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Decolorization of Reactive Blue 19 Dye by *Bacillus megaterium* Isolated from Soil

Reaktif Blue 19 Boyasının Topraktan İzole Edilen *Bacillus megaterium* Tarafından Renginin Giderimi

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ABSTRACT

The principle aim of this study was to decolorize Reactive Blue 19 dye with *Bacillus megaterium* (*B. megaterium*) and to determine the effect of some parameters such as pH, temperature, initial dye concentration, nitrogen and carbon sources on decolorization. With this purpose, a new isolate identified as *B. megaterium* by genotypic (16S rRNA sequence) characterization, was found effective on Reactive Blue 19 decolorization. Glucose (20g/L) was found as the most suitable carbon source for Reactive Blue 19 decolorization with a yield of 92%. When the effect of nitrogen sources on decolorization was investigated, the highest dye removal rate was found as 91% which was obtained in shake flask containing yeast extract (10 g/L). In trials determining pH effect on dye removal; it was found that *B. megaterium* enables decolorization of Reactive Blue 19 at neutral pHs with a high percentage of 91%. P values were calculated for all parameters and found as $p<0.05$. Results of FTIR analysis showed that decolorization of Reactive Blue 19 by *B. megaterium* occurred via biodegradation. When chemical and physical wastewater treatment methods were compared to the biological methods, usage of microbial sources such as *B. megaterium* is considered as an efficient and economical alternative.

Key Words

Reactive Blue 19, *Bacillus megaterium*, decolorization, biodegradation.

ÖZ

Bu çalışma da Reactive Blue 19 boyasının *Bacillus megaterium* (*B. megaterium*) ile renginin giderimi ve pH, sıcaklık, başlangıç boya konsantrasyonu, azot ve karbon kaynağı gibi parametrelerin renk giderimi üzerindeki etkisinin belirlenmesi amaçlanmıştır. Bu amaçla, izolasyonu yapılan ve genotipik olarak (16S rRNA sekansı) *B. megaterium* olarak tanımlanan, izolatın Reactive Blue 19'un renk gideriminde etkili olduğu bulunmuştur. %92 giderim ile glukoz en uygun karbon kaynağı olarak saptanmıştır. Azot kaynağının etkisi araştırıldığında %91 ile en yüksek giderim oranı maya özütünde tespit edilmiştir. Önemli parametrelerden bir olan pH'ın etkisine bakıldığında nötral pH değerinde *B. megaterium*' un Reactive Blue 19 boyasını renk giderimin %91 oranında gerçekleştirildiği belirlenmiştir. FTIR sonuçlarına göre Reactive Blue 19'un renk gideriminin biyodegradasyon ile gerçekleştiği gözlemlenmiştir. Kimyasal ve fiziksel atık su arıtım metodları biyolojik metodlarla karşılaştırıldığında *B. megaterium* gibi mikrobiyal kaynaklar etkili ve ekonomik bir alternatif olarak değerlendirilebilir.

Anahtar Kelimeler

Reactive Blue 19, *Bacillus megaterium*, dekolorizasyon, biyodegradasyon.

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INTRODUCTION

Due to high amount of water used in dyeing processes, the textile industry is the most important generator of liquid pollutants. Also, dyes are used in cosmetics, textile industry, pharmaceuticals and printing. Discharge of untreated dye containing effluents causes environmental problems such dyes show toxic effect on organisms, create aesthetic problems, reduce photosynthetic activity by means of decreasing light penetration [1]. Physico-chemical methods have been used to treat dye bearing effluents, these methods include activated carbon adsorption, advanced oxidation, electrolysis, coagulation, flocculation, etc. All these methods often economically unfeasible and may cause secondary pollution [1–3]. Since dyes show resistant chemical characteristic, the removal of dyes from the environment is very difficult through conventional methods. Therefore, it is not possible to treat these dyes via classical methods due to their stability with resistant azo bonds, resistance to aerobic degradation, heat, and light [4, 5]. Thus, it is seen that reactive dyes are discharged into the environment without being treated up to the ratio of 90 %. These types of dyes cannot be decolorized with chemical and physical methods led up to the idea that biological treatment methods are usable applications in eliminating environmental pollutions. Many conducted studies show that microorganisms are capable of eliminating a variety of synthetic dyes in aerobic and anaerobic conditions via biosorption, bioaccumulation, and biodegradation [6–9]. Conducted studies show that complex organic compounds are degraded by various enzymes such as laccase (EC 1.10.3.2) [10], lignin peroxidase (EC 1.11.1.14) [11], NADP-DCIP reductase(EC 1.6.99.3) [12] and tyrosinase (EC 1.14.18.1) [13]. It is known that some bacteria with their enzymes turn azo dyes into aromatic amines through anaerobic reduction or breakage of azo bonds and they do this via anaerobic degradation. Although azo bonds are resistant to degradation by the bacteria in aerobic conditions, some aerobic bacteria species are reported to break these bonds with azoreductase (EC 1.7.1.6) enzyme which tolerates oxygen [14]. The Reactive Blue 19 (RB-19), has a highly stabilized aromatic anthraquinone structure. Because of these properties, it is very resistant to chemical oxidation [15].

