Tripathi et al | Article 9556
J Pure Appl Microbiol. 2024;18(4):2558-2569. doi: 10.22207/JPAM.18.4.29
Received: 15 May 2024 | Accepted: 12 September 2024
Published Online: 16 November 2024

P-ISSN: 0973-7510; E-ISSN: 2581-690X

RESEARCH ARTICLE OPEN ACCESS

Physicochemical Investigations of Textile Wastewater and Process Parameter Optimization for Bio-decolorization of Congo Red Dye by *Pseudomonas aeruginosa* MT-2 Strain

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Abstract

Pollution caused by dyes is a major environmental threat, posing adverse impacts on humans, animals, and plants. Therefore, the remediation of such pollutants is essential to protect the environment. This study aimed to conduct physicochemical and bacteriological analyses of textile wastewater to isolate and identify potential native bacterial strains for the decolorization of Congo red dye. Physical and nutritional process parameters were optimized to achieve maximum decolorization. The biological and chemical oxygen demands of the analyzed textile waste water were found to be above the recommended limits. In this study, 19 Congo red -decolorizing bacteria were isolated, with one bacterial culture capable of growing at a higher dye concentration of 300 mg/L. This bacterium was characterized biochemically and genetically (using 16S rRNA sequencing) and identified as the *Pseudomonas aeruginosa* MT-2 strain. A maximum decolorization of 94.0% was achieved at an initial dye concentration of 150 mg/L, 35°C, and pH 8.0 under static conditions. The bacterial culture also showed resistance to heavy metals such as arsenic, lead, and chromium. The biodegradation of Congo red dye was confirmed through UV-vis spectral analysis and Fourier transform infrared spectrophotometry. The findings of this study demonstrate the high remediation potential of the MT-2 strain, making it suitable for possible use in dye biodecolorization at contaminated sites.

Keywords: Biodecolorization, Congo Red, FT-IR, Optimization, Physicochemical Factors, 16S rRNA

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Citation: Tripathi M, Shukla S, Singh R, et al. Physicochemical Investigations of Textile Wastewater and Process Parameter Optimization for Bio-decolorization of Congo Red Dye by *Pseudomonas aeruginosa* MT-2 Strain. *J Pure Appl Microbiol.* 2024;18(4):2558-2569. doi: 10.22207/JPAM.18.4.29

INTRODUCTION

Industrial developments have played a crucial role in the global economy, but they have also led to a massive increase in waste production, which is harmful to all living entities in the biosphere.[1,2] The discharge of effluents can be hazardous to the ecosystem, yet a significant quantity of textile effluents is still being released directly into the environment.[3] Dyes used in various industries are identified as a primary source of water pollution.[4,5] Synthetic dyes are commonly employed to color a wide range of industrial products and are extensively used across different industries.[6,7] Dyes are mainly classified based on various criteria, including their structures and applications.[8] Congo red is a type of azo dye, and its remediation methods have been studied by researchers.[9-11]

Azo dyes contain a characteristic azo bond (-N=N-) that makes them resistant to degradation through natural processes. The effluent discharged from textile industries contains harmful and hazardous chemicals that are difficult to remove from the environment.[12] The constant growth of the textile industry has led to an increased rate of effluent production.[13] The discharge of untreated effluent directly into the environment results in decreased oxygen levels in water, necrosis of flora, and other ecological impacts. Additionally, untreated effluent poses threats to humans and animals, with adverse effects on human health, including skin irritation, respiratory problems, gastrointestinal disorders, and blood clotting.[14,15] Untreated effluent also exhibits high levels of BOD, COD, salinity, and intense coloration.[16] For the safety of the environment and living organisms, it is essential to remove these dyes before their discharge. Currently, several approaches are being used to remove dyes from wastewater.[17] These methods, including physical and chemical approaches, have certain drawbacks, such as high costs and the need for large quantities of chemicals and energy.[8,18,19] Consequently, they are not widely applicable for dye decolorization.[20] Therefore, there is a need for alternative methods that are safer and more cost-effective than conventional techniques for dye removal.

