Original Article

PPTCC 1015S Strain of *Bacillus cereus* as an Effective Medium for Removing Azo Dyes; Acid Blue 113 and Acid Orange 7

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Abstract

Background: Bio-remediation methods of organic pollutants are promising due to their high efficiency, low cost, and green chemistry. These methods are effective alternatives to traditional elimination methods.

Methods: *Bacillus cereus* PTCC 1015S strain was used to eliminate acid blue 113 (AB113) and acid orange 7 (AO7) from aqueous solutions. The bacteria were activated in the Luria Bertani Broth (MILLER) culture medium. The effective parameters such as incubation time and temperature, pH of dye solution, dye concentration, and the bacterial inoculation quantity on decolorization were investigated. Kinetic and thermodynamic studies were done to clarify the different aspects of the introduced method. Also, the reliability of the method to remove studied dyes was investigated in dye factory wastewater samples.

Results: The first-order kinetic model was the best model to describe the kinetics of decolorization. The obtained thermodynamic data showed that the elimination process was endothermic ($\Delta H^0 = +29.03, 30.53$) while it was spontaneous ($\Delta G^0 < 0$) for both studied dyes and so entropy change ($\Delta S^0 = +109.63, 116.32$) was an effective force in the decolorization. Under optimized conditions, *B. cereus* PTCC 1015S showed high average removal; 98.5 and 99.3 % for AB113 and AO7 respectively in dye model solutions. The removal efficiency was more than 85 % for both studied dyes in wastewater spiked samples.

Conclusions: The *B. cereus* PTCC 1015 strain was capable of being exploited in the removal of AB113 and AO7 dye molecules as an affordable and eco-amiable method for the treatment of aqueous samples contaminated with the studied azo dyes.

Keywords: Dye elimination, Bio-remediation, Bacterial medium, Wastewater

Introduction

Nowadays avoiding the production and use of xenobiotic compounds is an impossible task. The great concern regarding this subject is the uncontrollable and undesirable discharge of these materials into the environment.[1] Almost all commercially available dyes are synthetic compounds, except for some rarely inorganic pigments. It has been estimated that 7×10^7 tons of synthetic dyes are consumed per year in different industries.[2] It has also been reported that dyeing industries' processes commonly lead to the release of 10% to 15% of total used dyes into the environment and these amounts can be reached 50% in some traditional coloring methods.[3] Indeed, in the modern world, we are faced with an environmental problem that can be named dye effluents.[4,5]

The assortment of dye compounds belonging to various classes now exceeds 3000 within the market, with close to half of them classified as azo dye compounds.[6] These particular dyes are characterized by the presence of one or more azo groups (—N=N—) within the molecular structure, which establish connections between organic components, usually including at least one aromatic nucleus.[6] Because of high biological, chemical, and photocatalytic sustainability, and high resistance, azo dyes are widely used in a number of industries including food, cosmetics, paper printing, and textile. Thus, the possibility of releasing the azo dyes into environmental resources is more than the other classes.[7]

Numerous techniques with varying degrees of efficiency have been used for the purification of water contaminated with azo dyes. These methods encompass electrocoagulation,[8] photodegradation,[9] ozonation,[10]

membrane processes,[11] electrochemical oxidation,[12] and adsorption.[13] The main problems of the methods are consuming high energy and producing a great amount of sludge which cause secondary contamination that requires safe disposal.

Recently, there has been a surge in interest regarding biological decolorization due to its environmental adaptability, minimal sludge production, and reduced water consumption. Biological-based decomposition methods can be more efficient and effective alternative to physicochemical methods especially if it produces non-toxic final products.[14] Different organisms including bacteria,[15] actinomyces,[16] fungus,[17] yeasts,[18] and biomass of plants[19,20] have the ability to decolorize various kinds of azo dyes in particular environmental conditions.

