the pH of the working environment and dye removal is shown in Table 4. When the pH of the

working environment is 3, the dye removal rate according to pH 5 is increased by 13.59% and it is increased by 14.32% according to pH 7.

### The effect of dye concentration on dye removal

The relationship between dye concentration of the working environment and dye removal is shown in Table 4. When the initial dye concentration was 100 ppm, the dye removal rate according to 150 ppm increased by 4.87%. The dye removal rate increased by 7.25% compared to 50 ppm when dye concentration was 100 ppm.

### The binary effect of salt and pH on dye removal

The effect of combinations of different levels of pH and NaCl on decolorization is statistically significant. Figure 2 gives the profile graphic for this interaction. According to

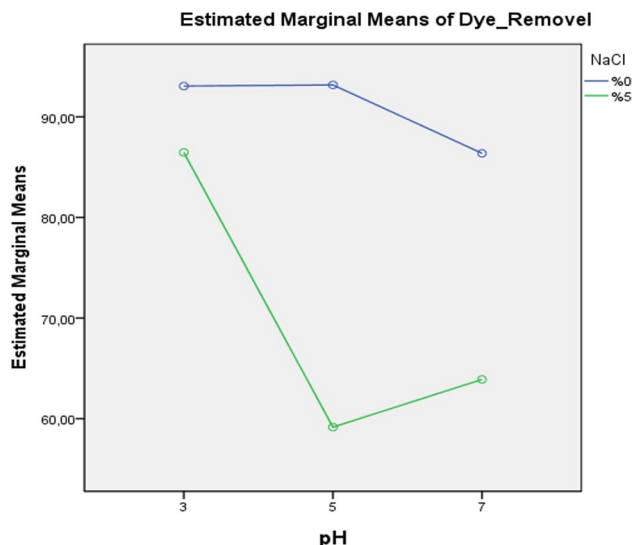


Fig. 2 The profile graph of pH and salt interaction

Table 4 The relationship between pH and dye removal

		N	Subset			
				1	2	3
pH	Tukey HSD <sup>a,b</sup>	7	24	75.1361		
	Tukey HSD <sup>a,b</sup>	5	24		76.1631	
	Tukey HSD <sup>a,b</sup>	3	24			89.7595
Dye concentration (mg/L)	Tukey HSD <sup>a,b</sup>	50	24	77.1433		
	Tukey HSD <sup>a,b</sup>	150	24		79.5189	
	Tukey HSD <sup>a,b</sup>	100	24			84.3965

Means for groups in homogeneous subsets are displayed. Based on observed means. The error term is Mean Square (Error) = 1.879 a. Uses Harmonic Mean Size = 24.000

b. Alpha = 0.05

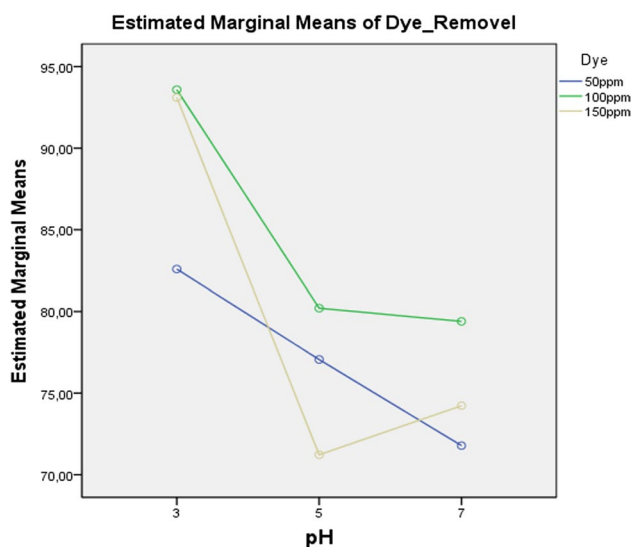


Fig. 3 The profile graph of pH and dye concentration interaction

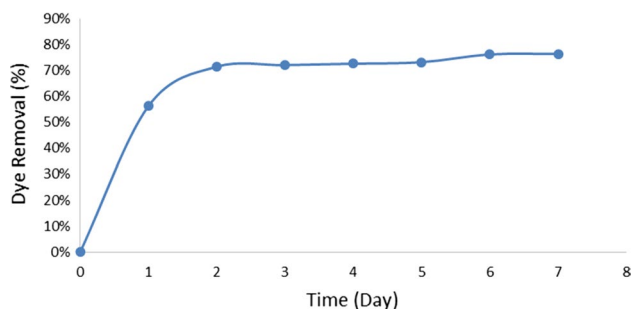


Fig. 4 The effect of incubation period on dye removal at optimal conditions

Fig. 2, the decolorization is at the highest level when NaCl is 0% and the pH level is 3 or 5.

### The binary effect of pH and dye concentration on dye removal

Figure 3 gives the profile graphic for the interaction of pH and dye concentration. The decolorization is at the highest level when the dye concentration is 100 ppm and the pH level is 3. It is statistically significant in all other bilateral interactions. All three and four interactions are statistically significant.

### The effect of incubation time on dye removal

The results of the experiments to determine the best time of decolorization at optimal conditions are given in Fig. 4. As seen in Fig. 4, it was determined that the best decolorization rate has occurred at the end of 2 days of incubation. *A.*

*niger* removed 98.97% of RBB after 2 days of incubation at 100 ppm dye concentration. There wasn't any significant change in the decolorization rate from 2 to 7 days.

