



## Azo dye decolorization by using four different psychrotolerant *Bacillus* species

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### Abstract

Since the early ages, human beings have used colored materials to leave marks on their environment and the following generations. In time, paints, which gained great importance with painting the objects and the items they used, brought environmental problems with them. Synthetic paints/dyes used to meet the increasing need over time had to be eliminated from the environment. Nowadays, using bacteria is considered as a good solution for eliminating dyes that contain azo groups. These dyes are used in large quantities and difficult to decompose. The main goal of this study was to decolor six different (amido black 10B, evans blue, janus green, methyl orange, methyl red, and orange G) azo dyes by use of four different psychrotolerant *Bacillus* (*Bacillus* sp, *B. cereus*, *B. mycoides*, and *B. subtilis*) isolates. According to the obtained results, it was determined that the psychrotolerant *Bacillus* isolates could remove colors of the six azo dyes by presenting high decolorization activity (up to 98%).

### Keywords:

Azo dyes; *Bacillus* spp., Color removal, Decolorization, Psychrotolerant

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### Introduction

Today, azo dyes are used today in many fields, including textile, plastic, and paper industries (Sudha et al., 2014). Dye-contaminated water, especially from the dyeing processes in the textile industry, is discharged with the waste water of the textile factories. The dyeing of wastewater results from dyeing the textile products, adding prints on the products, and washing the products. The paint level of water depends on the density and the structure of the dyestuff (Fu & Viraraghavan, 2000). Discharging textile industry wastewater to the environment causes undesirable environmental conditions that are not suitable for human, animal, and plant health

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(Chung & Stevens, 1993). This may result in putting many wild species in danger of being extinct. When natural dyes are compared to synthetic dyes, the use of synthetic dyes is increasing day by day because they have different color choices. Synthetic paints are used in many fields such as cosmetics, food, and textiles (Aksu, 2005). The majority of commercially used synthetic dyes are toxic and carcinogenic and consist of azo dyes that can cause changes in genes (Seesuriyachana et al., 2007). These dyes have significant accumulation properties in nature because they are resistant to disintegration thanks to their azo bonds. Azo compounds are chemically represented as  $R-N=N-R'$ , where  $-N=N-$  is the azo group, and the R or R' can be either aryl or alkyl compounds (Chung, 2016). Azo dyes are stable in acidic and alkaline conditions, do not decompose in an oxygenated environment, and are resistant to heat and light. Due to these features, azo dyes cannot be purified and/or eliminated by standard treatment methods. Sometimes, a reactive dye cannot be eliminated from water and discharged to the waste water drainage system by contaminating the water in a high concentration that could be 90% (Lucas et al., 2007). In addition to both physical and chemical methods, various removal techniques can also be used to remove these dyes (Sun et al., 2007). Among these methods, biological methods have gained more popularity recently. Primarily, use of microorganisms, which can break down azo dyes in aerobic or anaerobic conditions, have been preferred (Jamie & Siddique, 2019).

It is clear that the investigation of the effects of azo dyes on public health will be one of the most important strategies in preventing many diseases, especially cancer (Chung, 2016). In this study, it was aimed to use psychrotolerant bacteria in the removal of azo dyes color that could be found in waste waters. For this purpose, methyl red, methyl orange, orange g, amido black 10b, evans blue, janus green paints were used and color removals in these dyes were measured using a spectrophotometer with the help of four different and psychrotolerant *Bacillus* species.

## Materials and Method

### *Bacillus* Strains Used in This Study

In this study, four different strains belonging to the *Bacillus* genus were used. These are: *B. subtilis*, *B. mycoides*, *B. cereus*, and *Bacillus* sp. strains. All strains were obtained from Özlem Barış, who works at Atatürk University, Faculty of Science, Department of Biology, Microbiology Laboratory. These bacteria were isolated from the caves of Erzurum province and their development temperatures were determined as 7 - 45 °C and their optimum growth temperature was 30 °C (Barış, 2009). The cultures used in this study were grown on TSA media by incubating them at 30 °C for 24 hours.

### *Azo Dyes* Used in This Study

In this study, six different azo dyes were used. These are Amido Black 10B (EC Apparatus Corporation), Evans Blue (Merck, 5191531), Janus Green (Merck, 9743040, CI Nr 11050), Methyl Orange (Merck, 123602), Methyl Red (Merck, 5279510, CI Nr13020), and Orange G (Merck, 5202928, CI Nr 16230). All of the used dyes are commercially available, and obtained by purchasing them from a local distributor.

