RESEARCH ARTICLE

Study the Ability of the Newly Isolated Thermophilic Bacterium (*Geobacillus thermoleovorans*) Strain Ir1 (JQ912239) in Decolonization and Degradation of Azo Dyes (Methylene Blue and Acid Orange G)

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ABSTRACT

The synthetic dyes whose molecules contain two adjacent nitrogen atoms between carbon atoms are remarkable in the industrial area, called azo dyes, capable of resisting degradation and accumulating in different environmental habitats at high concentrations. Biodegradation of these dyes by bacterial groups is an eco-friendly method to eliminate these dyes from industrial effluents. *Geobacillus thermoleovorans* strain Ir1 (JQ912239) was used to test its ability to degrade methylene blue and acid orange G. Primarily, and the bacterium was grown on an LB medium containing 250 mg/L of the dyes. The optimum dye concentration, temperature, and pH were determined. Results showed that this bacterium was capable of decolorizing the dyes entirely within 5 days. Optimum conditions for degradation were 400 mg/L of dye concentration at 55°C and pH7 for methylene blue and 200 mg/L of dye concentration at 55°C and pH7 for acid orange G. This strain is capable of decolonization of these dyes within few days under optimum conditions.

Keywords: Azo dyes, Degradation, *Geobacillus thermoleovorans,* Synthetic dyes,

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INTRODUCTION

Commonly, the colored substance generally applied in aqueous solutions because of their great affinity to water is Dyes. The color contribution happens primarily by a chromophore group available in its chemical structure and is widely important in the textile, paper, leather, or food industry. Usually, petroleum by-products and earth minerals are used to prepare various synthetic dyes like reactive dyes, azo dyes, etc., in the textile industry due to their high solubility in water.[1,2]

According to hydrophobicity, there are 2 types of azo dyes: (i) hydrophobic azo dyes, which are taken up and reduced inside the cell, and (ii) hydrophilic ones, which are reduced outside the bacterial cell. And different azo dyes are widely available for commercial use. Due to the non-fluorescent nature of azo dyes, fluorescent probes are used to track its pathway attached with the azo dye by the alkyl bond. In textile azo dyes, synthetic dye, reactive dye, acid dye, sulfur dye, basic dye, oxidation dye, anthraquinone dye, acridine dye, and many other different colorants are used. The main reasons why different types of azo dyes are used in the dying process are their different usage purposes, viz. cellulosic fiber, protein fiber, and synthetic fiber. As all the dyes do not get fixed to the fiber when dying, some unfixed dyes are released through effluent, causing pollution.[3] Though the use of dye has been an integral part of the socio-economic component since 2000 BC, the use of synthetic dye becomes predominant only after the industrial revolution. It becomes an essential part of textile industries, producing 900,000 metric tons of dyes produced annually.[4,5]

The *Geobacillus* species are characterized by the rod, Gram-positive cells, aerobic or facultatively anaerobic, spore-forming, capable of surviving and growing at high temperatures, and the genus contains nearly twenty species that are excessively heterogeneous and request reclassification. Due to the genus power to employ and to produce a wide range of substances, in addition to high temperatures resistance, they are believed as perfect agents for many biological processes. They represent a source of an extensive array of thermostable enzymes: amylases, lipases, pectinases, β-galactosidases, endonucleases, etc. Applications of bacteria and their enzymes range from the food industry and medicine to molecular biology and bioremediation. New studies concerning *Geobacillus* properties and fields of use are carried out every year.[6]

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G. thermoleovorans strain Ir1 was isolated from oil-contaminated soil in Iraq with high capability to employ the aromatic compounds.[7] This work was to test this bacterium's ability to decolorize and degrade methylene blue and acid orange G and use the UV spectrophotometer and HPLC analysis to emphasize this capability under optimum conditions.

MATERIALS AND METHODS

Dyes and Chemicals

Chemicals and solvents used in all experiments were analytical grades; the textile dyes were obtained from Himedia, India.

The tested microorganism

G. thermoleovorans strain Ir1 (JQ912239) was used; it is a novel strain capable of utilizing different aromatic compounds. It was isolated in a previous study from oil-contaminated soil in Iraq.[7]

Culture of *G. thermoleovorans* Strain Ir1 (JQ912239)

G. thermoleovorans strain Ir1 (JQ912239) was preserved within a silica gel. To revive the culture, 0.1 mg of bacterium isolate was added into 500 mL of LB medium provided with 0.1 mL $MnCl_2$ in a conical flask, and the culture was incubated in a shaker incubator at 65°C and 150 rpm for one to two days. After the incubation period, a loopful culture was streaked on LB broth and incubated at 65°C for one day. A single colony was picked up with a sterile loop, transferred into LB broth flasks, and incubated at the same conditions. The bacterium was stained with Gram +ve stain, and the purity was checked by microscopic examination.

