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Decolorization of a Textile Azo Dye, Novacron Red SB, by Naturally Occurring Bacteria

Dipankar Dewanjee [1], Jannatul Ferdousi [1], Tasneem Chowdhury [1], Sawkat Ara Pinki [2], Md. Imranul Hoq [1,*]

1. Department of Microbiology, University of Chittagong, Chittagong - 4331, Bangladesh; E-Mails: dipankar.dewanjee@gmail.com; ferdousi.mbio@std.cu.ac.bd; tasneem.mbio@cu.ac.bd; imran.mbio@cu.ac.bd
2. Flinders University, Australia; E-Mail: pink0048@flinders.edu.au

* **Correspondence:** Md. Imranul Hoq; E-Mail: imran.mbio@cu.ac.bd

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Abstract

Bangladesh faces devastating surface and groundwater pollution due to the discharge of untreated or poorly treated wastewater streams from textile industries. These effluents contain highly toxic azo dyes and numerous other chemicals that pose serious risks to public health. The biological treatment, based on the activity of naturally occurring bacteria, has exhibited enormous potential as an alternative method of decolorizing azo dyes besides the conventional physicochemical treatment. This study aimed to isolate natural bacteria from industrial wastewater outlets and assess their efficiency in decolorizing the azo dye Novacron Red SB. Initially, we selected five efficient isolates, which we further screened, and finally selected three isolates as strong dye decolorizers. We also conducted a comprehensive investigation to characterize the isolates morphologically, culturally, physiologically, and biochemically, which identified the organisms as *Bacillus megaterium*, *Klebsiella aerogenes*, and *Corynebacterium rathyi*. We have also configured the optimal conditions for maximum dye decolorization by varying dye concentration of 0.005-0.010%, exposure time of 24-72 h,

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and co-substrates such as glucose, peptone, and ammonium acetate, applying a wide temperature range of 25-45°C and a pH range of 4-9. Optimization studies exhibited distinct decolorization profiles: *B. megaterium* achieved maximum decolorization of 79% at pH 6.5, *K. aerogenes* exhibited 75% decolorization at pH 8, while *C. rathyi* showed 67% removal at pH 7 and all at 37°C. Notably, all the bacteria reduced the dye better at 0.005% dye concentration after 72 h of incubation in the presence of ammonium acetate as a co-substrate at 37°C and pH 6.5-8. The outcomes of this study thus indicate a high potential of natural bacteria in treating textile dye effluents.

Keywords
Textile dye; decolorization; biological treatment; optimization

1. Introduction

Environmental pollution is a critical global challenge, and uncontrolled industrialization without proper effluent treatment over the past few decades has worsened the situation in Bangladesh [1]. Textile industries, along with a few others, represent a cornerstone of the country's economy [2]. Every day, millions of liters of wastewater containing diverse inorganic and organic chemical compounds are released from numerous textile dyeing, washing and printing mills into public drains that ultimately flow into rivers. Processing of 1 kilogram of textile material in conventional finishing operations typically requires close to 100 liters of water [3, 4]. It is estimated that more than 10,000 different dyes and pigments are used in industries, with global annual production of synthetic dyes exceeding 7×10^5 tons [5]. In Bangladesh, a major share of dye waste is discharged into the environment, leading to alterations in chemical oxygen demand (COD), biological oxygen demand (BOD), and pH. Such severe pollution is largely linked to synthetic dyes' diverse use. Azo dyes dominate synthetic dye production, representing roughly 60-70% of all dye classes. The complex structures of synthetic dyes make them resistant to biodegradation [6]. These dyes persist in the environment for extended periods due to their complex structures. However, not all dyes fully adhere to fabrics during dyeing, leading to 10-50% of unused dye being discharged directly into wastewater due to process inefficiencies [7]. Furthermore, groundwater may be contaminated from leaching of metals. It is noteworthy that certain dyes exhibit high toxicity and mutagenic properties while reducing light transmission, thereby impairing photosynthetic processes, and the consequent oxygen deficit compromises the water's suitability for drinking, recreation, and crop irrigation [8]. Many synthetic azo dyes and their degradation intermediates are toxic, and in some cases these compounds are carcinogenic to humans and other animals [9]. Taking these consequences into consideration, the industries should properly treat their effluents before releasing them into the environment. To treat these effluents, several primary, secondary, and tertiary treatment processes like flocculation, trickling filters and electrodialysis are used, but unfortunately these treatment methods are not very effective at removing all those dyes and toxic chemicals [10]. Textile effluent treatment employs physical-chemical methods for dye removal and toxic material mineralization. But these approaches applied for the treatment of dye-containing wastewater have high operating costs and produce undesirable byproducts [11]. Similarly, physicochemical techniques used to

remove heavy metals cause secondary contamination, sludge production, and additional expenses [12]. In contrast, biological processes have received increasing interest as a viable alternative due to their cost-effectiveness and eco-friendly nature [13]. Microorganisms, such as bacteria and algae, in particular, can detoxify a wide range of industrial pollutants that include heavy metals and dyes [14, 15]. Naturally occurring bacteria, such as *Proteus mirabilis* and *Pseudomonas luteola* have shown promising results for azo dye degradation under anoxic conditions of less than 0.5 mg/L dissolved oxygen [13]. Different trophic groups of bacteria can achieve a higher degree of degradation and even complete mineralization of dyes under optimum conditions [16]. Bacterial breakdown of azo dyes initiates with enzymatic reduction and azo bond cleavage under static or anaerobic conditions, generating colorless but toxic aromatic amines which subsequently undergo multistep aerobic or anaerobic bioconversion into simpler, non-toxic compounds. Our research group explored the tremendous potential of naturally occurring bacteria in reducing toxic hexavalent chromium (Cr-VI) to the less toxic trivalent form (Cr-III) from tannery effluent [17]. Recently, we demonstrated the potential of native bacterial strains in the decolorization of textile azo dyes [18].

This study focuses on isolating and identifying bacterial strains with the ability to decolorize Novacron Red SB, an azo dye that is more widely used locally and that may require different optimal conditions than other dyes. Furthermore, we examined how different physical parameters and nutrient conditions influenced the bacterial strain's ability to decolorize the dye.

1.1 Novelty Statement

Hussain et al. [19] provided a foundation for research on Novacron Red SB decolorization, but our study advances research on it in several ways. We used dye concentrations that are typical of actual textile effluent. We also isolated bacteria with different decolorization profiles. We performed a more precise pH optimization (4-9 at 0.5 intervals) to determine the true optimum. Interestingly, we found ammonium acetate to be the most effective co-substrate, which differs from the earlier finding of peptone. We also used statistical analysis to validate all comparisons. Finally, we tested our optimized conditions in real textile effluent, which was not performed previously. Together, these improvements offer a more comprehensive foundation for using these bacteria in wastewater treatment.