In this study, it was aimed to determine the most effective isolate from the waste environment to provide biodegradation of Reactive Blue 19 dye, used in the textile industry. The effect of different incubation parameters (carbon and nitrogen sources, temperature, pH and dye concentration) and enzymes which are known to play an important role on decolorization was also investigated.

MATERIALS and METHODS

Dye

Reactive Blue 19 dye was obtained from Piko Kimya Ltd. Co. İstanbul/Turkey. The stock dye solution was prepared with the concentration of 50 mg/L and sterilized in an autoclave for 15 minutes at 121°C 1 atm and appropriate amounts were added to the media. After autoclaving, there was no change in the stock dye solution.

Microorganism

Microorganisms used in this study were isolated from soil (Ankara-Mamak region). Identification of isolates based on 16s rRNA sequence analysis was conducted by Bioeksen R&D Technologies Ltd. The stocks of microorganisms were kept in Luria-Bertani/glycerol medium (20 % glycerol) at -20°C.

Decolorization Studies

Decolorizing studies were carried out in 250 mL shake flasks in 100 mL GYP(Glucose-Yeast-Peptone) medium. The composition of GYP medium was peptone 10 g/L, yeast extract 5 g/L, glucose 20 g/L. 1 mL of suspension from 0.5 McFarland turbidity of bacteria in fresh culture passages and 1 mL of Reactive Blue 19 solution were added to sterilized medium and incubated left for incubation for 48 hours at 37°C and 150 rpm. To determine the effect of different carbon sources on decolorization, five different sources such as glucose, lactose, maltose, galactose, and mannitol were assayed. To determine the effect of nitrogen sources on decolorization, ammonium sulfate, ammonium nitrate, urea, peptone, and tryptone were used. Temperature (25-50°C) and pH (4-8) parameters were studied to examine the effect of environmental factors on the removal of the tested pollutant. Provided that all physiological conditions were kept constant, 50-500 mg/L of dyes were analyzed in order to examine the effect of dye concentration. Uninoculated flasks containing Reactive Blue 19 were used as control samples and the flasks, which were used in the series of experiments were prepared triplicate.

Determination of the amount of Reactive Blue 19

After 48 hours of incubation, the culture liquid was centrifuged for 5 minutes at 10285 g at 25°C. The supernatant was compared with control medium via Shimadzu UV-1700® (Japan) spectrophotometer measurement at 557 nm. The percentage of decolorization was measured with this formula: Decolorization (%) = [(ODi-ODf)/ODi]×100, where, ODi: initial absorbance of the dye, ODf: final absorbance of the dye at any time interval.

Determination of Enzyme Activities

The activities of laccase (EC 1.10.3.2), lignin peroxidase (EC 1.11.1.14), manganese peroxidase (EC 1.11.1.13), and tyrosinase (EC 1.14.18.1) were measured by using the supernatant after the bacteria culture was centrifuged. Laccase activity was monitored by spectrophotometrically ABTS(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) as a substrate at 420 nm [16]. Lignin peroxidase (LiP) activity was monitored via spectrophotometrically in veratryl alcohol oxidation with the presence of H2O2 at 310 nm wavelength as Tien and Kirk specified [17]. Manganese peroxidase (MnP) activity was measured via oxidation of 2,6-dimethoxyphenol (DMP). The reaction was started by addition of H2O2 and was monitored spectrophotometrically at 469 nm at 25°C [18]. Tyrosinase activity was measured via oxidation of L-DOPA. Absorbance changes were examined via reading optical density of the reaction against reagent blank at 475nm after the reaction mixture was incubated at 28°C for 15 minutes [19]. One unit of enzyme is defined as 1µmol substrate, the amount of enzyme required to convert the product in 1 min. For measuring the activity of azoreductase which is an intracellular enzyme, culture liquid was centrifuged at 10285 g at 25°C for 15 minutes and the supernatant was removed. Pellets were sonicated 5 times for 15 seconds with 10 seconds intervals in ice by sonicator to provide the disintegration of the cells [4]. After pre-incubation for 4 minutes at 30°C, the reaction was started by addition of NADH. The activity was calculated by tracking the amount of decrease in color density at 482 nm.