## Discussion

The experimental design is to observe and interpret the variations in the output of the process, with changes in factors affecting the process, to improve the performance of a process.

The experimental design aims to separate significant factors from insignificant ones that impact the experiments and to achieve the optimum values of important parameters affecting the process. It can be easily used to investigate the interaction between factors and to increase the information obtained from the experiments. The most important feature of experimental design is to minimize time and cost (Montgomery 2009). Factorial designs within the experimental design are the most effective designs used to examine the effect of two or more factors. All factor combinations that affect the output in factorial designs have been experimented and all effects that may occur are investigated. There were some studies about the decolorization activity of fungal strains in the literature (Namdhari et al. 2012). However, there was not any study about biodegradation of dyes fungal strains including factorial experiment design. Previous studies emphasized that full factorial design was a useful tool to determine optimal conditions for pollutant removal (Fazli et al. 2010; Özbay et al. 2013; Malkoç 2017; Labena et al. 2021) by adsorption or biosorption mechanisms. Fazli et al. (2010) optimized Reactive Blue 19 biosorption by macrofungus *Ganoderma* sp. using response surface methodology (RSM). They showed that the decolorization performance of the macrofungus was raised in optimal conditions determined by RSM easily, comparing with non-optimized conditions. Similarly, in the current study, the reactive dye biodegradation performance of the fungus *A. niger* was increased in optimal conditions using full factorial design methodology. Also, Malkoç (2017) studied the Remazol Red dye biosorption by living *Aspergillus terreus* biosorbent and reported that pH and temperature were the most significant factors for dye biosorption. The results of dye biodegradation studies were fitted with the previous study focused on dye removal by *A. terreus* by biosorption mechanism.

Recent studies focused on the negative effects of water pollution and showed the interactions of pollutants with living organisms or nucleic acids (Congur 2021). For instance, Lellis et al. (2019) emphasized the main effect of textile dye pollution on the human's executive molecule DNA and also the importance of bioremediation technologies to overcome textile dye pollution in water.

Many studies from past to present have shown the success of using fungi in bioremediation (Chulhwan et al. 2004; Nerud et al. 2004; Yang et al. 2011; Gül 2013). Yang et al. (2011) reported that a fungal strain called *Penicillium* sp. removed 98.23% of Malachite Green dye at 25 mg/L concentration. Gül (2013) reported that the filamentous fungal strain called *R. arrhizus* removed 100% of Remazol Blue, 100% of RBB, and 71.83% of Remazol Red in 2, 3, and 8 days, respectively. Chulhwan et al. (2004) reported that the fungal strain named *Trametes versicolor* KCTC 16,781 decolorized 98.4% and 99.0% of acid yellow and acid blue 350 dyes after 2 days, respectively. In another study, it was reported that *P. ostreatus* strain f6 decolorized 69, 96, 75, and 88% of Eosin Yellowish, Evans Blue, Phenol Red, and Poly B-411 (20 ppm) during a 14-day treatment, respectively (Nerud et al. 2004). In this study, *A. niger* removed 98.97% of RBB after 2 days of incubation at 100 ppm dye concentration. As seen, the previous studies showed that fungi were the excellent biologic materials to remove textile dyes from wastewaters as alive forms (Nerud et al. 2004; Yang et al. 2011; Gül 2013). The usage of living fungal strains in decolorization technologies ensures some advantages such as not to waste energy for the preparation of biomass (harvesting, drying, and storage) used as biosorbent (Yang et al. 2011). Also, it was reported that these living fungal forms showed maximum dye removal capacity at their optimal conditions for their metabolisms in the same studies. Gül (2013) showed that the filamentous fungus (*R. arrhizus*) performed maximum anionic dye removal at pH 3 at which the fungal surface was positive due to the lower pH value than the isoelectric point. Similarly, the fungus called *A. niger* is also a filamentous fungus but a halophilic/halotolerant fungi differently from *R. arrhizus* and the maximum decolorization occurred at pH 3 by *A. niger*. The results of the current study were fitted with the previous study's results. The decolorization rate by halophilic and halotolerant bacteria mostly depends on the type, molecular weight, and substitution groups of the dye (Amoozegar et al. 2011). It was reported that the halophilic/halotolerant bacteria decolorized azo structured dyes successfully (Amoozegar et al. 2011). Also, Gül (2013) the textile dye decolorization performance of filamentous fungus *R. arrhizus* using different classes or types of dyes and reported that the decolorization performance was strongly related to the chemical structure of the dye molecule. The decolorization experiments in this study indicated that the halophilic/halotolerant fungi achieved a successful decolorization rate of an azo dye RBB. The results of this study showed that the fungal strain called *A. niger* adapted in harsh conditions, which was similar to the conditions of textile wastewater, was

an effective and efficient biologic material to be used in the biological wastewater treatment process.

## Conclusion

The optimum conditions were determined according to the results of this study. The optimal dye concentration, pH, molasses dosage were determined as 100 ppm, 3, and 10 g/L for the most effective decolorization rate of RBB by *A. niger*, respectively. In conclusion, the fungal strain isolated from hypersaline environments can be efficiently used for the removal of textile dyes.

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## Declaration

**Conflict of interest** The authors declare that they have no conflict of interest.

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