2. Materials and Methods

2.1 Collection of Samples

Effluent samples were collected from different locations of the drain of Clifton Apparels Ltd. (Composite), 179, Baizid Bostami Road, Nasirabad Industrial Area, Chattogram, Bangladesh, in sterile glass bottles. To maintain sample integrity, each sample was placed in an insulated box with frozen refrigerant packs immediately after collection and preserved at 4°C upon arrival in the lab.

2.2 Isolation of Bacteria

Bacterial strains were isolated by repeatedly streaking the collected samples onto nutrient agar (Hi-Media, India) plates. Distinct colonies were selected for isolation based on colony morphology. Each distinct colony was carefully picked up and transferred onto a nutrient agar slant. Slants

containing different colonies were prepared, sealed in a polythene bag, and stored at 4°C for subsequent analysis. To maintain the isolates' characteristics, subculturing was performed at 3-week intervals.

2.3 Observation of Dye Decolorizing Ability

Bacterial isolates were inoculated into Minimal Salt Medium (MSM) supplemented with 0.005% Novacron Red SB dye [20]. For comparison purposes, a negative control was prepared by adding Novacron Red SB azo dye to minimal salt medium (MSM) without inoculating bacterial isolates. These experiments were carried out in the dark to eliminate any potential photodegradation of the dye. Both the tested and control samples were incubated at 37°C for 72 h under shaking conditions. After incubation, dye decolorization in tested samples was assessed visually by comparison with the negative control (Figure 1d). To reduce bias, two independent observers assessed decolorization, and only isolates showing clear color reduction were selected. The reduction in color intensity was considered as the ability of the strain to decolorize Novacron Red SB, which was preserved for further analysis.

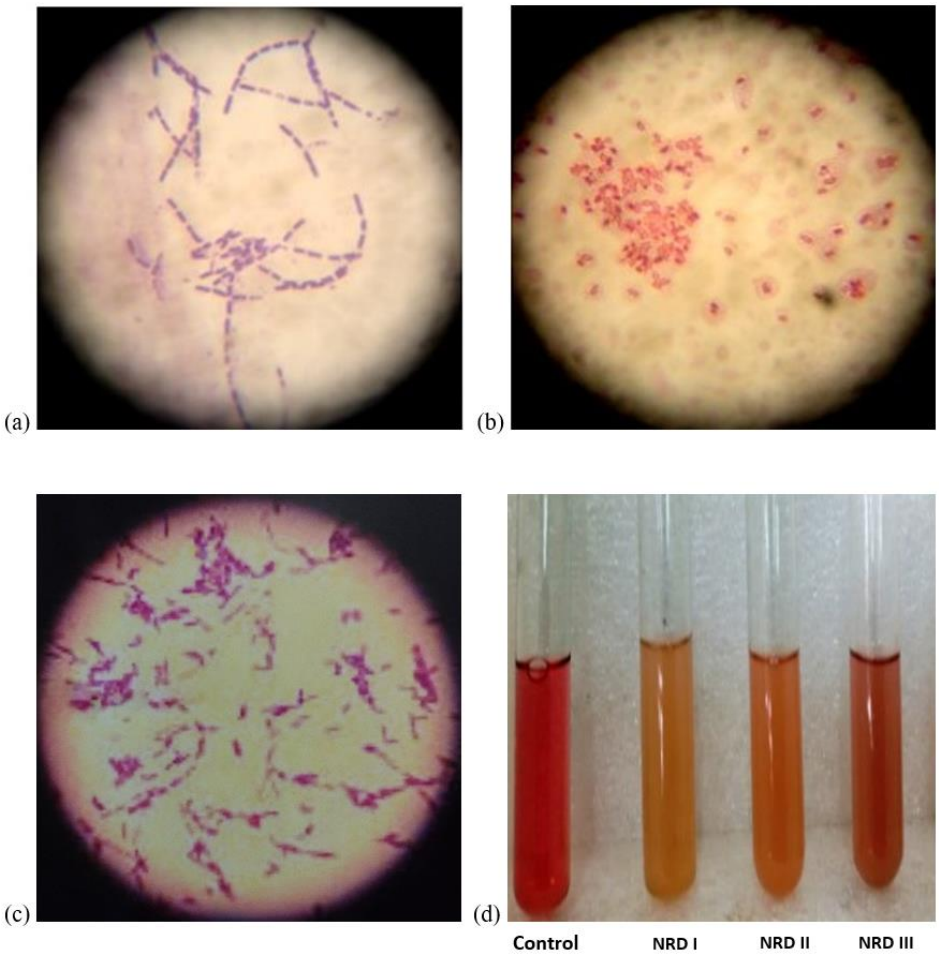


Figure 1 Microscopic images of vegetative cells following Gram-staining of bacterial isolates NRD I, II, III from textile dye effluent are shown in (a), (b), and (c), respectively, where Gram-staining was performed by consequent exposure of a fixed film with crystal violet solution for 30 sec, Lugol's iodine for 30 sec, 95% ethanol for 20 sec and safranin solution for 10 sec. (d) Visualization of dye decolorization by the respective isolates.

2.4 Identification of Selected Isolates

We selected isolates showing significant dye decolorization for identification. Cultural properties, microscopic examinations and conventional biochemical tests i.e. motility test, Indole test, Voges-Proskauer test, methyl-red test, Simmons' citrate test, nitrate reduction test, catalase test, starch hydrolysis test, gelatin hydrolysis test, oxidase test, urease test, triple sugar iron test, and hydrogen sulfide production test, were performed. All microscopic, physiological and biochemical observations were compared with standard descriptions from Bergey's *Manual of Systematic Bacteriology*, 2nd edition (2001-2012) [21] and the bacterial isolates were presumptively identified up to species level.

2.5 Assessment of Dye Decolorization

The bacterial isolates were cultured in MSM broth (pH 7) and incubated until mid-logarithmic phase. The inocula were adjusted to optical densities at 625 nm (OD_{625}) of 0.002 for Gram-positive strains and 0.008 for Gram-negative strains, as measured using a UV-Visible spectrophotometer (Shimadzu UV-VIS 1800, Japan). OD_{625} was used as a relative turbidity measure; viable cell numbers were not independently calibrated.

We used these OD values consistently across all experiments. A 0.015% solution of Novacron Red SB (prepared in distilled water) was centrifuged at 6000 rpm for 10 min for the determination of the maximum wavelength (λ_{max}) of the dye to remove any undissolved dye particles or suspended impurities. The OD of the dye was measured in the range of 400-700 nm using a UV-Visible spectrophotometer (PG Instruments - UK, Model: T 80+ UV-VIS), and its maximum absorbance (λ_{max}) was determined at 510 nm. The dye solution of 0.0003-0.01% at 2-fold dilution was diluted in MSM. After centrifugation, the OD of each dilution was measured at 510 nm, which was identified as λ_{max} of the dye. Novacron Red SB decolorizing efficiency of the isolates was calculated from the values of OD at 510 nm and presented as percent (%) decolorization = $(A_i - A_t) \times 100/A_i$. Here, A_i and A_t are the OD at initial and treated time, respectively.