### ***Dye Concentrations Used in This Study***

The dyes used in this study were studied in 5 different concentrations. These concentrations are determined as 10, 20, 50, 100, and 200 mg/L. The concentrations used in this study were determined by considering previous studies (Mishra and Sharma 2014; Lalnunhlimi & Veenagayathri 2016; Al Hamadi 2017). The dyes are prepared in flasks containing 250 ml of TSB. Preparation of 10, 20, 50, 100, and 200 mg/L concentrations were done as follows; 2.5, 5, 12.5, 25, and 50 mg of each dye were weighed for each concentration, respectively. The powdered dyes were dissolved with some distilled water and each of them was transferred to a different flask. Then, 7.5 g of TSB was weighed and transferred to the flask as instructed by the manufacturer. Distilled water was added slowly in small amounts to complete it to 250 mL.

All *Bacillus* strains, enlivened in petri prior to use in TSB. TSBs that contain different concentration of the six different azo dyes, are prepared for use by suspending the bacteria aseptically in 10 mL tubes ( $1-2 \times 10^8$  CFU per tube). Subsequently, the samples taken from cultures, which were incubated for 72 hours at 30 °C, were centrifuged at 2,000 rpm for 15 minutes and dye removal was determined by measuring the absorbance values of supernatants (Barış, 2009; Shah, 2014; Guadie et al., 2017). All concentrations were tested as duplicates.

### ***Determination of the Azo Dyes Removal Rates***

In order to determine the rate of decolorization of the used azo dyes, Shah (2014) and Guadie et al. (2017) method has been partially modified and used. Briefly, cultures were centrifuged at 2,000 rpm for 15 minutes. The absorbance values of the obtained supernatants at the wavelength at which the dyestuff give the maximum absorbance were read in the spectrophotometer. Janus green, methyl red, evans blue, amido black 10B, methyl orange, and orange G were read at 605, 430, 615, 618, 466, and 480 nm, respectively. Azo dye removal percentage was determined by calculating the difference between the negative control's (NC) absorbance value and the positive control (PC) concentration's absorbance value after incubation. TSB medium without addition of any dyes and bacteria was used as a blank.

Azo dye decolorization calculation formula:

$$\text{Decolorization \%} = \frac{\text{NC Abs.} - \text{PC Abs.}}{\text{NC Abs.}} \times 100$$

## **Results**

### ***Azo Dye Removal Results of *B. cereus****

Color removal results of all tested azo dyes that was made by *B. cereus* presented in Table 1. The color reduction rates of janus green azo dye at concentrations of 10, 20, 50, 100, and 200 mg/L were calculated to be 22%, 17%, 21%, 10%, and 8%, respectively. Likewise, the same concentrations of the methyl red azo dye were decolorized by 31%, 28%, 26%, 43%, and 36%, respectively. Similarly, the same concentrations of the evans blue azo dye were decolorized by 58%, 80%, 53%, 68%, and 0%, respectively. No results were obtained at 200 mg/L concentration of evans blue. Additionally, amido black 10B azo dye was also tested at the same five concentrations and the decolorization rates were 11%, 38%, 65%, 73%, and 63%, respectively.

Methyl orange and orange G azo dyes were tested at only 50, 100, and 200 mg/L concentrations. Their color removal rates were 37%, 70%, and 35%; and 72%, 58%, and 36%, respectively.

#### ***Azo Dye Removal Results of B. subtilis***

Color removal results of all tested azo dyes that was made by *B. subtilis* presented in Table 2. The decolorization rates were ranged between 3-67%. The color reduction rates of janus green azo dye at concentrations of 10, 20, 50, 100, and 200 mg/L were calculated to be 56, 25, 3, 24, and 8%, respectively. Also, the same concentrations of the methyl red azo dye were decolorized by 13, 35, 19, 22, and 37%, respectively. In the same way, the same concentrations of the evans blue azo dye were decolorized by 67, 60, 32, 37, and 10%, respectively. As well, amido black 10B azo dye was also tested at the same concentrations and the decolorization rates were 35, 42, 17, 21, and 8%, respectively. Methyl orange and orange G azo dyes were tested at only 50, 100, and 200 mg/L concentrations. Their decolorization rates were 41, 39, and 25%; and 57, 54, and 45%, respectively.