FTIR Analysis

After decolorization of Reactive Blue 19, bacteria culture was centrifuged at 10285 g at 25°C for 15 minutes. After centrifugation metabolites were extracted from the supernatant by adding an equal volume of ethyl acetate. Fourier Transform Infrared spectroscopy (Thermo Fisher Scientific, Nicolet iS10, Saltam, MA, USA) was performed by using the rotary vacuum Evaporated sample (extracted to after 24 h decolorization

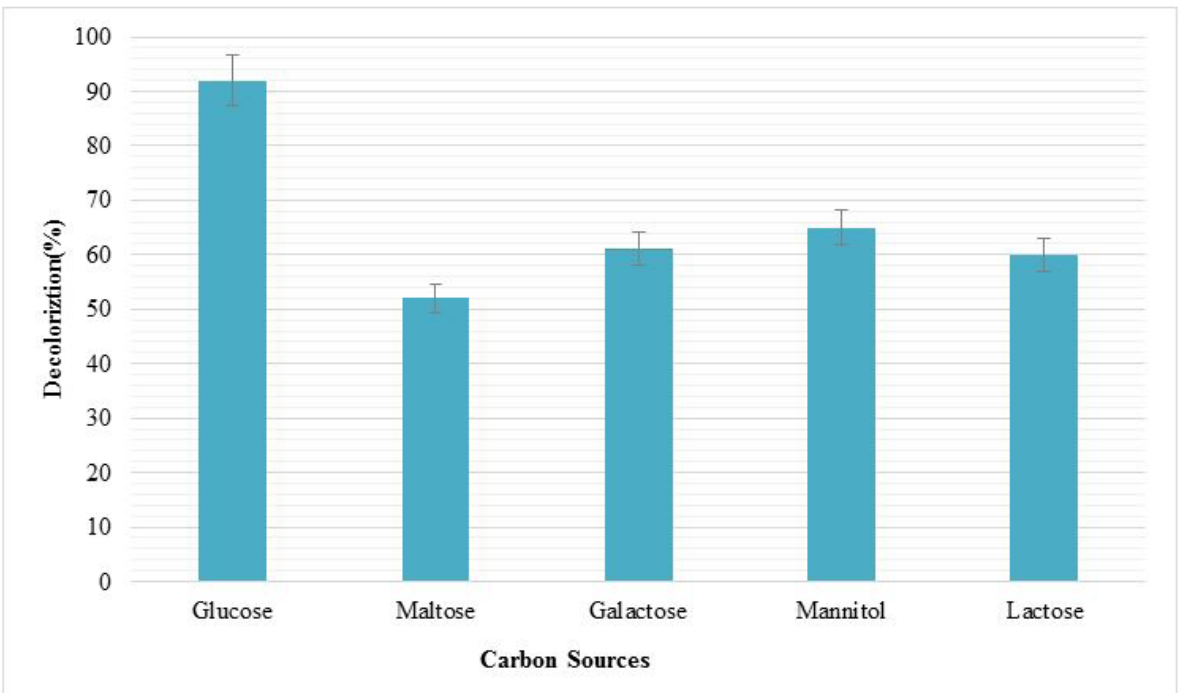

Figure 1. The effect of different carbon sources (20 g/L) on RB19 decolorization after 48 hours of incubation (other physiological conditions were kept constant).