Various bacteria strains have been used in the decolorization process of azo dyes. In fact, cleavage of the $-N=N-$ bond of azo dyes is the first step of bacterial degradation. Bacteria spanning distinct trophic groups have demonstrated the ability to facilitate the decolorization of azo dyes under various conditions, including anaerobic (methanogenic), anoxic, and aerobic environments. Various bacterial strains, such as *Pseudomonas*,[21] *Enterobacter*,[22] *Citrobacter*,[23] *Pseudomonas aeruginosa*,[24] and *Shewanella*,[25] have been employed individually for the biodegradation of dye compounds belonging to diverse classes. Moreover, specific Bacillus species, including *Bacillus cereus* KM201428,[26] *Bacillus cereus* BWL1061,[27] *Bacillus megaterium*,[28] and *Bacillus subtilis*,[29] have been harnessed to effectively eliminate assorted azo dyes from wastewater derived from coloring processes.

In this study *Bacillus cereus*, PTCC 1015S strain was introduced as a highly effective biological medium to eliminate and biodegrade of two frequently used azo dyes namely AB113 and AO7 in aqueous solutions. The effective parameters of the decolorization process were studied. Kinetic and thermodynamics of the elimination processes were investigated and the yield of the proposed elimination method at the optimum conditions was determined in the real dye wastewater samples.

Methods
Chemicals and Reagents
AB113 (FW: 681.65, λ_{max}: 558 nm nm) and AO7 (FW: 350.3, λmax: 484 nm) with high degree of purity were obtained from AlvanSabet Co. Hamadan, Iran (Figure 1). The standard strain of *Bacillus cereus* PTCC 1015 was brought from the Collection Center of Iranian Scientific and Industrial Research Organization, Tehran, Iran. Luria Bertani Broth (MILLER) culture medium was supplied from Merck Co. Darmstadt, Germany and used as medium to activate bacteria. HCl 0.1 M and NaOH 0.1 M solutions were obtained from Merck Co, Darmstadt, Germany which was used to adjust the pH of dye solutions. Aqueous stock standard solutions of studied dyes at concentrations 250 mg/L were prepared by the dissolution of the proper amount of dye in deionized and distilled water. Working standard solutions were made daily through serial dilutions of the stock solutions with double distilled water prior to analysis. The concentrations of the dyes were determined at maximum of wavelengths based on relative calibration curve obtained at the optimum conditions. The stock dye solutions were stored in a dark place away from direct light.

Instruments
Incubator shaker (Azma Sanat, 240 ax model, Iran) was applied to shake and maintain dye solutions in given conditions. The samples were weighted using an analytical balance (Citizen, CY 205, China). A hot plate stirrer (Metrohm, Switzerland) was used for stirring. A laboratory centrifuge (Heraeus, Labofuge 400 model, Germany) was applied to enhance the phase separation. The pH estimations were done using pH/Ion meter model-682 (Metrohm, Switzerland) and absorption studies were carried out utilizing Jusco (UV-Visible spectrophotometer model V-670, Japan).

Dyes Removal Procedure
The removal capability of *B. cereus* PTCC 1015 was examined in a batch process to eliminate AB113 and AO7. Taking into consideration the effective parameters on the dye removal efficiency, the studied dyes were eliminated as below conditions:

20 mL of bacterial inoculation was added to the dye solution reaching to 200 mg/L at pH = 7. Then, the solution was shacked under incubator shaking to facilitate the contact of dye molecules with bacterial body for 10 hours at 37 °C. Afterward, bacterial inoculation was separated by centrifuging the dye solution sample. AB113: the pH of dye solution (50 mg/L) was adjusted at 7 in the presence of 15 mL of bacterial inoculation. Next, under incubator shaking the dye solution was shacked for 15 hours at 37 °C. Then, the dye solution was separated from bacterial
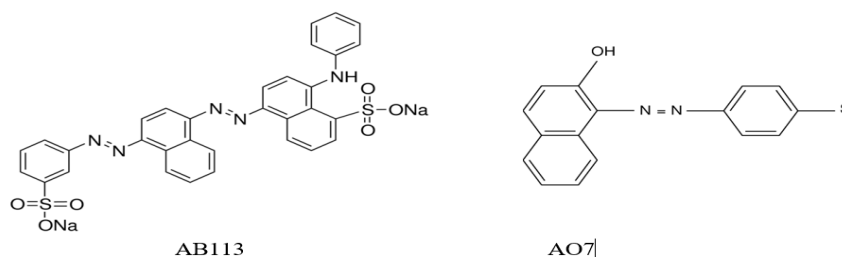

Figure 1. Molecular Structures of AB113 and AO7 Dye Compounds

inoculation by centrifuging. The concentration of studied dyes was determined by using a calibration curve obtained from a UV-Vis spectrophotometer.