#### ***Azo Dye Removal Results of B. mycoides***

Decolorization results of all tested azo dyes that was made by *B. mycoides* presented in Table 3. The decolorization rates were ranged between 7-80%. The color removal rates of janus green azo dye at concentrations of 10, 20, 50, 100, and 200 mg/L were calculated to be 80, 52, 24, 44, and 14%, respectively. The same concentrations of the methyl red azo dye were decolorized by 78, 53, 12, 7, and 32%, respectively. Similarly, the same concentrations of the evans blue azo dye were decolorized by 68, 69, 32, 35, and 12%, respectively. In addition, amido black 10B azo dye was also tested at the same five concentrations and the decolorization rates were 49, 47, 46, 25, and 17%, respectively. Both methyl orange and orange G azo dyes were tested at only 50, 100, and 200 mg/L concentrations. The color removal rates of these two dyes were 25, 23, and 31%; and 55, 43, and 44%, respectively.

#### ***Azo Dye Removal Results of Bacillus sp.***

Spectrophotometrically measured color removal results of all tested azo dyes that was made by *Bacillus* sp. presented in Table 4. The decolorization rates were ranged between 3-98%. The color removal rates of janus green azo dye at concentrations of 10, 20, 50, 100, and 200 mg/L were calculated to be 18, 12, 3, 21, and 5%, respectively. The same concentrations of the methyl red azo dye were decolorized by 65, 68, 46, 9, and 35%, respectively. Likewise, the same concentrations of the evans blue azo dye were decolorized by 94, 86, 35, 40, and 3%, respectively. Furthermore, amido black 10B azo dye was also tested at the same five concentrations and the decolorization rates were 98, 87, 59, 54, and 36%, respectively. Both methyl orange and orange G azo dyes were tested at only 50, 100, and 200 mg/L concentrations. The color removal rates of these two dyes were 47, 33, and 8%; and 66, 58, and 47%, respectively.

Table 1. Decolorization of six azo dyes by *B. cereus*

Used azo dyes (wavelength)	Controls	Concentrations (mg/L)					
		0	10	20	50	100	200
Janus Green (605 nm)	NC	0.040	0.134	0.185	0.494	0.793	0.902
	PC		0.105	0.155	0.394	0.718	0.831
Methyl Red (430 nm)	NC	0.287	0.174	0.248	0.276	0.501	0.982
	PC		0.121	0.180	0.206	0.288	0.637
Evans Blue (615 nm)	NC	0.035	0.080	0.093	0.199	0.235	0.333
	PC		0.034	0.019	0.095	0.077	0.334
Amido Black 10B (618 nm)	NC	0.107	0.047	0.056	0.073	0.129	0.241
	PC		-0.042	-0.035	-0.026	-0.0035	0.09
Methyl Orange (466 nm)*	NC	-0.068	NA	NA	0.207	0.291	0.506
	PC		NA	NA	0.133	0.088	0.331
Orange G (480 nm)*	NC	0.094	NA	NA	0.258	0.418	0.771
	PC		NA	NA	0.073	0.176	0.321

NC: negative control (distilled water + TSB + azo dye)

PC: positive control (distilled water + TSB + azo dye + psychrophilic *B. cereus*)

\* The results of methyl Orange and Orange G dyes could not be determined at concentrations of 10 and 20 mg/L.

Table 2. Decolorization of six azo dyes by *B. subtilis*

Used azo dyes (wavelength)	Controls	Concentrations (mg/L)					
		0	10	20	50	100	200
Janus Green (605 nm)	NC	0.040	0.134	0.185	0.494	0.793	0.902
	PC		0.060	0.140	0.442	0.604	0.835
Methyl Red (430 nm)	NC	0.287	0.174	0.248	0.276	0.501	0.982
	PC		0.121	0.180	0.206	0.288	0.637
Evans Blue (615 nm)	NC	0.035	0.080	0.093	0.199	0.235	0.333
	PC		0.027	0.038	0.136	0.149	0.300
Amido Black 10B (618 nm)	NC	0.107	0.047	0.056	0.073	0.129	0.241
	PC		0.031	0.033	0.061	0.102	0.217
Methyl Orange (466 nm)*	NC	-0.068	NA	NA	0.207	0.291	0.506
	PC		NA	NA	0.124	0.180	0.383
Orange G (480 nm)*	NC	0.094	NA	NA	0.258	0.418	0.771
	PC		NA	NA	0.111	0.194	0.428

NC: negative control (distilled water + azo dye)

PC: positive control (distilled water + azo dye + psychrophilic *B. subtilis*)

\* The results of methyl Orange and Orange G dyes could not be determined at concentrations of 10 and 20 mg/L.