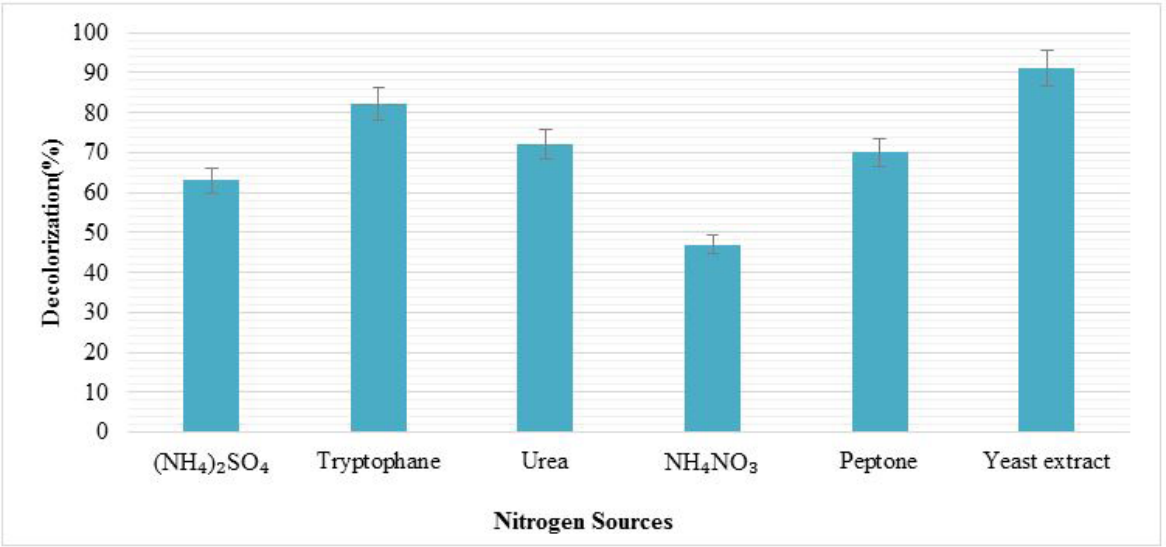

Figure 2. The effects of different nitrogen sources (10 g/L) on RB19 decolorization after 48 hours of incubation (other physiological conditions were kept constant).

period) dissolved in ethyl acetate and compared with that control dye. The FTIR analysis was done in the mid-IR region of 400-4000 cm^{-1} with 16-scan speed.

Statistical Analysis

The results were analyzed using SPSS (Statistical Package for the Social Sciences) version 23.0 and expressed as mean ± standard deviation (SD). The differences between the control and inoculated samples were analyzed using one-way ANOVA and Post Hoc Tests (LSD and Duncan). Differences at $P<0.05$ were considered to be statistically significant.

RESULTS and DISCUSSION

Determination of Microorganism Species

The microorganism isolated was determined as *B. megaterium* (GenBank accession number: KC609020.1) according to the 16S rRNA analysis by Bioeksen R&D Technologies Ltd.

The Effect of Different Carbon and Nitrogen Sources on the Decolorization of RB19 Dye

Nitrogen and carbon sources have a significant effect on dye removal processes, which take place in microorganisms. Since microorganisms have different metabolic characteristics, uptake into cells of these sources leads to a variation of decolorization processes. Referring to the chemical structure of the dyes, they appear to be carbon deficient. Therefore, the biodegradation process is difficult to take place without an extra carbon source [20]. Organic nitrogen sources are required for NADH regeneration [21]. Carbon sources provide energy for the growth of microorganisms and function as an electron donor for disintegration the bonds in the structure of dye [22].

According to the shown in Figure 1, when glucose was used as carbon source, decolorization occurred with a high rate as 92% ($p<0.05$) Besides that, when maltose, galactose, mannitol, and lactose were used, Reactive Blue 19 dye was decolorized up to 60%. Analyzing the effect of nitrogen sources on decolorization, the highest productivity was with the use of yeast extract as seen in Figure 2.

In a study done by Singh et al.(2014) stated that decolorization of Acid Orange dye by *Staphylococcus hominis* RMLRT03 took place when the maximum amount of carbon and nitrogen sources occurred in the environment [22]. In that study, decolorization in the environment with glucose yielded the best rate with the percentage of 89.81%, while the highest yield was obtained with yeast extract as 93.24%. These results were similar to the data found in the current study. When Ayyasamy et al.(2015) investigated the effects of decolorization of Remazol Golden Yellow dye, unlike this study; yielded the highest rate by using starch and lactose [23].