2.6 Optimization of Dye Decolorization

To determine the optimal conditions for Novacron Red SB decolorization, various physicochemical parameters were studied. These physicochemical parameters are co-substrate, temperature, pH, time, and dye concentration. To identify the best-fitting parameters, we conducted several pilot experiments. All experiments were performed under shaking conditions at 120 rpm using a 5% (v/v) inoculum of a 24-hour-old bacterial culture. For example, 5 mL of culture was added to 95 mL MSM, and the same 5% (v/v) ratio was maintained for other working volumes. A control was run to examine any abiotic degradation. Each experiment was conducted in triplicate.

0.005% (50 mg/L) and 0.010% (100 mg/L) Novacron Red SB dye concentrations were applied to determine the condition that provided maximum decolorization. Other environmental factors were kept constant, i.e., the medium's pH was set at 7 and the temperature at 37°C. We repeated the experiments at 24 h intervals to determine the maximum decolorization.

To assess the effect of carbon-rich co-substrates on bacterial decolorization, glucose, ammonium acetate and peptone at a specific concentration (4 g/L) were added separately in MSM medium with 0.005% (50 mg/L) dye concentration and 72 h incubation period. These co-substrates were selected

based on their frequent use in the literature and the objective was to compare their effectiveness in supporting decolorization by the isolates. Controls (MSM medium with co-substrate but without dye) were used to zero the spectrophotometer before measuring each sample.

The medium was tested at pH 4-9 at 0.5-unit intervals, with a 0.005% dye concentration and 4 g/L co-substrate concentration (ammonium acetate) and a 72 h exposure time. We used controls (medium with dye, no bacteria) at each pH tested. We accounted for any absorbance reduction observed in the controls.

Different temperatures i.e. 25°C, 37°C and 45°C, were used to determine the optimum temperature and 0.005% dye concentration, 4 g/L co-substrate concentration (ammonium acetate), and pH 7 of the medium, and 72 h exposure time were applied.

2.7 Confirmation of Optimized Condition

Decolorization efficiency of the dye by bacterial isolates was examined to assess the potential of the optimized condition to work in real textile effluent under laboratory conditions. The experiment was conducted by adding 4 g/L ammonium acetate as a co-substrate to the effluent, adjusting the pH, sterilizing the effluent by filtration through a sterile 0.22 µm filter and then co-incubating with the bacterial isolates for an incubation time 72 h at 37°C. In contrast, another textile effluent sample was taken and sterilized without pH adjustment or the addition of any co-substrate. This sample was then incubated with bacterial isolates at 25°C (effluent temperature) for 72 h, which was considered an unoptimized condition. The dye decolorizing ability of the bacterial strains under optimized conditions was evaluated by measuring the absorbance reduction at 510 nm using spectrophotometry.

2.8 Statistical Analysis

All experiments were performed with three independent biological replicates per treatment and time point (n = 3). Data are presented as mean values; error bars represent standard deviation (SD). Statistical analyses were performed using GraphPad Prism version 11. We inspected distribution and variance graphically and observed no obvious violations. For the time-course experiments, separate two-way ANOVAs were performed for each bacterial strain to evaluate the effects of conditions and incubation time (24, 48, and 72 h) and their interaction. The treatment factors were: pH (4.0-9.0) for the pH experiments; dye concentration (50 mg/L, 100 mg/L) for the dye experiments; temperature (25°C, 37°C, and 45°C) for the temperature experiments; co-substrates (glucose, peptone, ammonium acetate) for the co-substrate experiments. For the optimized vs. unoptimized comparison, the factors were condition (optimized vs. unoptimized) and bacterial strain (*B. megaterium*, *K. aerogenes*, and *C. rathyi*). For the pH data at 72 h, we also performed separate one-way ANOVAs for each strain to compare pH levels, followed by Tukey's HSD test. Following a significant ANOVA result ($p < 0.05$), Tukey's HSD test was used for all pairwise multiple comparisons. We set statistical significance at $p < 0.05$. Different letters above the bars denote statistically significant differences between experimental groups, while shared letters denote no statistically significant differences.

3. Results

3.1 Physical Characterization of Textile Effluent

Initial analysis of the textile effluent samples showed a high load of pollution markers. The samples exhibited pH values ranging from mildly acidic to neutral. The effluent temperature measured around 25°C. Other physicochemical properties were recorded (Table 1).

Table 1 Physicochemical properties of textile dye effluent.

Property	Value
pH	7.2
Temperature (°C)	25
COD (mg/L)	1100
BOD (mg/L)	420
TDS (mg/L)	1310

Note: COD, chemical oxygen demand; BOD, biological oxygen demand; TDS, total dissolved solids.

3.2 Isolation and Identification of Dye Decolorizing Bacteria

This study utilized Novacron Red SB dye-containing effluent from Clifton Apparels Ltd. (Composite) of Chattogram city as a source of naturally occurring bacteria. The repeated streak plate method was applied until an identical single colony appeared. Five distinct bacterial strains were selected based on their colony properties.

We investigated the dye-decolorizing ability of the isolates in MSM. A 0.005% concentration of Novacron Red SB dye was added to the medium, with pH maintained at 7.2 (equivalent to the effluent pH). The inoculated medium was incubated for 72 h at 37°C, and the reduction in color intensity was observed by naked eye. To reduce bias, two independent observers assessed decolorization, and only isolates showing clear color reduction were selected. Among the 5 isolates, NRD I, NRD II, and NRD III exhibited clear signs of decolorization at 72 h and were selected for further investigation.

The microscopic, cultural, physiological and biochemical characteristics of the selected strains were observed (Table 2, Table 3 and Table 4) and compared with the standard descriptions of Bergey's *Manual of Systematic Bacteriology*, 2nd edition (2001-2012) [21], and the organisms were presumptively identified as *Bacillus megaterium* (NRD I), *Klebsiella aerogenes* (NRD II), and *Corynebacterium rathyi* (NRD III). Figure 1 presents their microscopic views.

Table 2 Microscopic observations of Novacron Red SB decolorizers.

Isolate	Vegetative Cell		Staining Properties		
	Shape	Arrangement	Gram	Acid fast	Spore
NRD I	Rod	Single or in short chain	+	-	+
NRD II	Rod	Single	-	-	-
NRD III	Short rod	Single or in pair	+	-	-

+, positive; -, negative; NRD I indicates *B. megaterium*; NRD II indicates *K. aerogenes*; NRD III indicates *C. rathyi*.

Table 3 Colony morphology and growth patterns of Novacron Red SB decolorizers on nutrient agar plate and slant.

Isolate	Form	Color	Elevation	Margin	Surface	Slant Character
NRD I	Irregular	Creamy-white	Convex	Entire	Smooth	Filiform
NRD II	Irregular	Yellow	Raised	Undulate	Rough	Beaded
NRD III	Irregular	Yellow	Flat	Entire	Smooth	Spreading

Table 4 Physiological and Biochemical characteristics of Novacron Red SB decolorizers.