Congo red removal through adsorption is also a significant approach.[21,22] Among several modern techniques, microbial dye decolorization and degradation stand out as promising methods. This approach is considered inexpensive and environmentally friendly for dye remediation.[20] Bacteria have been recognized as efficient agents for dye decolorization by degrading organic pollutants, such as dyes, and using them as energy and carbon sources for their growth and multiplication. Notably, numerous bacterial strains are highly tolerant to harsh conditions and exhibit rapid growth.[23] The use of microorganisms, especially bacteria, offers advantages such as ease of cultivation. Bacteria can remove dyes from effluent either through adsorption or enzymatic degradation. Myco-remediation is another biological strategy used to treat dye contamination.[24]

Bacterial dye decolorization has been extensively studied by researchers. Kishor *et al*.[25,26] reported on the dye degradation capabilities of bacterial strains that are resilient, prevalent, and rapidly growing. Other studies have demonstrated the efficiency of bacterial dye degradation. For example, Ikram *et al*.[27] investigated the degradation of Basic Orange 2 dye by *Escherichia coli* and observed 89.88% decolorization. In another study, Hanis *et al*.[28] showed 83.12% decolorization of Congo red by *Bacillus* sp. at 25 ppm, pH 7.55, and a temperature of 30°C over 5 days. Additionally, Sarkar *et al*.[29] investigated the decolorization and degradation of Congo red textile azo dye by *Chryseobacterium geocarposphaerae* and reported 96.52% decolorization within 12 hours.

This study aims to analyze the physicochemical characteristics of wastewater and isolate a potential bacterial culture for effective biodecolorization. The effects of various factors, including culture conditions, pH, temperature, and carbon and nitrogen sources, on the biodecolorization of Congo red by the indigenous *Pseudomonas aeruginosa* MT-2 strain were also investigated.

MATERIALS AND METHODS

Sampling

The dye-containing effluent was collected from the discharge location of power looms in Tanda, Ambedkarnagar, Uttar Pradesh, India, and stored in sterile bottles.

Physicochemical parameters analyses

The physicochemical parameters were assessed according to the American Public Health Association (APHA)[30] guidelines, and the values were reported as the mean ± SD of three replicates. Temperature was measured at the sample collection site using a thermometer. The pH of the samples were measured using an electronic pH meter (ELICO). To measure the biological oxygen demand (BOD), the initial dissolved oxygen (DO) of the sample was recorded, and BOD bottles containing the sample were incubated in a BOD incubator at 20°C. After 5 days, the DO was measured again, and BOD was estimated by subtracting the final DO from the initial DO. The dichromate reflux method was used to determine the chemical oxygen demand (COD) of the sample, following APHA guidelines. For measuring total dissolved solids (TDS), the gravimetric method was employed: 10 mL of the sample was filtered through Whatman filter paper, and the filtrate was fully dried and weighed to calculate the TDS content. Fluoride levels were estimated using the SPADNS method, in which the sample was mixed with SPADNS and acid reagent. The absorbance of the resulting solution was then measured at 570 nm using a spectrophotometer, and The fluoride concentration was determined by extrapolating from the values of standard solutions.

Isolation of Congo red dye-decolorizing bacteria

The bacteria responsible for dye decolorization were isolated from contaminated soil using the pour plate technique on solid minimal salt medium (MSM) amended with Congo red dye at a concentration of 50 mg/L. The plates were incubated at 35°C for 72 hours. Bacterial colonies on the MSM agar plates were repeatedly streaked onto the same medium, resulting in the isolation of pure bacterial strains. These pure strains were then tested on MSM agar plates with dye concentrations ranging from 50 to 500 mg/L to determine which bacterial strain exhibited the most efficient dye decolorization properties.

Identification of potential Congo red dye-decolorizing bacterium

Morphological, molecular (16S rRNA gene sequencing), and biochemical analyses were employed to identify the potential isolate(s). After 24 hours of incubation at 35°C, the morphology of the bacterial strain MT-2 was determined by examining colony features through bacterial staining and performing a catalase test. The selected bacterial isolate was further characterized using standard methods. Molecular characterization was performed using the 16S rRNA gene sequence amplification technique at the Cytogene Research Lab, Lucknow (India), to identify and compare bacterial diversity from complex microbiomes. The obtained sequence was then submitted to the DNA Databank of Japan to obtain an accession number. The nucleotide sequences were aligned using Clustal W software, and the neighbor-joining (NJ) method was employed to construct a phylogenetic tree.

Culture conditions

First, the bacterial inoculum was prepared. The dye decolorization experiments were conducted in liquid minimal salt medium, inoculated with a bacterial culture containing $3.1x10^6$ CFU/mL, and incubated at 35°C for 96 hours. The dye content and cell density were periodically examined every 24 hours, and quantitative analysis of dye decolorization was carried out spectrophotometrically at 495 nm. All experiments were performed in triplicate.