Table 3. Decolorization of six azo dyes by *B. mycooides*

Used azo dyes (wavelength)	Controls	Concentrations (mg/L)					
		0	10	20	50	100	200
Janus Green (605 nm)	NC	0.010	0.134	0.185	0.494	0.793	0.902
	PC		0.027	0.089	0.379	0.448	0.782
Methyl Red (430 nm)	NC	0.272	0.174	0.248	0.276	0.501	0.982
	PC		0.040	0.119	0.244	0.468	0.672
Evans Blue (615 nm)	NC	0.009	0.080	0.093	0.199	0.235	0.333
	PC		0.026	0.029	0.137	0.155	0.295
Amido Black 10B (618 nm)	NC	0.045	0.047	0.056	0.073	0.129	0.241
	PC		0.024	0.030	0.040	0.097	0.201
Methyl Orange (466 nm)*	NC	0.051	NA	NA	0.207	0.291	0.506
	PC		NA	NA	0.156	0.225	0.351
Orange G (480 nm)*	NC	0.083	NA	NA	0.258	0.418	0.771
	PC		NA	NA	0.118	0.239	0.434

NC: negative control (distilled water + azo dye)

PC: positive control (distilled water + azo dye + psychrophilic *B. mycooides*)

\* The results of methyl Orange and Orange G dyes could not be determined at concentrations of 10 and 20 mg/L.

Table 4. Decolorization of six azo dyes by *Bacillus sp.*

Used azo dyes (wavelength)	Controls	Concentrations (mg/L)					
		0	10	20	50	100	200
Janus Green (605 nm)	NC	-0.016	0.134	0.185	0.494	0.793	0.902
	PC		0.111	0.163	0.482	0.634	0.862
Methyl Red (430 nm)	NC	0.517	0.174	0.248	0.276	0.501	0.982
	PC		-0.061	0.071	0.150	0.460	0.589
Evans Blue (615 nm)	NC	0.021	0.080	0.093	0.199	0.235	0.333
	PC		0.005	0.026	0.134	0.142	0.324
Amido Black 10B (618 nm)	NC	0.035	0.047	0.056	0.073	0.129	0.241
	PC		0.001	0.025	0.030	0.060	0.180
Methyl Orange (466 nm)*	NC	-0.061	NA	NA	0.207	0.291	0.506
	PC		NA	NA	0.119	0.169	0.485
Orange G (480 nm)*	NC	0.129	NA	NA	0.258	0.418	0.771
	PC		NA	NA	0.090	0.176	0.409

NC: negative control (distilled water + azo dye)

PC: positive control (distilled water + azo dye + psychrophilic *Bacillus sp.*)

\* The results of methyl Orange and Orange G dyes could not be determined at concentrations of 10 and 20 mg/L.

## Discussion

The importance of environmental pollution control is increasing. Dyestuffs may have toxic effects or even mutagenic and genotoxic effects when spread in the environment or mixed with soil, plants,

animals and eventually foods and drinks in various ways. In this regard, it is important to remove the dyes that can harm the environment and living ecosystems in a harmless way, especially biologically (Jo-Shu et al, 1989; Leelakriangsak & Borisut 2012). In the past two decades, biological elimination of the azo dyes is preferred due to their lower cost and does not create toxic products in environments, and it is the most recommended dye removal method by several studies (Silveria et al., 2003; Shah et al. 2013b; Boustanabadimaralan 2014; Sghaier et al., 2019).

Therefore, in this study, the color removal capability of four different psychrophilic/psychrotolerant strains belonging to *Bacillus* genus, which are natural dye removers, was investigated on six azo dyes. As presented in tables 1-4, the decolorization rates of the tested *Bacillus* species varied and the maximum rate was 98%.