The percentage of dye removal was calculated from equation 1:

$$dye\ removal\% = \frac{C_0 - C_t}{C_0} \times 100 \tag{1}$$

where C_0 (mg/L) and C_t (mg/L) are initial dye concentration and the dye concentration at time t respectively.

Results and Discussion

Decolorization in the Absence of the Culture Medium

By introducing *B. cereus* PTCC 1015 in the physiology serum without the presence of the culture medium, the decolorization of AB113 and AO7 at two short and long times (6 and 18 hours) was studied. The results indicated that in the absence of the culture medium, the decolorization did not take place and it did not lead to quantitative removal of the studied dyes. Indeed, the dye solution did not degrade (cleavage of structure) using inactivate *B. cereus* due to a lack of growth in non-nutritious serum solution. Thus, Luria Bertani Broth (MILLER) culture was selected (as the related protocol) for feeding and activation of the bacteria. Figure 2 shows the decolorization by using standard strain in the absence of the culture medium.

Optimizations

The important parameters on the removal of studied dyes were taken into consideration and optimized. For this aim, 10 ml of studied dyes with certain concentration was prepared and various amounts of bacteria inoculation were included in them. Then, dye removal yield was calculated at the different contact times, temperature, dye concentration, bacteria inoculation, and in various pHs of dye solutions.

Effect of Contact Time

The importance of the time factor is obvious in most chemical and biochemical removal processes of pollutants. In fact, as time proceeds, the removal interactions gradually tend to be complete due to the mass transfer effect that mainly determines the kinetic properties of the procedure. Figure 3 shows the effect of time on the elimination efficiency of the used bacteria. The dye removal efficiency was calculated by equation 1 in different incubation times. These results show that at the initial times up to four hours, the percentage of decolorization has not been significant for both dyes. It seems that up to four hours the bacteria population did not gain to adequate quantity to speed up the dye removal process. After that, the decolorization mechanism acted faster than the initial times up to 10 and 15 hours for AO7 and AB113 respectively. After this period, the process reached a steady state, and the corresponding curves did not show a significant increase
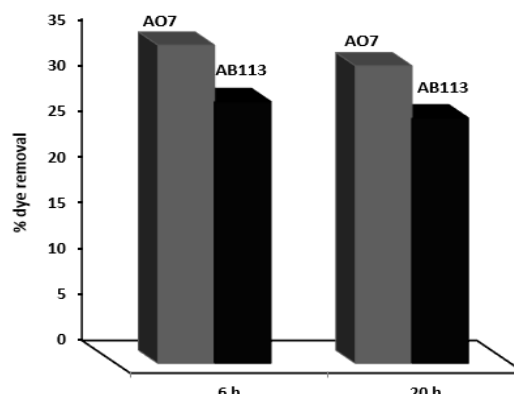

Figure 2. AB113 and AO7 Removal Efficiency of *Bacillus cereus* PTCC 1015 in the Absence of a Cultural Medium
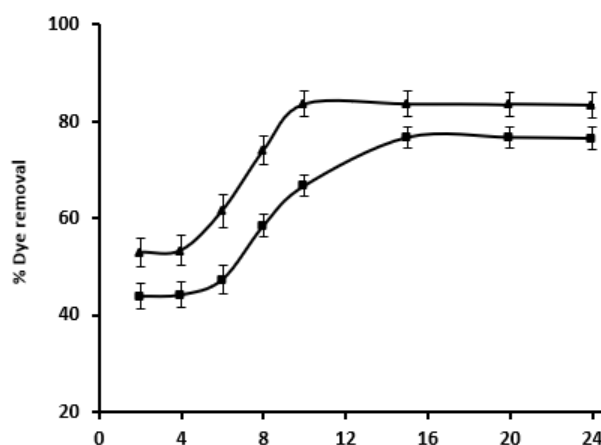

Figure 3. Effect of Exposure Time on the Removal Quantities of AB113 (■) and AO7 (▲). Dyes concentration was 25 mg/L

in removal. Therefore, 10 hours was selected as the optimal decolorization time for AO7 and 15 hours for AB113.