Decolorization Assay of the Azo Dyes

To study the bacterium's capability to utilize the dyes, one hundred milliliters of Luria-Bertani (LB) medium were dispensed in Erlenmeyer flasks (250 mL). This medium contains 250 mg/L of the dye (methylene blue, acid orange G) and sterilized by autoclaving. Subsequently, it inoculated with 1% of fresh bacterial culture and incubated under static conditions (65°C, pH 7) for 10 days. Control was made by inoculating flasks with the bacterium only. Every day, aliquots (5 mL) of the culture media were withdrawn, centrifuged at 10,000 rpm for 10 minutes at room temperature to separate the bacterial cell mass. For analysis of decolorization, the supernatant was used, and all the experiments were repeated in triplicates. The supernatant's absorption was measured at wavelength range for methylene blue (190–800) nm and (190–600) nm for acid orange G in a spectrophotometer. The supernatant was used for the analysis of decolorization. The percentage of decolorization (D%) was studied by the following:

Decolorization% = (Initial absorbance value - final absorbance value) / Initial absorbance value ×100.

Optimization of Decolorization conditions by *G. thermoleovorans* Strain Ir1 (JQ912239)

PH

The capability of *G. thermoleovorans* strain Ir1 (JQ912239) to degrade methylene blue and acid orange G at different Ph values (5, 6, 7, 8, 9, and 10) was determined supplementing LB broth flasks with 250 mg/L of the dyes. Then, cultures were incubated under static conditions (65°C) for three days. The suitable pH value was used later.

Temperature

To confirm the temperature of the bacterium to decolorize methylene blue and acid orange G, LB broth flasks supplemented with 250 mg/L of the dye were incubated under static conditions at (40, 45, 50, 55, 60, and 65)°C for three days. Then the optimal temperature was employed.

Concentration

To deduct the optimum concentration, the dyes were added at different concentrations (0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1) g/L. pH was adjusted to 7.0 and incubated under static conditions at 55°C for three days, and the optimal concentration was used.

Extraction

The inoculated flasks were extracted by using a separating funnel in the presence of a solvent (ethyl acetate), and one mL of culture supernatant was taken from cultures growing with methylene blue and others with acid orange G and extracted with 3 mL of ethyl acetate. Finally, the ethyl acetate solvent was evaporated, and the residue was dissolved in 1 mL ethanol.

UV- spectro assay

The samples were measured in ethanol with a Perkin Elmer double beam UV-Vis Spectrophotometer 117. Quartz cells (1 cm square) having 1.0 cm path length were used for the determination. Hydrogen discharge tungsten filament lamp was used as a light source, and maximum absorbance was recorded, and the assay was done at the Ministry of Industry and Minerals.

High-performance Liquid Chromatography (HPLC) Analysis

Luria-Bertani (LB) medium (100 mL) containing 400 mg/L of methylene blue and 200 mg/L of acid orange G were inoculated with the bacterium and incubated at 55°C, pH 7.0 three days under static conditions. After incubation, the bacterial cells were harvested by centrifugation at 13000 rpm for 10 minutes at 4°C. HPLC analyzed the resulting cell-free supernatant for degradation products at Ministry of Industry and Minerals. The mobile phase consisted of 0.1% formic acid: acetonitrile 60:40% (v/v), and the analyses were performed on (Shimadzu LC- 10 AVP binary delivery. Pump. Monitor by LC-10A UV-Vis spectrophotometer Japan, Icoyota) system; UV detection at 250 nm and the flow rate were 1 mL/min in C18 column (50×4.6 mmvd, 3mm particles size).

RESULTS AND DISCUSSION

Decolorization Assay of Azo Dyes

Experiments were completed by the static condition at 65 °C for 10 days to test the ability of the bacterium to decolorize methylene blue and acid orange G, e. The results appeared that this strain could decolorize both dyes, yielding 99 % decolorization, after 8 days of incubation, as shown in Figure 1.