Name of the Test	Behavior of Isolates		
	NRD I	NRD II	NRD III
Motility	-	+	-
Indole	-	-	-
Voges-Proskauer	+	+	+
Methyl-red	-	-	-
Simmons' Citrate	+	+	-
Nitrate Reduction	-	-	+
Catalase	+	+	-
Oxidase	-	-	-
Urease	-	-	-
Triple Sugar Iron	a/nc	k/a	k/nc
H2S Production	+	-	+

+, positive; -, negative; a/nc, acidic slant, no change; k/a, alkaline slant, acidic butt; k/nc; alkaline slant, no change.

3.3 Influence of Environmental Parameters on Dye Decolorization

The decolorization of Novacron Red SB was investigated against a number of parameters e.g., dye concentration, time, co-substrate, pH and incubation temperature.

Figure 2 shows that decolorization efficiency was significantly higher at 0.005% than at 0.010% dye concentration after 72 h. However, post-hoc analysis (Two-way ANOVA followed by Tukey's test) revealed that this concentration-dependent effect was significant only for *B. megaterium* and *K. aerogenes* ($p < 0.05$), and not for *C. rathyi* ($p > 0.05$).

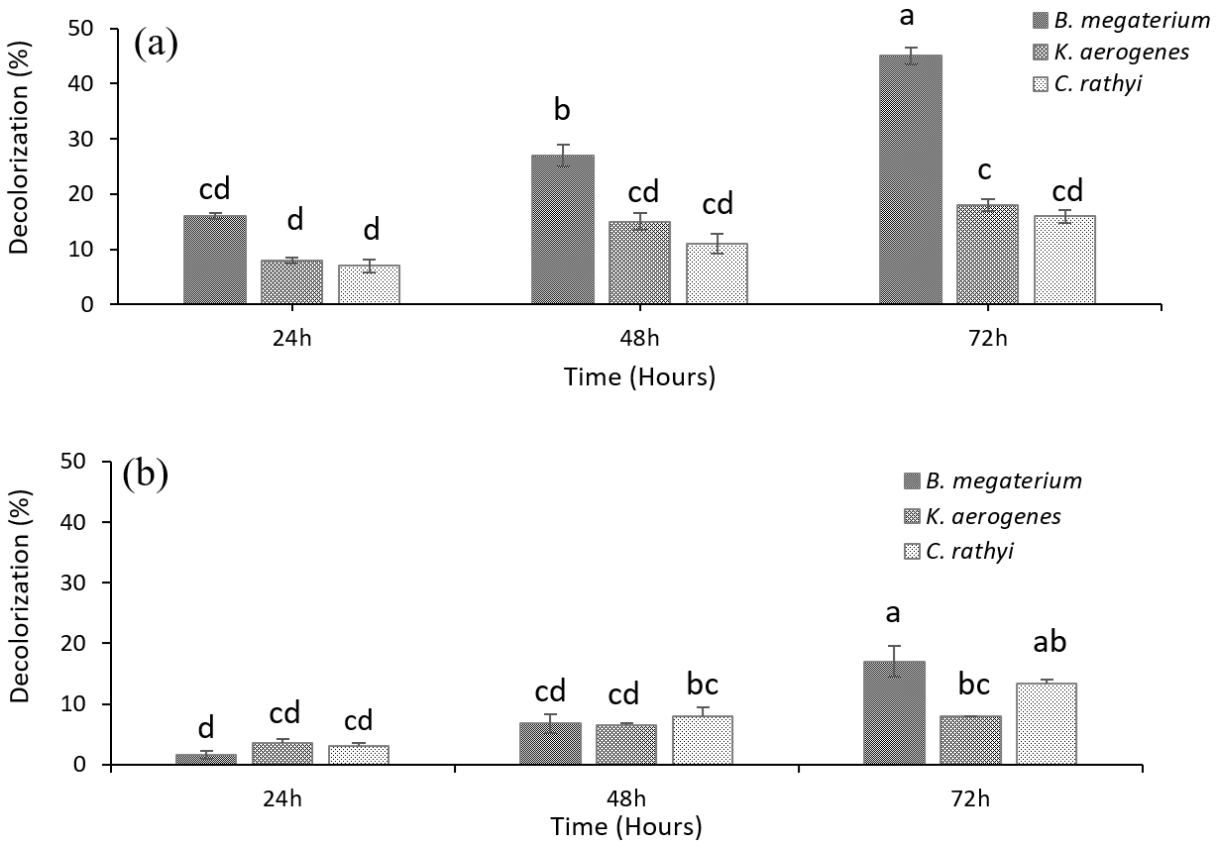


Figure 2 Effect of Novacron Red SB concentration on its decolorization with time by isolates from textile dye effluent. The experiment was conducted by inoculating MSM broth, pH 7 supplemented with 0.005% (a), 0.010% (b) of Novacron Red SB, incubating at 37°C, and measuring the absorbance at 510 nm in a UV-VIS spectrophotometer at 24 h interval. Here, different letters above the bars denote statistically significant differences between experimental groups, while shared letters denote no statistically significant differences (Two-way ANOVA followed by Tukey's HSD post-hoc test, $p < 0.05$).

In our study, *B. megaterium* consistently showed high decolorization across most conditions tested. All of them decolorized the 0.010% dye solution to a lesser extent. In this study, the highest dye decolorization was observed after 72 h.

Among the co-substrates tested for efficient dye decolorization, the type of co-substrate had a significant overall effect ($F(2,72) = 360.1$, $p < 0.001$, $\eta^2 = 0.437$). Post-hoc comparisonsshowed that ammonium acetate was a significantly better co-substrate than both glucose ($p < 0.001$) and peptone ($p < 0.001$) (Figure 3). There was no statistically significant difference between glucose and peptone.