Effect of pH

The molecular structure of AB113 and AO7 includes some functional groups namely sulfonic, hydroxyl, and amine that are surely affected by changing the pH of their solutions. Furthermore, altering the pH can modify the structure of the bacteria used, potentially causing structural degradation at certain pH levels. Therefore, the pH parameter was investigated across a wide range of values (pH 1-13), as it was anticipated to significantly impact dye removal efficiency for the reasons mentioned. The outcomes of this investigation are depicted in Figure 4.

As observed, the removal of the two studied dyes is minimal in an acidic environment. The acidic medium causes protonation (creation of positive charge) of functional groups and –NH, -OH, and sulfonic groups within the dye's molecular structure. With a rise in pH, the removal efficiency increases until a neutral medium is reached; however, in a basic solution, the corresponding curves indicate a decrease in decolorization. These observations underscore the significance of dye molecule surface charges in the decolorization process facilitated by *B. cereus*. Therefore, in further experiments, the pH of the dye solutions was adjusted to pH 7.

Bacterial Inoculation Effect

Figure 5 shows the decolorization of colors in the presence of four different volumes of bacterial inoculation. At the low amounts of bacterial inoculation, the removal processes have not fully been completed due to the need for higher amounts of *B. cereus* to eliminate the number of existing dye molecules. The results showed that most of the decolorization of both colors was increased by increasing the quantity of the bacteria and reached a maximum after 15 and 20 mL for AO7 and AB113 respectively. Thus, in further experiments, the cited inoculation volumes were used.

Incubation Temperature Effect

Another important factor is the incubation temperature. The impact of temperature on decolorization was evaluated in the range of 25-45˚C. The results showed that at 37 °C, *Bacillus cereus* bacteria had the highest decolorization ability for both studied dyes. Figure 6 shows that by increasing incubation temperature, decolorization increased up to 37 °C. At higher temperatures, the dye removal was decreased and it can be attributed to the changes in metabolic reaction rates in bacteria structure. Similar results have been formerly reported.[30]

Dye Concentration

Investigation of dye concentration was necessary to determine the removal capacity of the proposed elimination method. In fact, by increasing the concentration of dyes, the number of dye molecules increases. A high-capacity removal medium can be eliminated at a higher quantity of pollutant molecules, and concentration of the pollutant fully affect the yield of the process. Figure 7 shows the effect of the concentration of AB113 and AO7 on dye removal efficiency. After a given concentration, the efficiency of the removal process has been decreased and the maximum dye concentrations of dyes have been specified. The maximum dye removal of AB113 was determined at 50 mg/L while the same term was to be 200 mg/L for AO7. These results can be related to the more complicated molecular structure of AB113 related to AO7 and as well as its heavier molecular mass.

Kinetic Study

The kinetic study of removal processes can give useful information about the efficiency of the process and the probability of scale-up operation. Zero, first and second-order kinetic models (Table 1) were used to fit the obtained