Bacillus sp. can decolorize textile azo dyes (Acid red 2 and Acid orange 7), isolated aerobically, from the dye contaminated soil of the local dying houses in Nagpur (India).[8] In contrast, the newly isolated bacterial culture *Bacillus megaterium* ITBHU01 can decolorize the textile dye orange G with a decolorization efficiency of 95%. It suggests its application for decolorization dye-bearing industrial wastewaters.[9]

Optimization of Decolorization Conditions

Effect of pH

Luria-Bertani (LB) at different pH values (5, 6, 7, 8, and 9) was prepared to determine the suitable pH for decolorization. Figure 2 showed that the optimum pH for 99% of decolorization was 7. These findings agree with the studies in which high decolorization of methyl red was achieved by *Micrococcus* strain R3 in pH range of 6–8,[10] and the decolorization of Acid Orange dye by *Staphylococcus hominis* RMLRT03 strain was found in the pH range of 6–8.[11] Also, the neutral pH would be more favorable for color removal of the azo dyes and is suitable for industrial applications.[12]

Effect of Temperature

The bacterium was grown and incubated at (40, 45, 50, 55, 60, and 65) °C. Figure 3(a) shown that the optimum temperature for decolorization was achieved at 55°C after three days of incubation. It was found that the decolorization of the dye, acid orange G were studied with a temperature range of 20 to 50 °C, and the optimum temperature for *Pseudomonas putida* MTCC 102 was 37°C.[9] At the same time, the studies of bacterial consortium JW-2 showed a maximum 93% decolorization of Reactive Violet 5R at 37°C.[13]

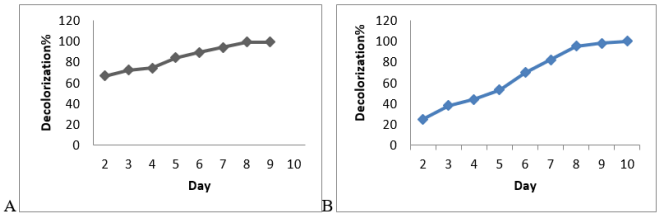

Figure 1: Decolorization percentage of azo dyes (250 mg/L) by *G. thermoleovorans* strain Ir1 (JQ912239) at 65°C for 10 days, under static condition, (λ max =480 nm). A): Methylene blue, (B): Acid Orange G.

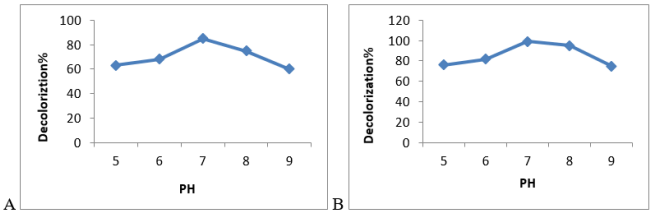

Figure 2: Effect of pH on decolorization percentage of azo dyes (250 mg/L) by *G. thermoleovorans* strain Ir1 (JQ912239) at 65°C for 3 days, under static condition, (λ max =480 nm). (A): Methylene blue, (B): Acid Orange G**.**

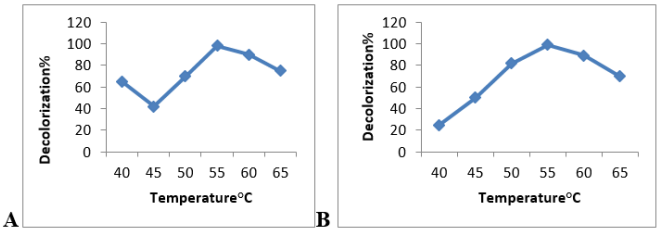

Figure 3(a): Effect of temperature on decolorization percentage of azo dyes (250 mg/L) by *G. thermoleovorans* strain Ir1 (JQ912239), under static condition, (λ max =480 nm). for 3 days and ph 7. (A): Methylene blue at 55°C (B): Acid Orange G at 55 °C.

Effect of Concentration

Different concentrations (0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1) g/L of both dyes were used to confirm the optimum concentration. Results in Figure 3(b) showed that the optimum concentrations for bacterial growth were 400 mg/L and 200 mg/L, in which the percentage of decolonization was 98% and 99% after three days of incubation for both methylene blue and acid orange G, respectively. In another study, it was concluded that the decolorizing dye potential of *Pseudomonas putida* MTCC 102 was quite high. It decolorizes the dye with better efficiency even at high concentrations of acid Orange G.[14-16] The use of biological methods by microorganisms in the removal of industrial dyes are widely considered due to their high efficiency and compatibility to the environment,[17] and by increasing methylene blue concentration, dye biodegradation efficiency of *Pseudomonas aeruginosa* gets increased (50–200 mg/L). A literature survey suggests that increasing the dye concentration gradually decreases the decolorization rate, probably due to the toxic effect of dyes on the individual bacteria and/or inadequate biomass concentration and blockage of active sites azoreductase by dye molecules with different structures.[18]