The Effect of pH on Decolorization of RB19 Dye

In a study conducted with *Bacillus sp.* and Reactive Blue 5, Liang et al. reported that the maximum rate of deco-

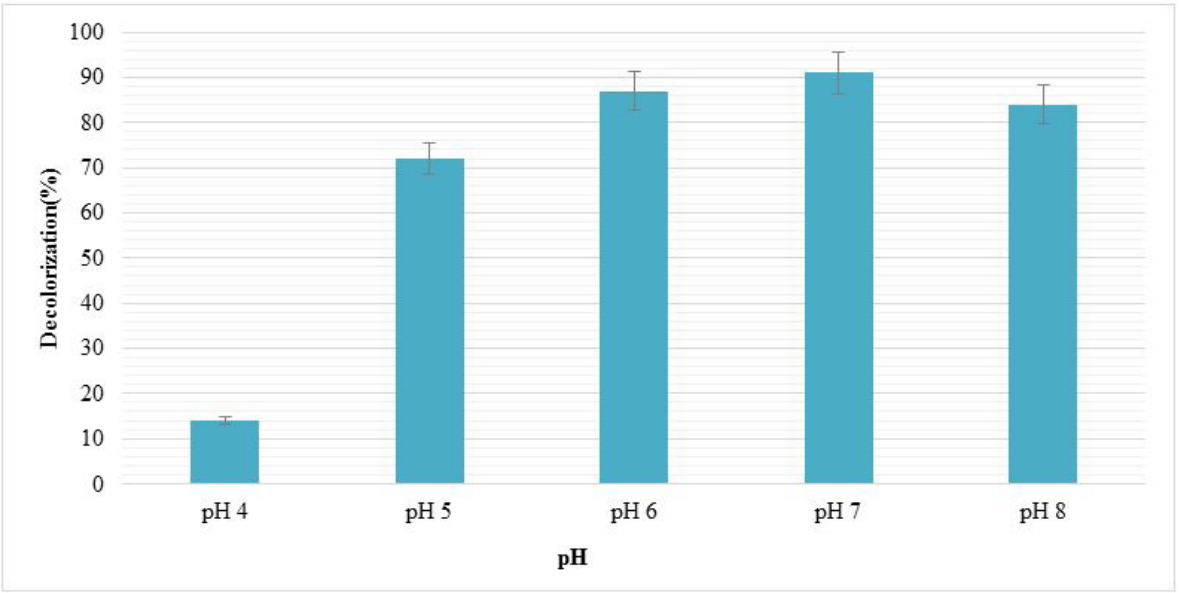

Figure 3. The effect of pH changes on decolorization of Reactive Blue 19 dye after 48-hour incubation (other physiological conditions were kept constant).

lorization as 60% at pH 7 [24]. In another study, Karatay et al. (2015) stated that the optimum pH for Remazol Blue decolorization by *B. megaterium*, *Micrococcus luteus* and *Bacillus pumilus* via Remazol Blue was 7. *B. megaterium* could decolorize Remazol Blue dye with the percentage of 60.8% at pH7; it was stated that Bacillus pumilus optimally decolorized Remazol Blue (76.7%) at pH 7 [25]. The results obtained in that study show similarity with the current study. Mahbub et al. (2015) found the optimum pH as 7 in their decolorization studies performed with *Bacillus* species [26]. As seen in Figure 3, optimum pH value was found as 7 and it was seen that ***B. megaterium*** could decolorize Reactive Blue 19 dye with a yield of 91 % ($p<0.05$). In the light of the acquired results, the decolorization process of Reactive blue 19 dye was higher in neutral pHs. pH is a very significant parameter for the growth of microorganisms and the removal of dyes from the environment. Referring to the literature, it is observed that bacterial decolorization occurs between pH 6 and 10 [27].