Effect of aeration

To determine the effect of aeration, 25 mL of MSM (pH 7.0) was added in 150 mL Erlenmeyer flasks and inoculated with the selected bacterial isolate. The flasks were incubated at 35°C under shaking conditions (150 rpm). To assess the effect of static conditions, 25 mL of inoculated medium was placed in screw-capped tubes and incubated at 35°C for 96 hours.

Effect of dye concentration

To observe the impact of different initial dye concentrations on bacterial decolorization, varying levels of dye (50-300 mg/L) were added to MSM broth and incubated for 48 hours under stationary culture conditions.

Effect of nutrients (carbon and nitrogen)

To identify the optimal carbon and nitrogen sources for the decolorization of Congo red dye, the effects of various carbon and nitrogen

sources on dye decolorization were assessed under the previously established optimal culture conditions.

Effect of inoculum size

To investigate the impact of inoculum dosage (1 to 6%, v/v) on dye decolorization, the bacterial inoculum was transferred to dye-containing MSM broth and incubated under stationary culture conditions for 48 hours.

Combined effect of initial pH and temperature

The combined effect of initial pH and temperature was assessed by preparing MSM broths with different pH ranges (7.0-9.0) using 0.1N HCl or 0.1N NaOH before sterilization. After sterilization, the MSM broths were inoculated with the optimal inoculum dosage and incubated at temperatures ranging from 25°C to 40°C for 48 hours.

Multi-metal tolerance test

To evaluate the multi-metal tolerance potential of the isolated bacterium, different concentrations (25-200 mg/L) of arsenic, chromium, lead, and mercury were added to nutrient agar medium and inoculated with the MT-2 isolate.

Measurement of Congo red decolorization

To determine the bacterial potential for dye decolorization, samples were collected from MSM media inoculated with the bacterial isolate. The samples were centrifuged at 10,000 rpm in a refrigerated centrifuge (4°C), and the residue was discarded. The supernatant was then analyzed using a spectrophotometer at 495 nm. The percentage of dye decolorization was calculated using the following formula[31,32]:

$$\% \text{ Decolorization of dye} = \frac{\text{Initial optical density - Final optical density}}{\text{Initial optical density}} \times 100$$

FT-IR analyses

samples before and after decolorization were analyzed to determine the biodegradation of the dye by studying the functional groups. The samples were measured across a wavenumber range of 500 to 4500 cm^{-1}.

Statistical analyses

All experiments in this study were conducted in triplicate. Statistical calculations, including standard deviation, were performed using The Microsoft Excel program.

RESULTS AND DISCUSSION

The discharge of colored wastewater from textile industries is a significant global environmental issue. Among the various components of textile effluent, dyes are the most influential in contributing to its toxicity. Bacteria play a crucial role in the biological remediation of azo dyes. In this study, various physical and chemical factors, such as oxygen levels, inoculum size, and nutrient supplementation, were evaluated to determine their impact on the efficiency of bacterial dye decolorization. Understanding each parameter that affects bacterial dye decolorization is essential for enhancing the effectiveness of the bioremediation process.

Physicochemical parameter analysis

The physical and chemical characteristics of colored discharge from powerlooms were analyzed following APHA methods. The results indicate that parameters such as biological oxygen demand (BOD), chemical oxygen demand (COD), and total dissolved solids (TDS) were found to be beyond the recommended threshold values set by international standards and other pollution regulatory bodies (Table 1). However, the pH and temperature were within the permissible limits. These findings suggest that the wastewater released from power looms is unsatisfactory concerning its physicochemical content, posing a potential environmental threat. Therefore, proper treatment of the wastewater is necessary before its discharge into the environment or water bodies.[18,32]

Isolation and screening of Congo red decolorizing bacteria

The sample was microbiologically examined, and the presence of dye decolorization activity was reported. Nineteen bacterial isolates were obtained on minimal salt agar (MSA) medium containing 50 mg/L of Congo red dye. To select the most potent Congo red decolorizing bacterium

among all isolated strains, the cultures were tested in MSA medium with varying dye concentrations (50-500 mg/L). The results showed that a single bacterial isolate was able to grow in the presence of up to 300 mg/L of dye with the addition of 0.4% (w/v) glucose, and this isolate was selected for further studies (Table 2). The findings revealed that increasing dye concentrations inhibited bacterial growth. Other researchers have reported similar results, indicating that higher concentrations exert a toxic effect on decolorization efficiency.[18,32,33] Congo red dye decolorization activity was observed in Petri plates (Figure 1).