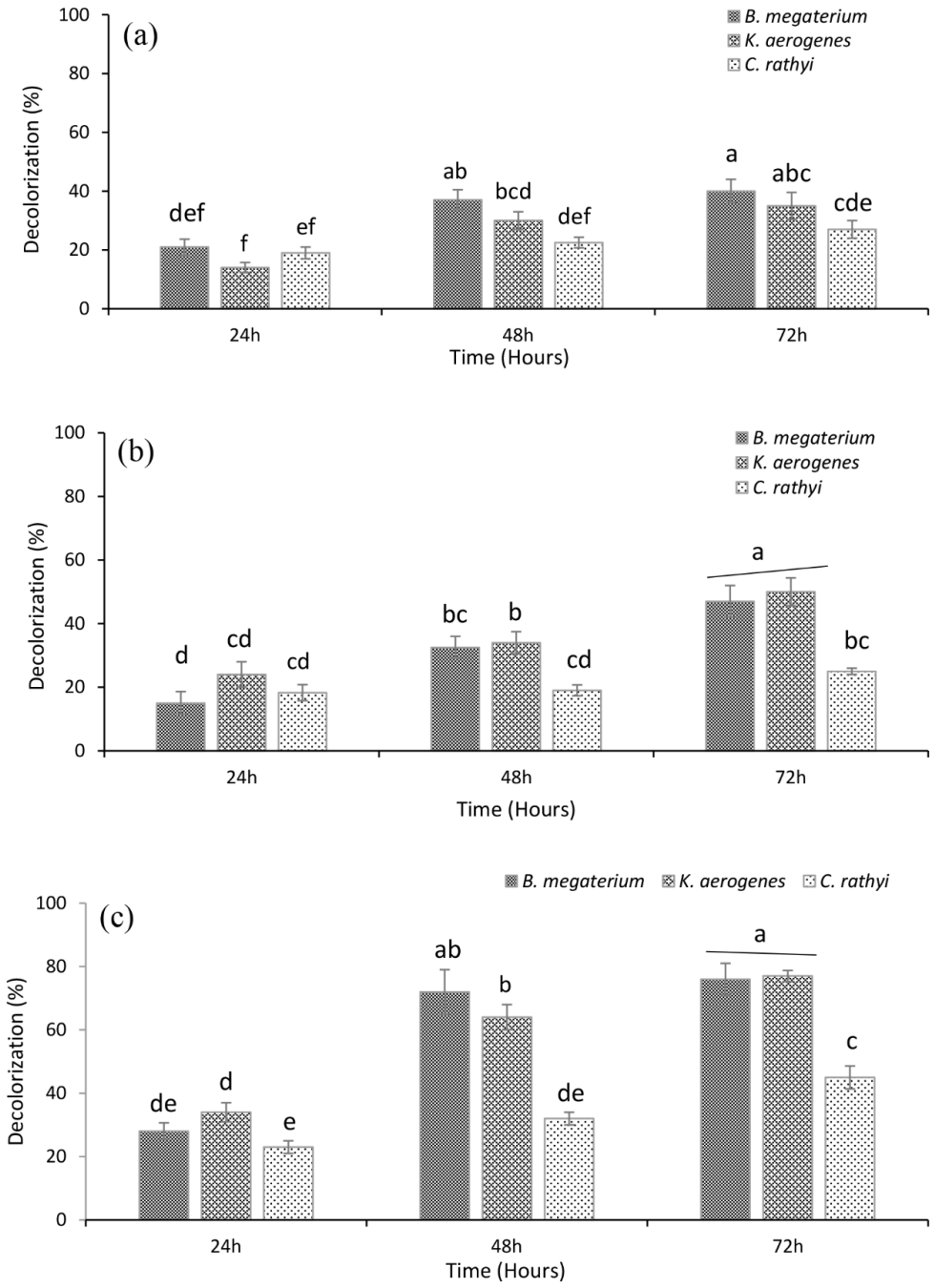


Figure 3 Effect of glucose, peptone, and ammonium acetate with time on decolorization of Novacron Red SB by isolates from textile dye effluent. The experiment was conducted by inoculating MSM broth, pH 7 supplemented with 4 g/L glucose (a), peptone (b), and ammonium acetate (c), incubating at 37°C, and measuring the absorbance at 510 nm in a UV-VIS spectrophotometer at 24 h intervals. Here, different letters above the bars denote statistically significant differences between experimental groups, while shared letters denote no statistically significant differences (Two-way ANOVA followed by Tukey's HSD post-hoc test, $p < 0.05$).

The decolorization of Novacron Red SB dye by the strains was examined in the pH range of 4-9 with 0.5 interval (Figure 4). Maximum decolorization activity was recorded at pH values of 6.5-8.0 (all p-values < 0.001). Among different pH levels tested for efficient dye decolorization, pH had a

significant overall effect for *B. megaterium* ($F(10,66) = 776.2$, $p < 0.001$), *K. aerogenes* ($F(10,66) = 539.0$, $p < 0.001$), and *C. rathyi* ($F(10,66) = 448.3$, $p < 0.001$) by two-way ANOVA, with significant time and pH × time effects also observed for all strains (all $p < 0.001$). As the pH × time interaction was significant, one-way ANOVA was performed at 72 h for each strain. Tukey's HSD showed that *B. megaterium* peaked at pH 6.5 (80.0 ± 2.65%); $F(10,22) = 403.8$, $p < 0.001$. pH 6.5 was significantly higher than pH 4, 4.5, 5, 5.5, 6, 7, 7.5, 8, 8.5, and 9 (each comparison, $p < 0.001$). *K. aerogenes* peaked at pH 8.0 (76.0 ± 3.00%); $F(10,22) = 345.7$, $p < 0.001$. pH 8.0 was significantly higher than pH 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8.5, and 9 (each comparison, $p < 0.001$). *Corynebacterium rathyi* peaked at pH 7.0 (67.0 ± 2.65%); $F(10,22) = 236.1$, $p < 0.001$. pH 7.0 was significantly higher than pH 4, 4.5, 5, 5.5, 6, 6.5, 7.5, 8, 8.5, and 9 (each comparison, $p < 0.001$).