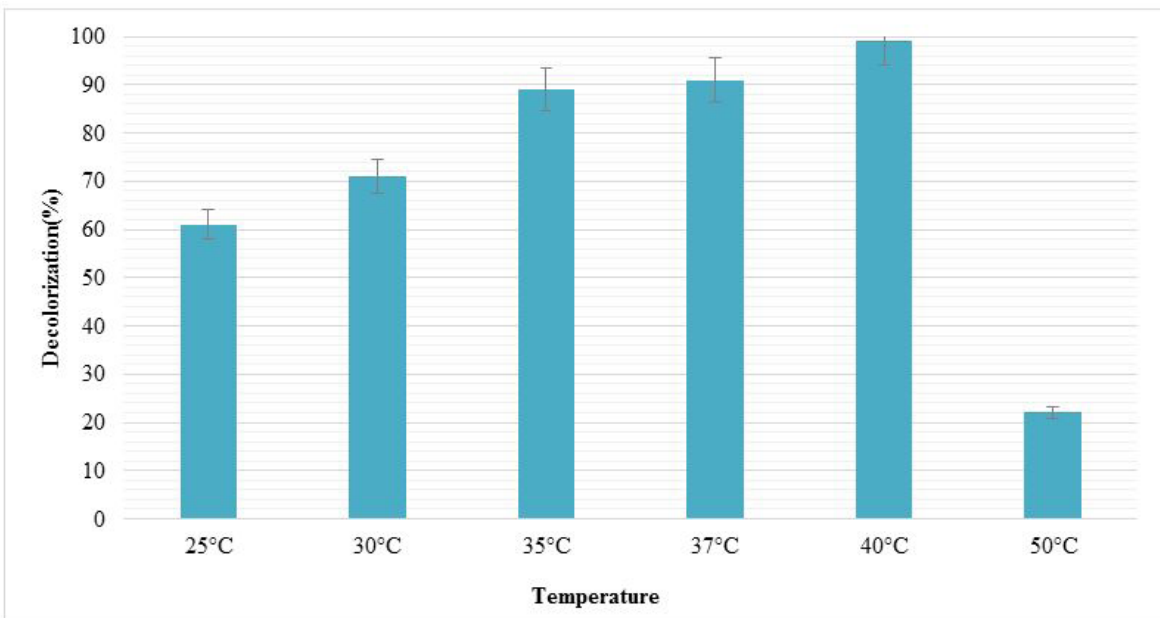

Figure 4. The effect of different temperature values on decolorization of Reactive Blue 19 colorant after 48-hour incubation (other physiological conditions were kept constant).

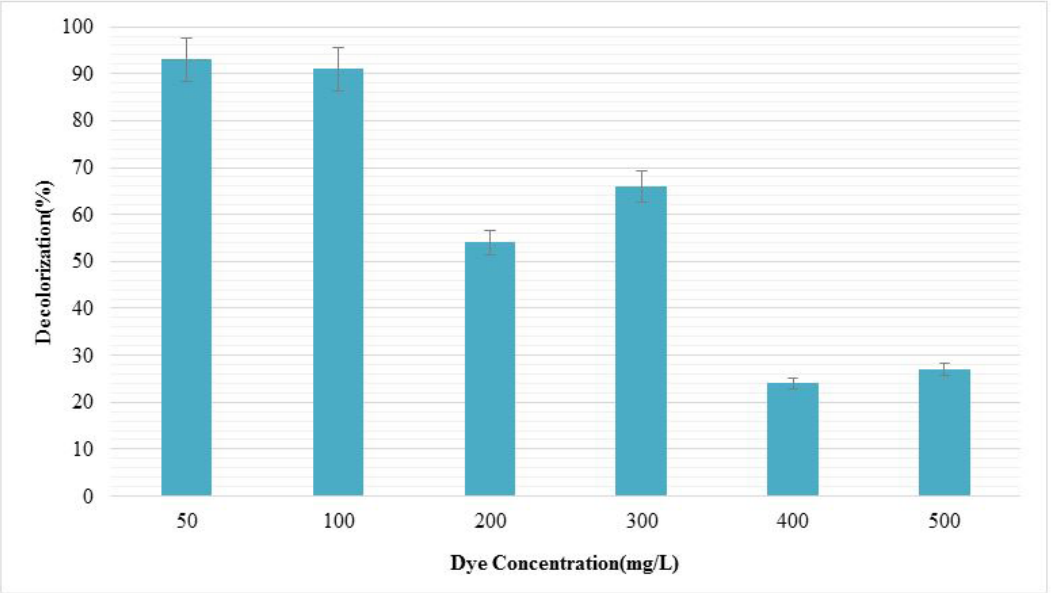

Figure 5. The Effect of Increasing Dye Concentration on Reactive Blue 19 (other physiological conditions were kept constant).

The Effect of Temperature on Decolorization of RB19 Dye

Mahbub et al. (2015) found that the optimum temperature was 35°C in their study for decolorization of Novacron Blue, Novacron Super Black azo dyes by *Bacillus* species in 2015. In that study, it was also stated that with the increase of temperature, the ratio of dye in the medium decreases and decolorization starts to decrease when the temperature is over 40°C [26]. As it is similarly seen in Figure 4, the efficiency of decolorization in *B. megaterium* increased when the temperature increased in the study ($p<0.05$). However, a rapid decrease was observed when the temperature is over 50°C.