Biochemical, morphological, and molecular characterization

The Congo red dye-decolorizing bacterium was identified by assessing its biochemical and morphological properties in our laboratory, with the results presented in Table 3.

The identification of the MT-2 isolate was confirmed through 16S rRNA sequence analysis. genomic DNA was extracted, and the 16S rRNA gene was amplified using PCR with specific primers.[34] The sequence was submitted to the DNA Data Bank of Japan (DDBJ), where it received The accession number LC720406. The isolate was identified as *Pseudomonas aeruginosa* MT-2. The Neighbor Joining (NJ) method was used to construct a phylogenetic tree (Figure 2).

The 16S rRNA gene is The most widely used marker for bacterial classification and identification.[35,36] This highly conserved gene is suitable for phylogenetic analysis at higher levels of taxonomy. In contrast, the internally transcribed spacer (ITS) region, which is highly variable, is used to differentiate isolates at lower levels of taxonomy.[34] Techniques based on 16S rRNA gene sequencing are considered more robust, reproducible, precise, and provide objective results.[35,36]

Effect of different process parameters for effective dye decolorization

Impact of aeration

The findings indicate that bacterial isolates achieved more efficient decolorization under stationary conditions compared to shaking culture conditions. A maximum of 98.2% Congo

Table 1. Physicochemical analyses of colored wastewater

Physico-chemical character	Permissible limit*	Observed value
Color	-	Red
Smell	-	Pungent
pH	6.0-9.0	7.8
Temperature	-	27°C
BOD	30 mg/L (in land surface water) 100 mg/L (Land irrigation)	165 ± 8.0
COD	250 mg/L	419 ± 13.0
TDS	1500 mg/L	2758 ± 41.0
Fluoride	-	1.9 ± 0.2

*Recommended by MOEF, CPCB and IS

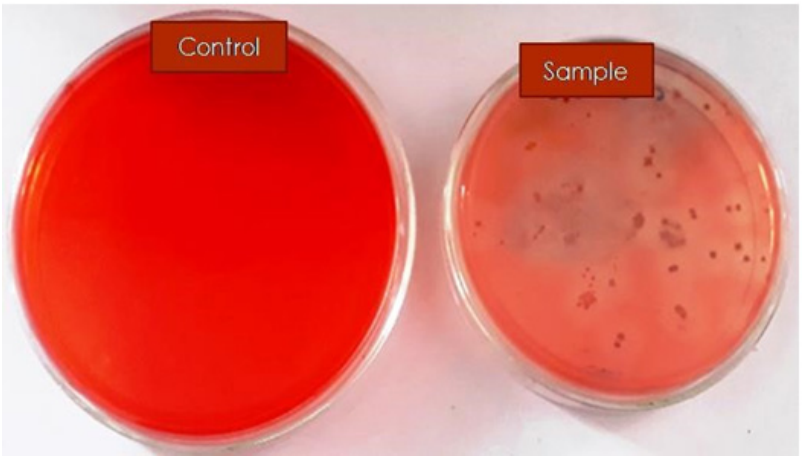

Figure 1. Isolation of Congo red decolorizing bacteria on MSA medium

red dye decolorization was achieved at 48 hours under static culture conditions at an initial concentration of 50 mg/L. In contrast, a maximum of 73.8% decolorization was observed under shaking culture conditions. The percentage of dye decolorization was significantly higher under static conditions. Similarly, Xie *et al*.[37] reported 95% decolorization of RB5 dye under stationary culture conditions for 24 hours. Lakshmaiah *et al*.[38] also found that the B5 strain achieved 61% decolorization of reactive blue 222 dye in 24 hours under stationary conditions. Other studies have also reported more efficient dye decolorization under stationary conditions compared to shaking conditions.[32,39] In aerobic environments, oxygen can act as a terminal electron acceptor instead of the azo dyes, potentially leading to lower decolorization rates.[40]

Table 2. Screening of the potential Congo red dye decolorizing bacterial isolate

Dye level (mg/L)	No. of bacterial isolates grown
50	19
100	3
150	2
200	2
250	1
300	1
350	-
400	-
450	-
500	-

Effect of dye concentration

The concentration and type of dye can significantly impact its remediation capability. It

Table 3. Morphological and Biochemical characteristics of *Pseudomonas aeruginosa* MT-2 isolate