*Bacillus cereus*, which was used in this study, has also been used by different researchers in dye removal process (Deng et al., 2008; Liao et al., 2013; Shah et al., 2013b; Madhuri et al., 2018). Deng et al. (2008), isolated *B. cereus* DC11 strain from active sludge left from the treatment of textile printing wastewater and tested it on 13 different azo dyes. The researchers observed that the strain they isolated had a high color-removing effect on all 13 azo dyes. Similarly, in our study, *B. cereus* was tested on removal of the six different azo dyes, and it was found to decolorize the used dyes 8 to 73% in different concentrations in all of these dyes. Likewise, Liao et al. (2013) determined that *B. cereus* HJ-1 strain was able to remove reactive black B azo dye. In another study, Shah et al. (2013b) tested azo dye removal with *B. cereus* and *B. megaterium* and they reported that the biological removal of azo dyes used in textile can be possible. Madhuri et al. (2018) showed that biodegradation of remazol red RB azo dye can be possible with *B. cereus*. The results of all the mentioned studies are consistent with the results of our study. In another similar study, *B. cereus* and *B. subtilis* were shown to remove azo dyes at the rates of 49% and 72%, respectively (Gomaa, 2016).

Leelakriangsak & Borisut (2012) tested *B. subtilis* azo-reductase activity on four different azo dyes and found that this bacterium was effective in azo dye removal by rates between 40-98%. This result is compatible with the current study. Results of this study indicated that the decolorization rate of *B. subtilis* on 6 different azo dyes varies between 8% and 67%. However, the difference in color removal between our study and Leelakriangsak & Borisut (2012) study could be resulted from different incubation temperatures. The researchers incubated their strain at 25 °C while we incubated them at 30 °C. In another study, Kumar et al. (2015) measured the biodegradative capacity of *B. subtilis* on four different azo dyes and showed that it was between 6 and 96%. These results are in accordance with the current study. Shah et al. (2013a) investigated the removal effect of *B. subtilis* ETL-2211 strain on the crystal violet dye and observed that it was effective at 35 °C. Unlike our study, although they kept the incubation temperature higher, they obtained similar results with our study.

In this study, it was determined that *B. mycoides* removed color in different concentrations of six different azo dyes at rates ranging from 3% to 80%. Mahmood et al. (2015) reported that bacteria can be effective in the consortium of six different bacterial species isolated from the textile wastewater zone in the removal of azo dye degradation, all of the four bacterial species that were used in this study are among the consortium. Although bacteria have not been individually tested, 66.7% of the bacteria in the used consortium are the same species that tested in this study. The results of Mahmood et al. (2015) can be considered as compatible with our study. Thakur et al.

(2012) found the presence of *B. mycooides* among the bacteria isolated from the wastewater systems of textile and dye factories. However, to best of our knowledge, spectrophotometric studies on azo dye removal by use of *B. mycooides* alone have not been carried out yet. This increases the importance of this study. Additionally, it was thought that this study will serve as a reference to the dye removal studies by use of *B. mycooides* that will be done in the future.

In the present study, the azo dye removal tests with *Bacillus* sp. suggested that this bacterium achieved a success rate of up to 98% in different concentrations. In contrast, Leelakriangsak & Borisut (2012) achieved negative results in their study in which *Bacillus* sp. tested on two different azo dye removal. The reason for this could be the use of different dyes for testing the bacterium. On the other hand, Guadie et al. (2017) tested *Bacillus* sp. CH12 strain biodecolorization property of on a textile azo dye and noted that *Bacillus* sp. had a high potential of decolorization activity. This result is coherent with the current study. In addition, Dawkar et al. (2010) determined that dye removal can be done within 24 hours by use of *Bacillus* sp. VUS strain. This result coincides with the results obtained from our study. In another study, Jaiswal et al. (2014) concluded that *Bacillus* sp. is effective in removing malachite green and indigo carmine azo dyes as a result of their experimental data, which suggested that the use of *Bacillus* sp. strains could be beneficial in removing azo dye from the environment. The majority of the studies mentioned above are in harmony with this study and show that *Bacillus* sp. has a strong azo dye removal potential.

In conclusion, the results obtained in this study revealed that the psychrotolerant *B. cereus*, *B. subtilis*, *B. mycooides*, and *Bacillus* sp. can be useful in the industry. However, there is a need for molecular and biochemical research involving more advanced techniques to illuminate the mechanisms related to the color removal capabilities of the used four different *Bacillus* spp. used in this study. It was thought that the findings obtained in our study can be taken as a basis for future studies.

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### **Author Contributions**

All author contributions are equal for the preparation research in the manuscript.

### **Data Availability Statement**

The data that support the findings of this study are available on request from the corresponding author.

### **Conflict of Interest**

The authors declare that they have no conflict of interest.

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