The Effect of Different Initial Dye Concentrations on Decolorization of RB 19 Dye

According to the results shown in Figure 5, the decolorization by *B. megaterium* decreased after 100 mg/L concentration. Govindwar et al. (2008) stated that remaining Red BLI concentration suppresses reproduction of *Pseudomonas sp.* SUK1 and reduced the decolorization rate. Decolorization was strongly inhibited in 1250 mg/L dye concentration. Furthermore, as the dye concentration increases, the time required for decolorization is reported to increase proportionately [28].

Karatay et al. (2015) studied dye concentration between 28.7-97.9 mg/L in their study which they conducted with *B. megaterium*, *Bacillus pumilus* and *Micrococcus luteus.* In that study, it was stated that *B. megaterium* could decolorize 50% of the dye when its concentration was less than 97.9 mg/L. This percentage decreased to 31.7% when its concentration was over 97.9 mg/L. *B. pumilus* removed the tested dye in all concentrations at the approximately the same rate. Similarly, the decolorization rate was observed to decrease with *M. luteus* when the dye concentration decreased [25].

Like present study, the conducted studies showed that increasing dye concentration decreased decolorization rate ($p<0.05$). The reason behind, why dye concentration decrease effect on decolorization increasing, that this is thought to be the toxic effect of increasing doses of colorants on microorganism and the blockage of enzymes by dyes [3].

Enzyme Activities

According to results of measurements of the study, laccase, manganese peroxidase, and lignin peroxidase, tyrosinase and azo reductase activities could not be found. Liang et al. (2013) reported that decolorization of Reactive Black 5 dye by *Bacillus sp.* was related with NADH depended on azoreductase [24]. It was indicated at Karatay et al.'s (2015) research that azoreductase enzyme was responsible for the decolorization of Remazol Blue dye by *Bacillus pumilus* and *B.megaterium* [25]. Despite the mentioned study, azoreductase activity was obtained by the method used in the current study. Further studies are needed to be done to clarify

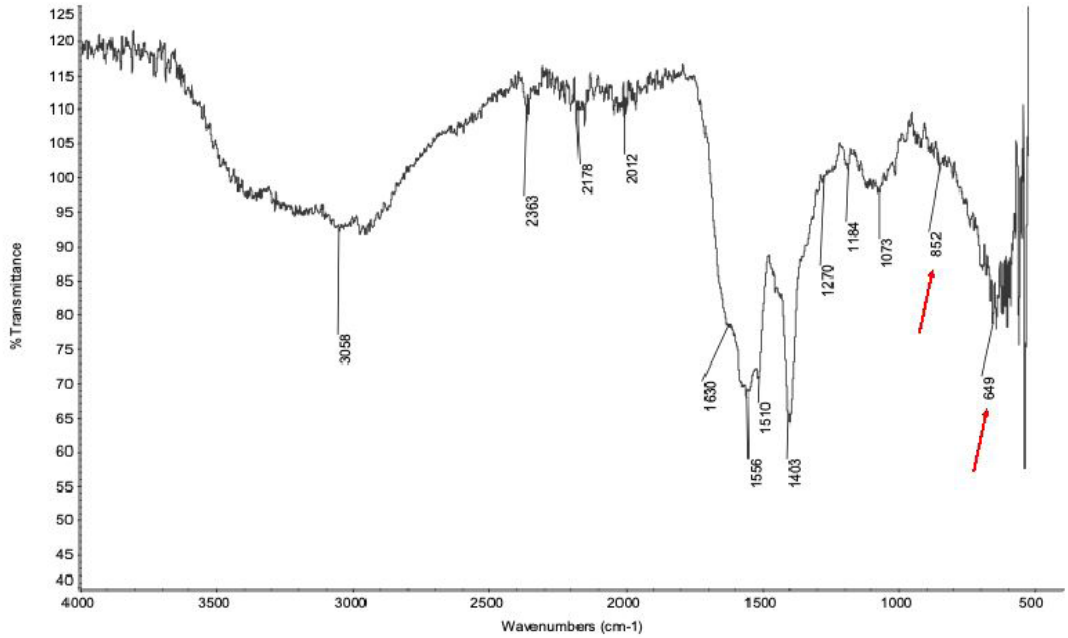

Figure 6. FT-IR spectra of standard dye (The red arrows indicate the aromatic rings).

the biodegradation mechanism of Reactive Blue 19 dye by *B. megaterium*.