Character	Observation
Shape	Small rods
Motility	-
Gram's reaction	Negative
Catalase	+
Spore	-
Production of H_2S	-
Glucose utilization by bacterium	+
Sucrose utilization by bacterium	+
Starch utilization by bacterium	+

+: for positive reactions, -: for negative reactions

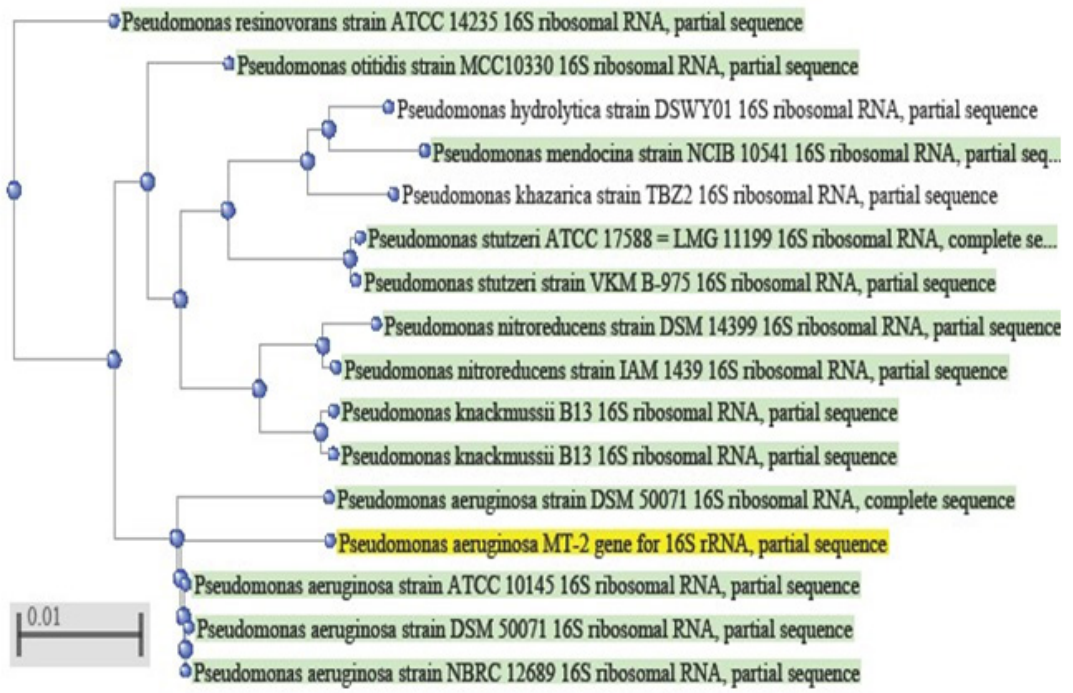

Figure 2. Phylogenetic tree construction of MT-2 strain using Neighbour Joining method

was observed that the extent of Congo red dye decolorization varied with different incubation times. The results revealed that increasing dye concentrations (50-300 mg/L) inhibited the extent of decolorization at each incubation period (24-96 hours). Maximum decolorization of 98.2% was achieved at 50 mg/L, followed by 95% at 100 mg/L, and 87.0% at 150 mg/L. the extent of dye decolorization decreased sharply as the dye concentration increased from 150 to 300 mg/L. However, there was only a marginal increase in the percentage of color removal as the concentration decreased from 150 to 50 mg/L. Consequently, further studies were conducted at an optimized initial concentration of 150 mg/L of Congo red dye. Microorganisms play a crucial role in the effective remediation of pollutants, offering a sustainable and environmentally friendly approach.[41-45] Researchers have reported that dye concentration affects the extent of biodecolorization. For instance, Pham *et al*.[23] noted that dye decolorization efficiency declined with increasing dye concentration. High dye concentrations can inhibit reductase enzymes responsible for dye degradation.[46]

Effect of carbon and nitrogen sources

Microorganisms require specific concentrations of carbon (C) and nitrogen (N) sources for optimal growth and metabolic activity.