UV Spectrophotometry Assay

UV spectrophotometry assay of the azo dyes before and after decolorization are shown in Figure 4. Results show the high ability of the bacterium to decolorize the toxic dyes. The UV spectrophotometry analysis indicated that methylene blue showed 98% decolonization and maximum absorbance at 209.00 nm before degradation and 261.00 nm after degradation. At the same time, acid orange G showed as much as 95% decolonization (degradation) and showed maximum absorbance at 264.50 nm before degradation and 388.00 nm. According to[19]they found that *Pseudomonas fluorescens* can degrade Direct Orange – 102, and the UV Spectroscopy before degradation showed maximum absorbance at 246.9 and 275.1 nm. While After degradation, the maximum absorbance was obtained at 247 nm and 276.7 nm.

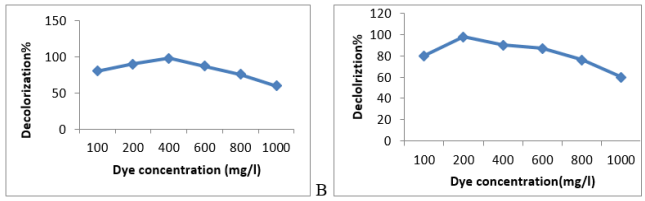

Figure 3(b): Effect of dyes concentrations on decolorization percentage by *G. thermoleovorans* strain Ir1 (JQ912239), under static condition, (λ max =480 nm). ph 7 and 55°C for 3 days. (A): Methylene blue, (B): Acid Orange G.

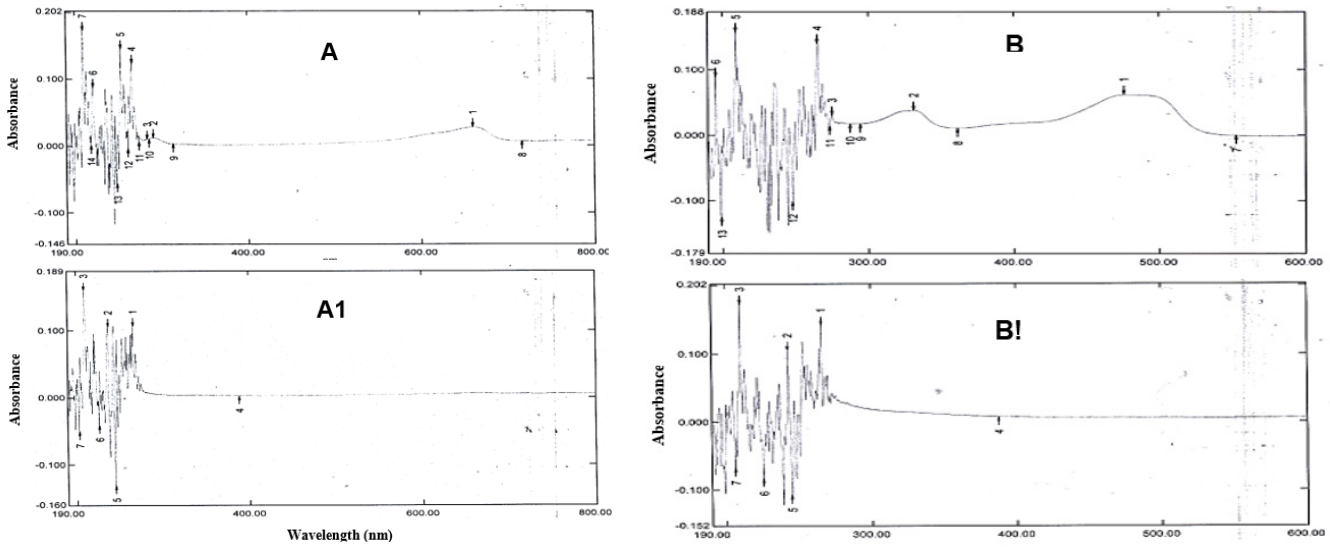

Figure 4: UV spectrum before and after degradation by *G. thermoleovorans* strain Ir1 (JQ912239), under static condition. (A): Methylene blue before degradation, (A1): Methylene blue after degradation at 55 °C for 3 days, (B): Acid orange G before degradation, (B1): Acid orange G after degradation at 55°C for 3 days.