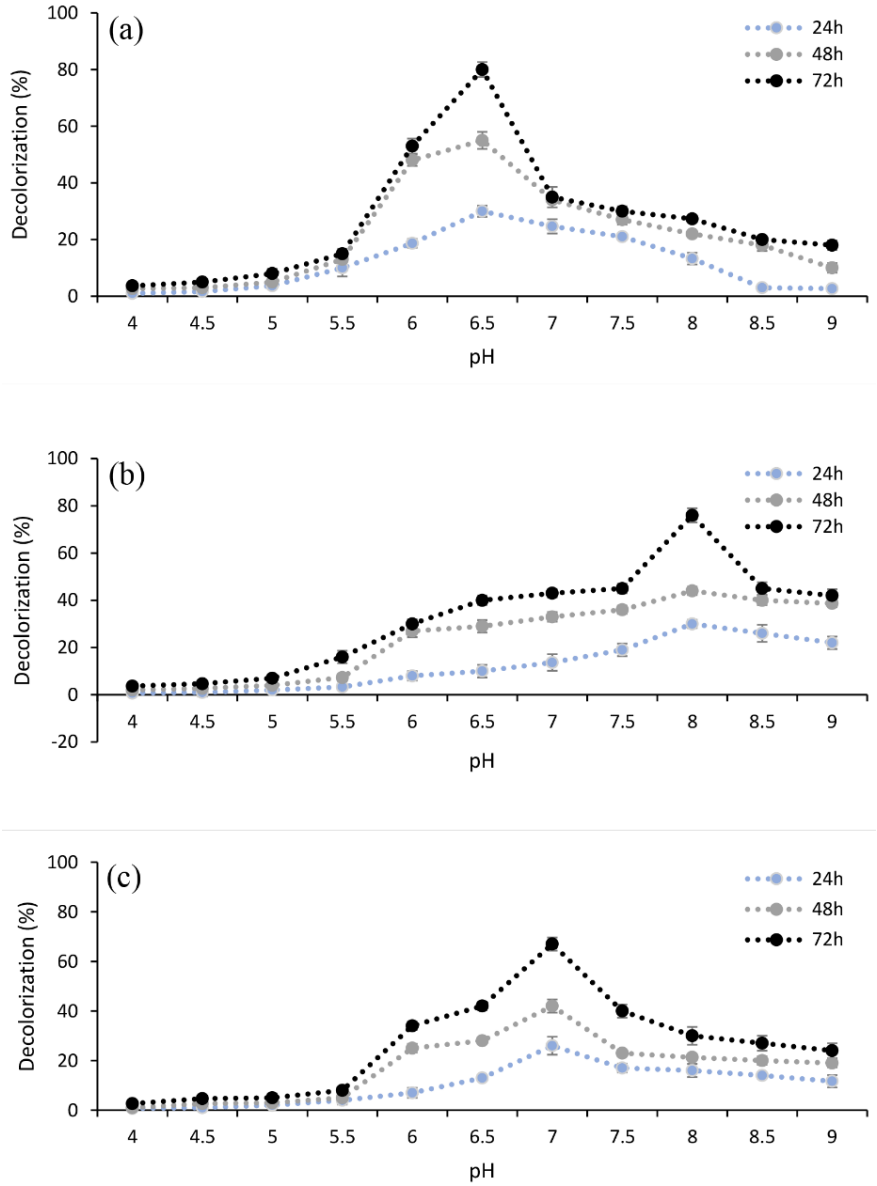


Figure 4 Effect of pH on decolorization of Novacron Red SB over time by *B. megaterium*, *Klebsiella aerogenes*, and *C. rathyi*. The experiment was conducted by inoculating MSM broth adjusted to pH 4-9 at 0.5 intervals with *B. megaterium* (a), *Klebsiella aerogenes* (b), and *C. rathyi* (c), followed by incubation at 37°C. Absorbance was measured at 510 nm using a UV-VIS spectrophotometer at 24 h intervals. Data are presented as mean

values; errors represent SD (n = 3). Statistical significance among pH levels was determined using two-way ANOVA followed by Tukey's HSD test.

Decolorization increased with temperature from 25°C to 37°C (Figure 5). A further increase in temperature to 45°C significantly impaired bacterial decolorization capacity. The optimum temperature for decolorization of Novacron Red SB dye by the bacterial strains was 37°C, with 85%, 80%, and 71% decolorization by *B. megaterium*, *K. aerogenes*, and *C. rathyi*, respectively. A two-way ANOVA on decolorization percentages after 72 h with temperature and strain as factors showed a significant effect of temperature ($F(2,18) = 619.1$, $p < 0.001$, $\eta^2 = 0.986$) and strain ($F(2,18) = 24.2$, $p < 0.001$, $\eta^2 = 0.729$), with no significant temperature × strain interaction. Decolorization at 37°C (mean = 78.7%) was significantly higher than at both 25°C (mean = 33.7%) and 45°C (mean = 17.3%). Tukey's HSD test revealed that decolorization at 37°C was significantly higher than at 25°C ($p < 0.001$) and 45°C ($p < 0.001$). Therefore, 37°C is the optimal temperature for decolorization.

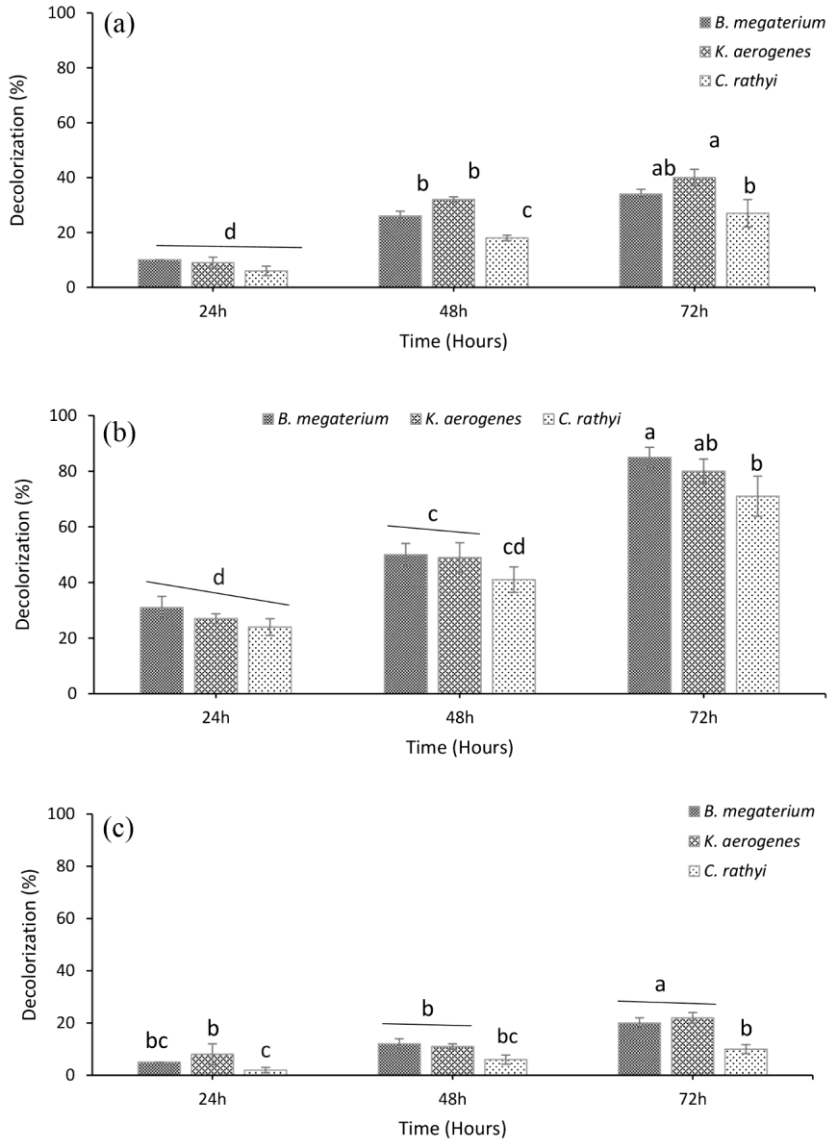


Figure 5 Effect of temperature on decolorization of Novacron Red SB over time by *B. megaterium*, *Klebsiella aerogenes*, and *C. rathyi*. The experiment was conducted by inoculating MSM broth, pH 7, at 25°C (a), 37°C (b), and 45°C (c) and measuring the

absorbance at 510 nm in a UV-VIS spectrophotometer at 24 h intervals. Here, different letters above the bars denote statistically significant differences between experimental groups, while shared letters denote no statistically significant differences (Two-way ANOVA followed by Tukey's HSD post-hoc test, $p < 0.05$).