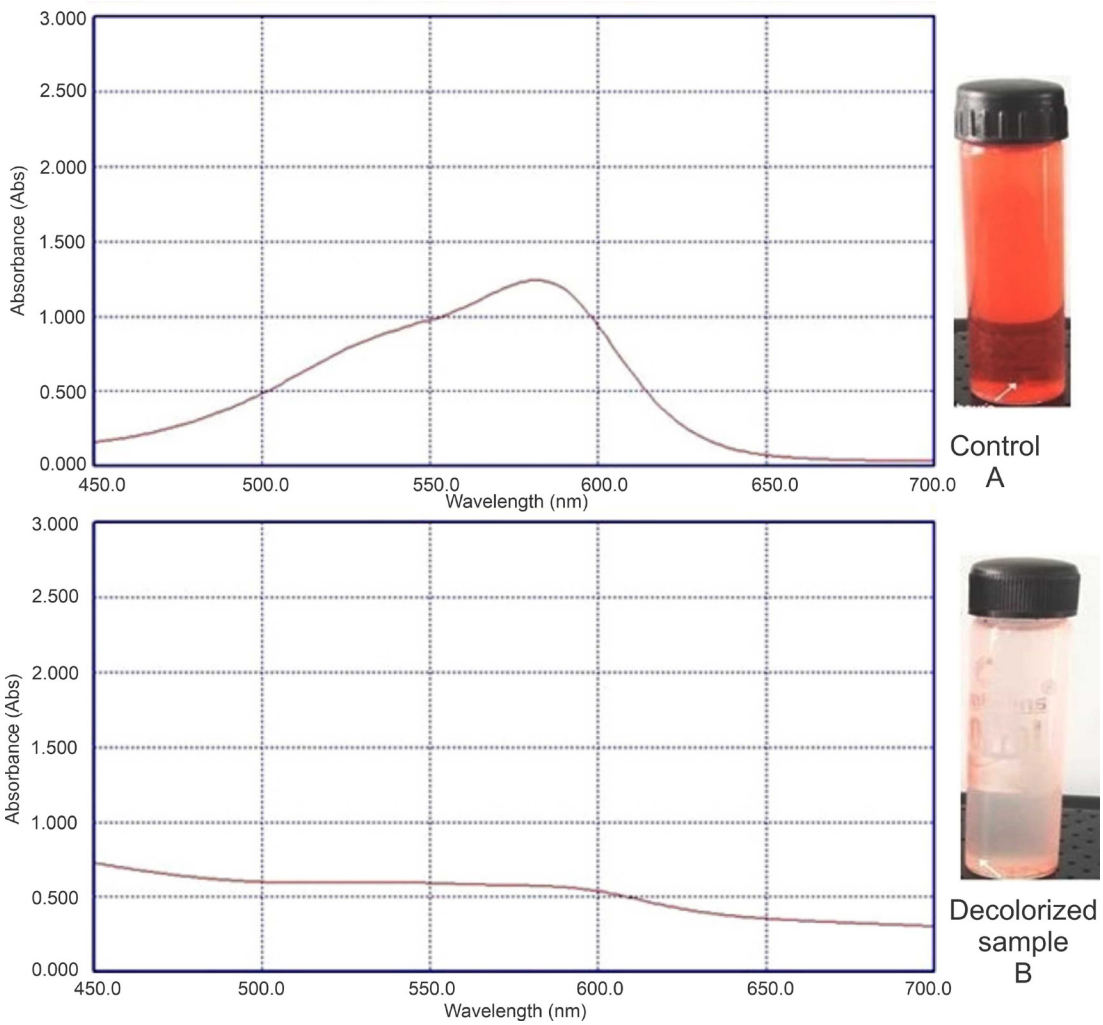

Figure 3. Biodegradation study of Congo red Dye: (A) UV-vis spectral analysis of control dye (B) UV-vis spectral analysis of bio-decolorized dye