Table 1. The Used Kinetic Models; Zero, First and Second-Order

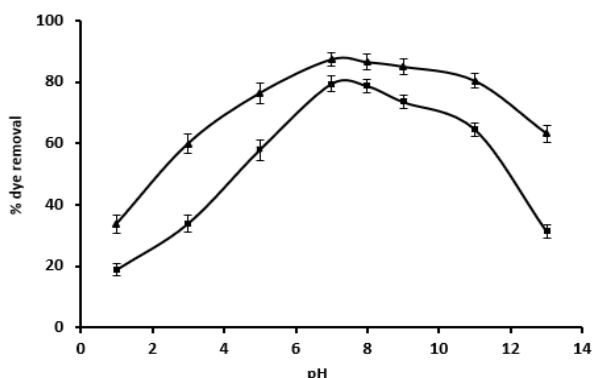| Kinetic Models	Linear Form	Plot	Rate Constant
Zero order	$C_t = C_0 - K_0 t$	(C_t) *vs* time	K_0 (mol/L min)
First order	$Ln(C_t) = ln(C_0) - K_1 t$	lnC_t *vs* time	K_1 (1/min)
Second order	$\frac{1}{C_t} = \frac{1}{C_0} + K2t$	$(1/C_t)$ *vs* time	K_2 (L/mol min)

Figure 4. Effect of pH of Dye Solutions on the Elimination Quantities of AB113 (■) and AO7 (▲). Dyes concentration was 25 mg/L
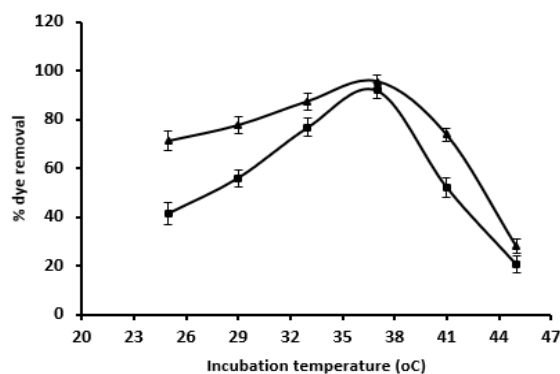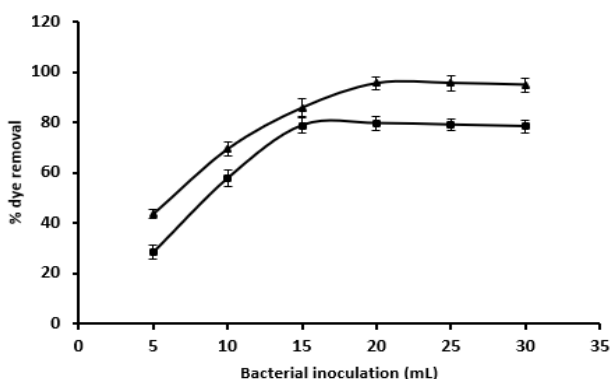

Figure 5. Effect of Bacterial Inoculation on the Removal Efficiency of *Bacillus cereus* for AB113 (■) and AO7 (▲). Dyes concentration was 25 mg/L

Figure 6. Changing of Removal Efficiency by Incubation Temperature for AB113 (■) and AO7 (▲). Dyes concentration was 25 mg/L
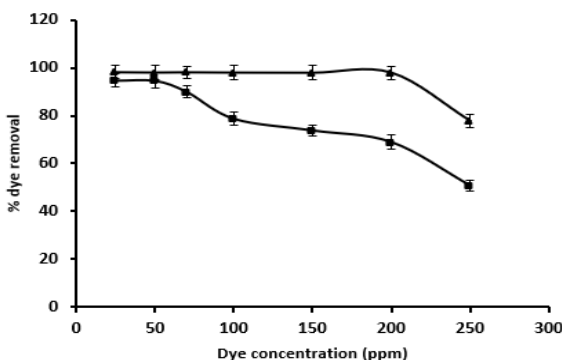

Figure 7. Effect of Dye Concentration on the Removal Efficiency of *Bacillus cereus* for AB113 (■) and AO7 (▲)

Table 2. The Obtained Rate Constants and Correlation Coefficients for Elimination of AB113 and AO7