FTIR Analysis of Decolorized Product

Results of FTIR analysis of dye control and sample obtained after decolorization showed various peaks. Reactive Blue 19 dye control showed peaks at 3440, 2947, 1603, 1572,1541, 1464, 1390, 1360, 1215, 1172, 1120, 1036, 993, 898, 864,775, 747,689,664 and 606 cm^{-1} (Fig. 6). Whereas the extracts after degradation by *B. megaterium* showed peaks at 3058, 2363, 2178, 2012, 1630, 1556, 1510, 1403, 1270, 1184, 1073, 852 and 649 cm^{-1} (Fig. 7)

The FTIR spectrum of extracted metabolites showed a significant change in positions of peaks when compared to control dye spectrum. Peaks of the wavenumber between 670 and 870 cm^{-1} at control dye samples correspond with aromatic rings. In *B. megaterium* extracted metabolites, aromatic rings had disappeared which means that the degradation of dye molecule is successful. The peak around 3440 cm^{-1}, which is represented to N-H vibration and 2947 cm^{-1} which is assigned to C-H stretching disappeared after the decolorization of Reactive Blue 19. As stated in the Ayed et al.'s study [29], the removal of dyes from aqueous solution can be due to adsorption to microbial cell or biodegradation. If the dye removal is based on biodegradation, a few absorbance peaks will disappear or a new peak will appear. According to current study, results showed that the mechanism of RB19 dye's removal by *B. megaterium* is related to biodegradation.

CONCLUSION

Chemical and physical methods used for the treatment of textile wastewaters can be costly, also cause environmental problems due to some chemical compounds formed after the treatment. However, treatment via biological methods is low-cost and environmentally friendly. In the present study, it was determined that the newly isolated *B. megaterium*, in optimum conditions, could decolorize Reactive Blue 19 dye used in textile industry. There are few studies about dye removal by *B. megaterium*. In these studies, *B. megaterium* had lower removal rate than found in the current study. In studies that are seen to be rare, the ones with the microbial consortium are also done. In these studies, which are made with more than one microorganism, including B.megaterium, the decolorization percentage rates are lower than the ones that have achieved in present study. These results, which are obtained and compared with the literature, add novelty to conducted study. This microorganism, which provides highly efficient decolorization is considered to be a good candidate for the treatment of textile industrial wastewater.

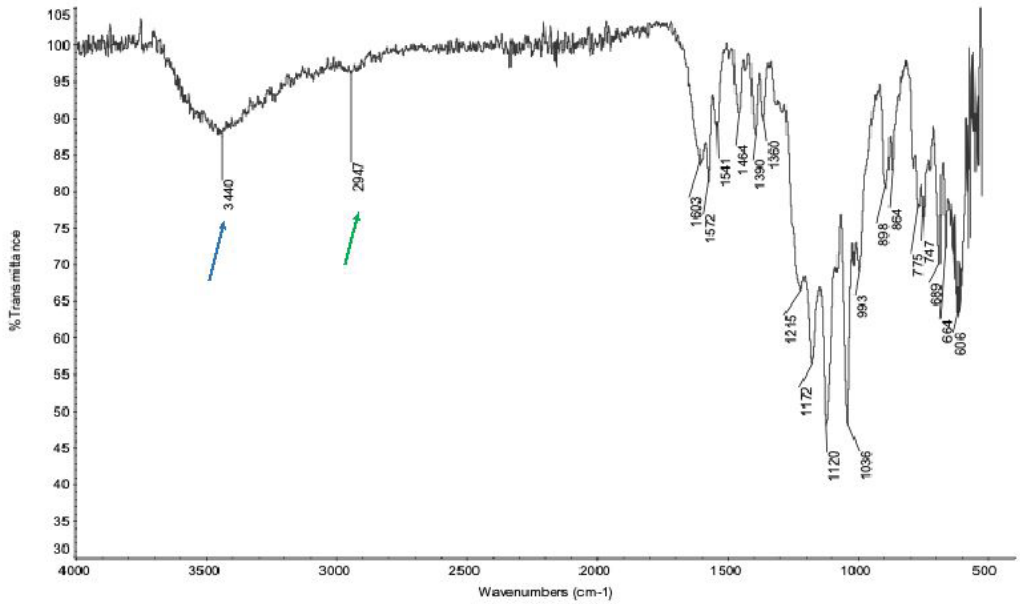

Figure 7. FT-IR spectra of dye degraded by *B. megaterium* (The blue arrow indicates the N-H vibration, a green arrow indicates the C-H stretching disappeared after the decolorization).

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CONFLICT of INTEREST

The authors have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript. On behalf of all authors, the corresponding author states that there is no conflict of interest.

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