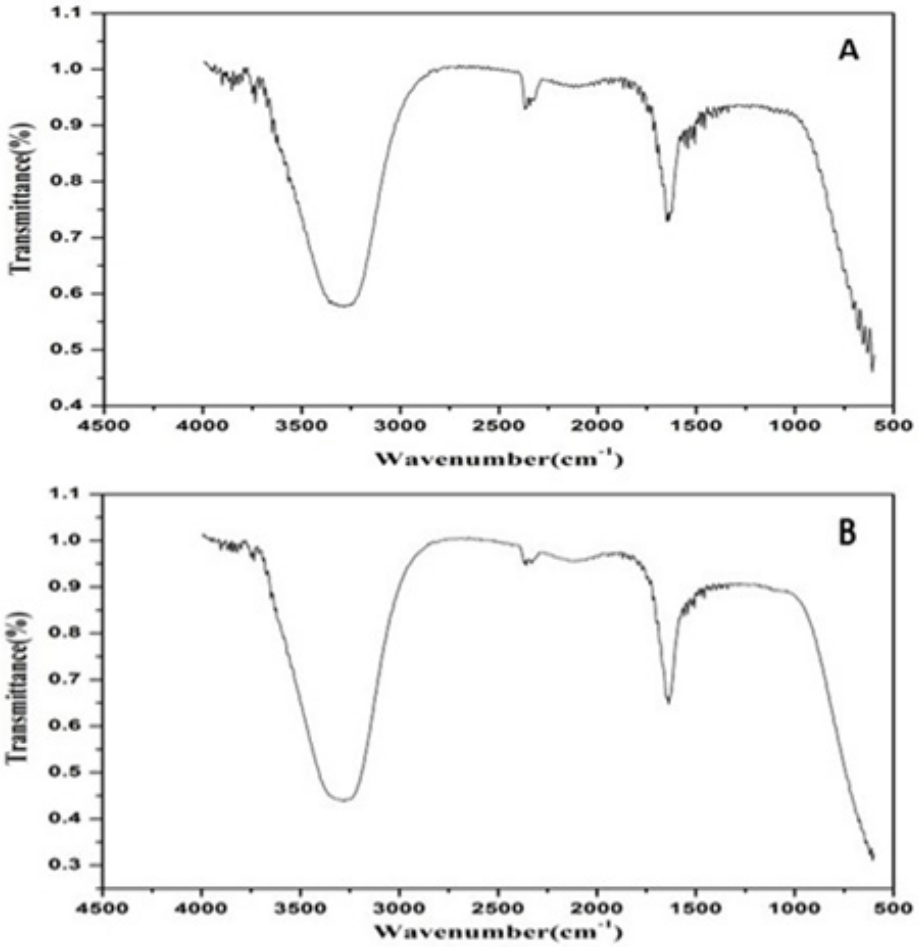

Figure 4. FTIR spectra of (A) Control dye, (B) Decolorized dye

The effect of various carbon sources (glucose, sucrose, and starch) and nitrogen sources (ammonium sulfate, ammonium nitrate, and peptone) on dye decolorization was evaluated under optimized conditions: an initial 150 mg/L Congo red dye concentration, static culture conditions, 4.0% (v/v) inoculum, pH 7.0, and 35°C over 48 hours of incubation. Among the carbon sources tested, the maximum dye decolorization (87.5%) was achieved using glucose as a co-substrate at a concentration of 4 g/L, followed by sucrose and starch. Among the nitrogen sources, ammonium sulfate proved to be the most effective, resulting in the highest dye decolorization (91.8%). The other nitrogen sources were ranked based on their dye-decolorization efficiency as follows: ammonium nitrate (89%) >peptone (68.2%). In a related study, Nasrin *et al*.[47] investigated the impact of various nitrogen sources, including peptone, yeast extract, and ammonium chloride, on the decolorization of four dyes by *Pseudomonas taiwanensis*. They observed improved results when yeast extract and peptone were used in combination with ammonium chloride, compared to using yeast extract and peptone individually. This suggests that combined nitrogen sources might enhance dye decolorization effectiveness.

Dyes with complex structures can be particularly challenging for microbes to degrade; hence, additional carbon sources are often necessary to meet their carbon requirements.[48] Liu *et al.*[49] also reported that using glucose as a carbon source enhances dye degradation due to

its easy uptake and rapid metabolism by bacteria, which supports bacterial growth and activity.

Effect of inoculum dose

The results indicated that dye decolorization improved with increasing inoculum dose (1.0-6.0% v/v) over the 48 hour incubation period. Maximum decolorization was achieved with a 4% v/v inoculum size. As the inoculum size increased from 1.0% to 4.0% (v/v), the extent of dye decolorization also increased correspondingly at each time point. However, further increasing the inoculum size from 4.0% to 5.0% did not significantly enhance the percentage of Congo red dye decolorization. An increase in inoculum size generally promotes microbial growth, as a higher number of cells can more effectively utilize enzymes and substrates.[50-52] However, beyond a certain point, additional increases in inoculum size do not necessarily lead to further improvements in decolorization, possibly due to reduced enzyme production or substrate availability.[53]

Effect of pH and temperature

The influence of pH (6.0-9.0) and temperature (25-40 °C) on dye decolorization was assessed. The MT-2 isolate achieved a maximum of 94.0% dye decolorization at 35°C and pH 8.0 under optimized conditions with an initial 150 mg/L dye concentration and no shaking. Deviations from these optimal values resulted in reduced decolorization efficiency. Both pH and temperature are crucial parameters as they directly affect the enzymatic activity of the microorganism. The optimal growth temperature for effective bio-decolorization of azo dyes is typically between 35°C and 45°C. However, decolorization efficiency declines above this range, likely due to enzyme denaturation.[54] Similarly, deviations from the optimal pH and temperature can reduce dye degradation efficiency due to enzyme inactivation and decreased bacterial growth.[55,56] Elevated temperatures can denature bacterial enzymes, rendering them ineffective.