Kinetic model	Constant	AB113 (mg/L)			AO7 (mg/L)		
		5	15	25	5	15	25
Zero order	K_0 (mg/L min)	0.0794	0.4334	0.8431	0.0823	0.3408	0.5969
	C_0 (cal)	4.83	14.9	26.76	4.18	11.88	20.09
	R^2	0.9920	0.9605	0.9490	0.9815	0.9276	0.9226
First order	K_1 (1/min)	0.0234	0.0651	0.0808	0.0304	0.0599	0.0653
	C_0 (cal)	5.05	19.49	39.25	4.48	14.73	25.79
	R^2	**0.9981**	**0.9957**	**0.9952**	**0.9994**	**0.9969**	**0.9987**
Second order	K_2 (L/mg min)	0.0071	0.0118	0.104	0.0117	0.0124	0.0087
	C_0 (cal)	5.59	53.76	21.73	5.4	26.15	105.2
	R^2	0.9870	0.9284	0.8928	0.9893	0.9716	0.9590

The bold format was used to show the best fit (First order Kinetic model)

decolorization data at different times.

Where C_0 is the initial concentration of the dye in the solution (mg/L) and C_t is the dye concentration in the solution (mg/L) at a given time. Table 2 shows the calculated rate constant of the bacterial removal process and R as the correlation coefficient of the least square method for three applied kinetic models. The obtained data showed that the first-order kinetic model was the best model to describe the kinetics of the dye removal process for both studied dyes (R^2 is more than 0.99). A fine fit of empirical data to the first-order kinetic model revealed that bacterial removal of AB113 and AO7 by the *B. cereus* used strain depended on the initial concentration of studied dyes. This result is in accordance with the reported results in similar studies.[31]

Thermodynamic of the Removal Processes
The thermodynamics of the proposed removal process was studied in an equilibrium condition. The related data were obtained at different temperatures to determine the basic thermodynamic terms including Gibbs free energy change (ΔG^0), enthalpy change (ΔH^0), and entropy change (ΔS^0) of the processes by using relevance equations as follows:

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \quad (2)$$

$$\Delta G^0 = -RTlnk \quad (3)$$

where T is temperature in kelvin, R is gases constant and k is equilibrium constant obtained from a combination of above-mentioned equations:

$$ln\frac{C0 - Ce}{Ce} = \frac{\Delta S^0}{R} + \frac{-\Delta H^0}{RT} \quad (4)$$

where C_e (mg/L) is concentration of dye at equilibrium state. From the plot $ln\frac{C0-Ce}{Ce}$ versus $\frac{1}{T}$ using Eq. 4, enthalpy and entropy changes of the decolorization process were calculated.

The values of thermodynamic parameters of the removing dye procedure are presented in Table 3. The

negative values of ΔG^0 show a favorable and spontaneous procedure for the investigated temperature range. The obtained data show that enthalpy change, ΔH^0, is positive (endothermic) which supports the results in Figure 6. Also, the positive values of ΔS^0 revealed that the procedure of dye removal has been accompanied by random interactions and happens. Furthermore, ΔG^0 values are more negative for AO7 than AB113 which may be related to the more simple structure of AO7 to decompose. Also, high amounts of ΔH^0 are a sign of the existence of a big interaction force like chemical interaction (bonds cleavage) that leads to the elimination of dyes. Altogether, the thermodynamic evaluation showed that the proposed elimination process can be done spontaneously, and produced entropy in the solution is its main promoter force.

The Proposed Method in the Real Sample
To investigate the removal capability of *Bacillus cereus*

Table 3. Thermodynamic Parameters in Removal Process of AB113 and AO7

	T (K)	ΔG^0 (KJ/mol)	ΔH^0 (KJ/mol)	ΔS^0 (J/mol K)
Acid blue 113	298	-3.64		
	302	-4.08	29.03	109.63
	306	-4.52		
	310	-4.95		
Acid orange 7	298	-4.13		
	302	-4.60	30.53	116.32
	306	-5.06		
	310	-5.53		

Table 4. The Removal Efficiency of AB113 and AO7 From Real Wastewater Samples

Dye	Add-Concentration (mg/L)	Concentration removed (mg/L)	Dye removal* %
AO7, AB113	50.0, 25.0	44.2, 21.6	88.4, 86.4
AO7, AB113	100.0, 50.0	86.8, 42.65	86.8, 85.3

*Average of three determinations.