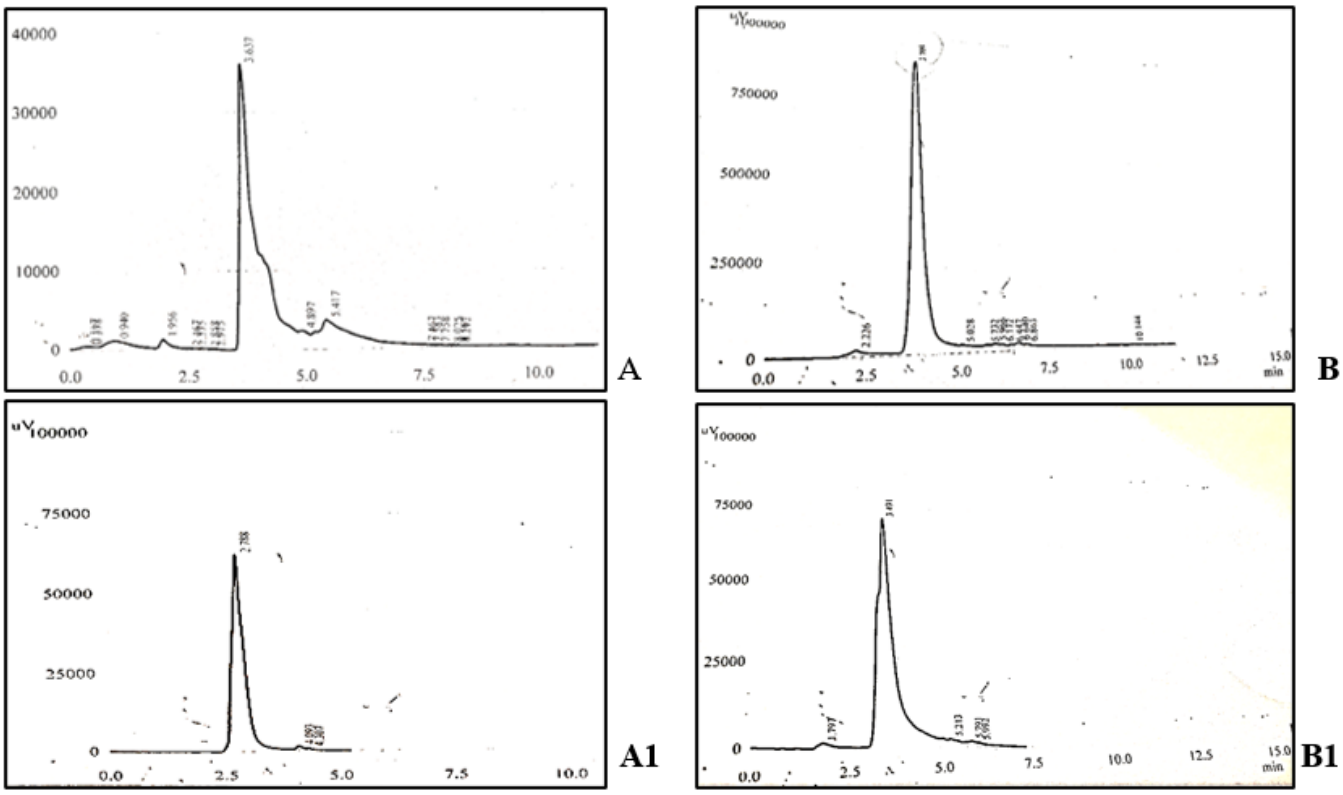

Figure 5: HPLC analysis of azo dyes before and after degradation by *G. thermoleovorans* strain Ir1 (JQ912239), for 3 days, under static condition. (A): Methylene blue before degradation, (A1): Methylene blue after degradation, (B): Acid orange G before degradation, (B1): Acid orange G after degradation with RT 3.6 and 3 respectively.

High-Performance Liquid Chromatography (HPLC)

The analysis of the standards and the supernatant of the culture treated with *G. thermoleovorans* strain Ir1 (JQ912239) appears in Figure 5. The bacterium showed high ability for the decolorization of azo dyes. The HPLC analysis indicated that the methylene blue showed as much as 98% consumption depending on the area of peaks eluted at 3.6 minutes. The HPLC analysis of the acid orange G showed 96% consumption depending on the area of peaks eluted at 3 min.

CONCLUSION

It can be concluded that the bacterial isolate *G. thermoleovorans* strain Ir1 (JQ912239) was able to degrade azo dyes (methylene blue and acid orange G). The optimum conditions for

degradation of these dyes are pH:7, 55°C and 400 mg/L for methylene blue, and pH:7, 55°C and 200 mg/L for acid orange G. This bacterium could be used in treating wastewater contaminated with these dye. Further studies could be done on the degradation of other azo dyes and the determination of genes and enzymes responsible for the degradation.

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