3.4 Optimized Condition Versus Unoptimized Condition

To validate the optimized conditions in the textile effluent, the pH of the effluent sample was first adjusted to 7 as the optimum. The pH of 7.0 was selected for this experiment because the textile effluent used in this study had a natural pH of 7.2, making pH 7.0 environmentally relevant for the effluent matrix. However, future studies should optimize pH for each strain individually before scaling up. Then the effluent was sterilized to avoid the effect of microbial contaminants in the sample and co-incubated with the bacterial isolates for the optimized time and temperature, 72 h and 37°C, respectively. All of these optimum parameters were combined and dye decolorization was performed against two solutions. One was a dye solution of known composition and the other was textile dye effluent containing a complex composition. Figure 6 illustrates the decolorization efficiency against these two solutions by the bacteria. All bacteria showed better decolorization under optimized conditions than under unoptimized ones. Among these bacteria, *B. megaterium* showed high decolorization under the optimized conditions.

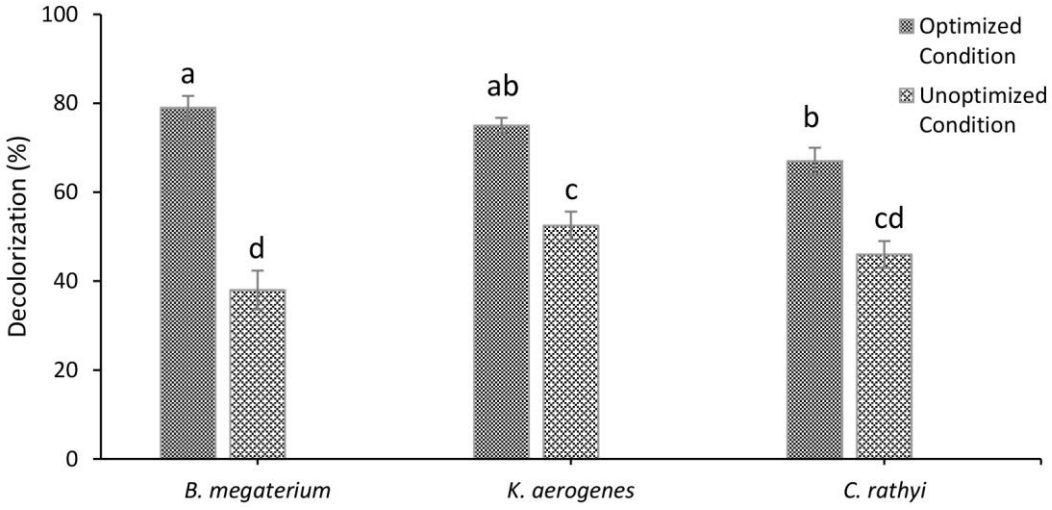


Figure 6 Comparative study of textile azo dye decolorization by *B. megaterium*, *Klebsiella aerogenes*, and *C. rathyi*. Under optimized conditions (by adding co-substrate 4 g/L ammonium acetate in the effluent, maintaining pH 7, and incubating at 37°C for a 72 h incubation period) and unoptimized conditions (no addition of co-substrate and not maintaining pH in the effluent and incubating at 25°C as the effluent temperature for 72 h). Here, different letters above the bars denote statistically significant differences between experimental groups, while shared letters denote no statistically significant differences (Two-way ANOVA followed by Tukey's HSD post-hoc test, $p < 0.05$).

The maximum decolorization occurred at 37°C and near-neutral pH (7.0) following a 72-hour incubation period.

4. Discussion

We investigated dye-decolorizing bacteria from textile effluents and the factors influencing the microbial decolorization of an azo dye, Novacron Red SB. Our analysis aimed to find out the conditions under which bacterial strains achieve maximum decolorization efficiency. Under the diverse range of environmental conditions and dye concentrations, we expected differences in microbial responses depending on dye concentration, pH, temperature, incubation period, and supplementation with additional carbon and nitrogen sources. We aimed to contribute to the existing literature by identifying effective bacterial strains with high textile dye decolorization efficiency and by examining the conditions that optimize their decolorization potential.

According to our results, the most critical factors for successful decolorization include incubation time, near-neutral pH, moderate temperature, and the presence of suitable co-substrates. Maximum decolorization was achieved after 72 hours of incubation (longer incubations are needed to confirm the true optimum), consistent with Ferdousi et al. [18] and Barathi et al. [22]. Ferdousi et al. [18] reported the maximum decolorization of the textile azo dye Novacron Blue after a 72 h incubation period. Barathi et al. [22] showed that with an increase in incubation period, the rate of decolorization increased, and after a certain point in time, it showed no further decolorization.

Furthermore, azo dyes lack sufficient carbon content, making their microbial breakdown challenging without supplemental carbon and nitrogen sources. Our results suggest that ammonium acetate significantly increases the decolorization rate, while glucose and peptone have a lesser effect on promoting the decolorization of Novacron Red SB. Similarly, Wang et al. [23] showed that external carbon sources act as electron donors, facilitating azo bond cleavage and reported that *Citrobacter sp.* achieved 90% decolorization of a different azo dye (Reactive Red 180) with glucose supplementation, compared to only 26.72% without it. Similar stimulation by glucose supplementation has been reported during the microbial degradation of other carbon-containing compounds [24]. In general, carbon sources serve as electron donors, facilitating azo bond cleavage during microbial degradation. Microbial metabolism of organic nitrogen generates NADH that functions as an electron donor in bacterial azo dye reduction pathways. While additional substrates enhanced dye breakdown, dye concentration also strongly influenced decolorization efficiency.

The efficiency declined at higher dye concentrations. This study shows that decolorization efficiency declines proportionally with increasing dye concentration. It might be due to the toxic effect of dyes on degrading microbes or blockage of the azoreductase enzyme's active site by dye molecules [25]. In contrast, the relatively low rate of dye decolorization at very low dye concentrations might be due to the remaining enzymatic system being unsaturated and involved in the reaction. As reported by Jadhav et al. [26] and Pearce et al. [27], decreased decolorization efficiency at very low concentrations occurs due to the lack of ability of the enzyme to recognize the substrate efficiently. Moreover, Çetin et al. [28] reported that higher dye concentrations require a longer time for removal of the dye. In addition to dye concentration, pH emerged as another key determinant of microbial decolorization performance.