Table 5. Comparison Some Similar Bio-remediation Methods to Eliminate AB113 and AO7

Dye Molecule	Microorganism	Time of Process (h)	Dye Removal %	Reference
AB113	*Citrobacter freundii, Moraxella osloensis, Pseudomonas aeruginosa*	22	90.0	[32]
	Pseudomonas aeruginosa, Bacillus flexus, Staphylococcus lentus and *Fenton oxidation*	-	97.0	[33]
	Biofilm reactor (Isolated microorganism from soil)	20	72.7	[34]
	Bacillus subtilis RMLP2	72	92.7	[35]
	Bacillus cereus PTCC 1015S	15	98.5a,	This study
AO7	Bacillus cereus MTCC 9777	96	68.5	[36]
	Brevibacillus panacihumi ZB1, *Lysinibacillus fusiformis* ZB2, *Enterococcus faecalis* ZL	2	98.0	[37]
	Bacillus sp	48	99.0	[38]
	Bacillus cereus PTCC 1015S	10	99.3	This study

PTCC 1015 in the real sample, a wastewater sample of Alvan CO (dye factory in Hamadan, west of Iran) was tested and spiked with two studied dyes. The wastewater sample was maintained at 4 °C to sediment all suspended particles. Then, the sample was genteelly filtered through filter paper, and different amounts of AB113 and AO7 dyes were added to 10 mL of wastewater solution. In optimum conditions, the removal quantity was calculated. The obtained results are shown in Table 4. As seen, the matrix of wastewater has not been a remarkable effect on the yield of the proposed dye degradation process.

Comparison with Other Studies
Diverse methods have been used to eliminate the dye compounds including AO7 and AB113. Bio-remediation procedures using microorganisms have been reported in literature. Table 5 shows some similar studies in comparison to our proposed method.

The data in Table 5 show that the obtained results through the introduced method for eliminating the studied dyes are better or comparable to the other similar methods, especially in terms of removal efficiency.

Conclusions
In this study, the *B. cereus* PTCC 1015 strain was introduced as a bio-degradation medium with a high potential in eliminating AB113 and AO7 as two frequently used azo dyes in different dye-related industries. The removal efficiency of the proposed method was studied under different experimental conditions including contact time, pH of dye solutions, bacteria inoculation, incubation time, and temperature. The optimum contact time, pH, temperature, bacterial inoculation, and dye concentration were found to be 10 hours, 7, 37 °C, 5 mL, and 200 mg/L respectively for AO7 elimination. The highest elimination of AB113 resulted at the time of 15 hours, pH 7, bacterial inoculation 10 mL, temperature 37 °C, and concentration 50 mg/L. A kinetic study showed that the speed of removal proposed method was dependent on initial dye concentration due to describing of the process by the first-order kinetic model. Also, the thermodynamic investigation revealed that the process was spontaneous and entropy of the removal system had a critical role on its promotion. Also, the efficiency of the used strain of *B. cereus* for eliminating studied dyes was proven in real wastewater samples.

Considering our data, it can be derived that the *B. cereus* PTCC 1015 strain is capable of being exploited in the removal of AB113 and AO7 dye molecules as an affordable and eco-amiable method for the treatment of aqueous samples contaminated with the studied azo dyes.

Authors' Contribution
Conceptualization: Bahram Ebrahimi, Bahareh Rahimian Zarif.
Data curation: Bahram Ebrahimi.
Formal analysis: Pardis Mokri.
Funding acquisition: Bahram Ebrahimi, Pardis Mokri.
Investigation: Pardis MoKri.
Methodology: Bahram Ebrahimi, Bahareh Rahimian Zarif.
Project administration: Bahram Ebrahimi, Bahareh Rahimian Zarif.
Resources: Pardis Mokri.
Software: Sirwan Mohammadiazar.
Supervision: Bahram Ebrahimi, Bahareh Rahimian Zarif.
Validation: Sirwan Mohammadiazar.
Visualization: Pardis Mokri.
Writing–original draft: Bahram Ebrahimi.
Writing–review & editing: Bahram Ebrahimi.

Ethical Approval
Not applicable.

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