Biodegradation of Congo red dye

The degradation of Congo red dye and other azo dyes led to the formation of non-toxic intermediates and products. The control sample exhibited maximum absorption at 495 nm, with a distinct peak observed at this wavelength (Figure 3). In contrast, no peak was detected in the inoculated, decolorized samples, indicating that Congo red dye was degraded by the *Pseudomonas aeruginosa* MT-2 strain. Several studies have reported similar findings for dye biodegradation. For instance, Dutta *et al*.[57] documented the degradation of brilliant green dye by the bacterium Achromo bacterinsolitus, isolated from forest soil in the Biosphere Reserve of Odisha. Pinontoan *et al.*[58] observed efficient decolorization of Trypan Blue dye by a bacterial isolate (TB2) from dye-contaminated sewage water, identified as Aeromonas caviae. Additionally, Xie *et al.*[59] reported that the bacterial strain MS-S2, found in the intestine of termites feeding on wood, was effective in bioremediating malachite green dye. Ullah *et al*.[60] also reported the remediation of Brown 703 azo dye by *Pseudomonas aeruginosa* from dye-containing effluent.

FT-IR analyses of decolorized dye

To confirm the decolorization of Congo red dye, Fourier transform infrared (FT-IR) spectroscopy was employed. A comparative analysis of the FT-IR spectra of the degraded and non-degraded dye samples was conducted. The FT-IR spectra of the Congo red dye control and the decolorized dye revealed differences in the presence of functional groups. The control dye exhibited peaks around 1600 cm^{-1}, 3300 cm^{-1}, and smaller peaks near 1400 cm^{-1} (Figure 4). In contrast, the FT-IR spectrum of the decolorized sample showed the disappearance of the peak near 1400 cm^{-1}. Sarkar *et al*.[29] also investigated FT-IR spectra for Congo red dye and observed several peaks in the control sample at 645 cm^{-1}, 1446 cm^{-1}, and 1740 cm^{-1}. However, the FT-IR spectra of the decolorized dye showed the absence of peaks at 1584 cm^{-1}, which correspond to the azo bond stretching vibration (-N=N-). This indicates the reduction and cleavage of the azo bond in Congo red dye.

Multi-heavy metal tolerance

The MT-2 isolate demonstrated resistance to arsenic (As), chromium (Cr), and lead (Pb) at an initial concentration of 50 mg/L.

However, it was unable to grow on nutrient agar supplemented with mercury (Hg), even at a 25 mg/L concentration. The isolate showed tolerance to a maximum of 100 mg/L of arsenic and 125 mg/L of lead. These findings suggest that the dye-decolorizing bacterium MT-2 has potential for use in remediating sites contaminated with heavy metals. Kishor *et al*.[25] investigated Congo red dye decolorization by the bacterial strain *Bacillus cohnni* (RKS9), which achieved 99% decolorization in 12 hours and removed 59.76% of cadmium, 40.51% of chromium, 52.71% of lead, and 26.51% of nickel.

CONCLUSION

Microbial remediation of dyes offers a cost-effective, eco-friendly solution for treating textile dye effluents. This study aimed to explore the dye remediation potential of microbes and enhance the efficiency of dye decolorization to mitigate environmental contamination. The MT-2 strain of *Pseudomonas aeruginosa*, isolated from colored wastewater, proved highly effective in decolorizing Congo red dye, achieving up to 94% discoloration under optimal conditions. This indicates that *Pseudomonas aeruginosa* MT-2 may be a promising candidate for *in situ* dye decolorization in textile industry wastewater. Additionally, the MT-2 strain's heavy metal tolerance makes it suitable for use in sites contaminated with heavy metals. Although various bacterial strains have demonstrated dye decolorization potential, further research on optimizing bioremediation processes and applying advanced strategies is essential for broader-scale implementation and success.

ACKNOWLEDGMENTS

None.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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