Deviation from the optimal pH impaired microbial performance, with maximum efficiency recorded between pH 6.0 and 8.0. pH is a key factor influencing cellular proliferation and multiple biochemical and enzymatic processes. Deviation from the optimal pH in either direction (acidic or alkaline) reduces decolorization efficiency. Olukanni et al. [29] reported a comparable pH optomun finding the highest decolorization of the azo dye Methyl Red by *Micrococcus* strain R3 within near-

neutral pH values of 6.0-8.0. Maximum dye decolorization typically occurs near neutral pH (6.0-8.0), with significantly reduced efficiency under highly acidic (pH < 4) or alkaline (pH > 9) conditions. Novacron Red SB is a reactive azo dye containing sulfonate ($-SO_3^-$) groups and is therefore predominantly anionic. Although adsorption of this anionic dye onto the bacterial surface may be enhanced under acidic conditions due to reduced electrostatic repulsion, maximum decolorization occurred at near-neutral pH, where bacterial growth and azoreductase activity are optimal. Therefore, microbial metabolism, rather than adsorption alone, primarily governs overall dye removal. At alkaline pH, the azo bonds will be deprotonated to negatively charged compounds and this results in obstruction of azo dye decolorization [30]. Beyond pH, temperature also played a crucial role in influencing the activity of dye-degrading enzymes and overall bacterial performance.

The decolorization of Novacron Red SB dye by the strains increased with an increase in temperature from 25°C-37°C but further increase in temperature to 45°C impaired bacterial decolorization capacity, indicating thermal sensitivity of the process. A similar temperature dependence was documented by Çetin et al. [28] for Reactive Red dye at 35°C using mixed microbial cultures. In many bacterial systems, the rate of azo dye decolorization rises with increasing temperature, but only up to a certain point. Afterwards, dye decolorization decreases. The reduction in decolorization rate at higher temperatures may be due to thermal deactivation of azo-reductase enzymes or loss of cell viability [31]. Thus, while general trends are comparable, direct comparisons across different dye structures and bacterial genera require caution.

Overall, our study indicates that microbial decolorization efficiency depends on concentration, incubation period, co-substrate availability, pH, and temperature. The optimum conditions (37°C, pH 7.0, 72 h) align with operational parameters commonly applied in biological wastewater treatment systems. We recommend Design of Experiments (DOE) for future studies to more comprehensively evaluate factor interactions and confirm the optimal conditions. Consequently, the selected isolates may represent promising candidates for the biological treatment of azo dye-containing textile effluents under laboratory conditions. However, further studies using multiple dyes and real wastewater under field conditions are required before their practical application can be confirmed. Moreover, future studies should include bacterial growth measurements (e.g., optical density or dry weight) to normalize dye removal relative to biomass.

4.1 Limitations

1. 16S rRNA sequencing was not performed for this study due to funding limitations. Future work should employ 16S sequencing to confirm species identity and explore genomic potential.

2. The “naked eye” observation was used only as a rapid, preliminary test to eliminate non-decolorizing isolates from the total isolated bacteria. We performed all subsequent quantitative experiments using spectrophotometric measurements at 510 nm.

3. Our study did not identify or quantify degradation products or metabolites, nor assess their ecotoxicity. Future studies should include enzyme activity assays, identification of metabolic intermediates, toxicity evaluation, and elucidation of the degradation pathway to provide a more comprehensive mechanistic understanding of the decolorization process.

4. Azo dye reduction can occur under aerobic conditions via alternative mechanisms (e.g., aerobic azoreductases, laccases, or non-enzymatic adsorption). All experiments were performed under aerobic shaking conditions, and the exact mechanism was not confirmed. Aerobic shaking

conditions excluded obligate anaerobic bacteria. Consequently, the decolorization efficiencies reported in this study represent only the capacity of aerobic and facultative anaerobic isolates. Future studies could employ anaerobic enrichment strategies to capture obligate anaerobes and compare their dye-degrading capabilities under anoxic conditions.

5. The relatively coarse temperature gradient and the use of only two discrete dye concentrations limited our ability to determine the precise optimal temperature and concentration for maximal decolorization.

6. The textile effluent was sterilized before inoculation to eliminate native microbial populations that would normally interact with the introduced strains in a treatment plant. Sterilization does not simulate real treatment conditions and so non-sterile validation is needed before scale-up.

7. Salinity or different ion concentrations, heavy metal concentrations, and exact dye concentration in effluent were not analyzed in this study. Future studies should include comprehensive physicochemical characterization to better understand the factors influencing microbial community composition.

5. Conclusions

Discharging textile dyes into water bodies poses risks to both human and animal health. The shift toward biological treatment methods for textile dye effluent has created ground for research regarding textile dye decolorization by microbes. Therefore, this study aimed to explore the use of bacteria for decolorizing textile dyes from textile effluent.

The findings show that bacteria isolated from textile dye effluents exhibit considerable decolorization capacity under the aerobic laboratory conditions tested. Among the 5 isolates, 3 isolates, presumptively *Bacillus megaterium*, *Klebsiella aerogenes*, and *Corynebacterium rathyi,* showed maximum decolorization. We examined their dye-decolorizing ability against several parameters, i.e., dose, time, co-substrate, temperature, and pH. The highest decolorization percentage was found using ammonium acetate as co-substrate at a 72 h incubation period at 37°C. Dye decolorization decreased with the increase in dye concentration. It was found that the dye decolorization gradually increased from acidic to neutral pH values and gradually decreased towards strong alkaline conditions.

Overall, it is evident that the dye decolorizing ability of all isolates was best at 37°C and under optimized conditions compared to unoptimized ones. These results suggest that the isolates are promising candidates for further investigation in controlled systems, but toxicity assessment and scale-up studies are required before considering practical application. The metabolites produced after dye degradation need to be analyzed in future research.

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Author Contributions

Conceptualization: Dipankar Dewanjee, Jannatul Ferdousi, Tasneem Chowdhury, Sawkat Ara Pinki, Md. Imranul Hoq. Data curation: Dipankar Dewanjee, Jannatul Ferdousi, Sawkat Ara Pinki. Formal analysis: Tasneem Chowdhury, Md. Imranul Hoq. Investigation: Tasneem Chowdhury, Md. Imranul Hoq. Methodology: Dipankar Dewanjee, Jannatul Ferdousi, Tasneem Chowdhury, Sawkat Ara Pinki, Md. Imranul Hoq. Resources: Tasneem Chowdhury, Md. Imranul Hoq. Supervision: Tasneem Chowdhury, Md. Imranul Hoq. Writing - original draft: Dipankar Dewanjee, Jannatul Ferdousi, Tasneem Chowdhury, Sawkat Ara Pinki, Md. Imranul Hoq. Writing - review and editing: Dipankar Dewanjee, Jannatul Ferdousi, Tasneem Chowdhury, Sawkat Ara Pinki, Md. Imranul Hoq.

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Competing Interests

None of the authors has competing interest.

Data Availability Statement

The data generated and analyzed during the current study are available from the corresponding author upon request.

AI-Assisted Technologies Statement

An artificial intelligence (AI) tool was used solely for language refinement of this manuscript. Specifically, ChatGPT was used to improve the readability and linguistic clarity of the English text. All scientific content, data interpretation, and conclusions were developed independently by the authors. The authors have thoroughly reviewed and edited the AI-assisted text to ensure its accuracy and accept full responsibility for the content of the manuscript.

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