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Research Article

Decolourization of Malachite Green Dye by Bacteria Isolated from Drainage Effluent

Sheetal Sahare[1,2], Ram Prasad Kushvaha[1], Renu Mishra[2], Urmila Meghwal[1] and Sunil Kumar Snehi[1]*

[1]Molecular Plant Virology Lab, Department of Microbiology, Barkatullah University, Bhopal (Madhya Pradesh), India

[2]Department of Botany and Microbiology, Sri Sathya Sai College for Women, Bhopal (Madhya Pradesh), India

***Corresponding Author:** Dr. Sunil Kumar Snehi, Molecular Plant Virology Lab, Department of Microbiology, Barkatullah University, Bhopal (Madhya Pradesh), India.

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Abstract

The widespread use of synthetic dyes, particularly malachite green (MG), in various industries has led to significant environmental pollution, necessitating effective and eco-friendly remediation strategies. This study focused on the isolation and identification of bacterial strains capable of degrading malachite green dye. A total of seven bacterial isolates were obtained from a drainage canal of New Market, Bhopal (Madhya Pradesh). Preliminary screening on dye-containing media identified two potent isolates Pseudomonas otitidis and *Aeromonas hydrophila*, exhibiting significant degradation capabilities. Biodecolorization Assay confirmed that isolate *Pseudomonas otitidis* achieved the highest decolorization efficiency of 80% in optimized condition (72 hours, 35°C), while Aeromonas hydrophila reached 76% under similar conditions for the malachite green dye. BLASTn analysis confirmed ≥ 99.86 and 100 percentage sequence similarity with known bacterial strains. Overall, the findings highlight *Pseudomonas otitidis* and *Aeromonas hydrophila* as promising bacteria for the bioremediation of malachite green contaminated environments, with *Pseudomonas otitidis* showing exceptional efficiency and potential for application in sustainable wastewater management strategies.

Keywords: Malachite Green; Decolorization; *Pseudomonas otitidis; Aeromonas hydrophila*; Wastewater Management

Introduction

Synthetic dyes are extensively utilized in the textile, food, cosmetic, and paper printing industries. Each year, around one million tons of synthetic dyes are produced globally, and nearly 280,000 tons of these dyes are discharged into textile effluents [1,2]. Because of their non-biodegradable nature, these dyes block sunlight penetration in water, hindering photosynthesis and thereby impacting aquatic plants and animals [3]. Dyes possess a stable structure that is resistant to biodegradation, and they exhibit toxic, mutagenic, and carcinogenic properties.

Malachite Green (MG) is a dark green, crystalline, water-soluble cationic (basic) dye, chemically known as N-methylated diaminotriphenylmethane [4]. Malachite Green is among the most widely used dyes across various industries, including dyeing, papermaking, pharmaceuticals, and cosmetics [5]. Several studies have reported the cytotoxic effects of Malachite Green on cells from various organisms, including humans. Moreover, its potential to induce carcinogenesis, teratogenesis, and mutagenesis has also been observed in human cells [4,6]. Various biological and physicochemical techniques have been applied to dye wastewater degra-

dation and degradation. Coagulation, flocculation, adsorption, ion exchange, precipitation, and photodegradation are examples of physico-chemical techniques that have been effectively used [7-9]. However, these methods have certain limitations, including high costs and the generation of sludge, which can lead to secondary pollution. Currently, biological approaches employing microorganisms are considered an attractive, eco-friendly, and cost-effective option for wastewater treatment [7]. Various bacterial strains have demonstrated high efficiency in the degradation and degradation of Malachite green dye including *Kocuria rosea* MTCC 1532, *Sphingomonas paucinabilis*, *Brevibacillus laterosporus*, *Pseudomonas* sp. DY1, *Klebsiella terrigena* PTCC, *Ochrobactrum* sp. JN214485, *Pseudomonas* sp. YB2, *Bacillus vietnamensis* sp. MSB17, *Pseudomonas veronii* JW3-6, *Stenotrophomonas maltophilia, Pseudomonas geniculata, Bacillus altitudinis, Bacillus subtilis,* and *Citrobacter freundii* [7,10-17]. In the present study, different bacteria were isolated from the drainage effluent, and their potential to degrade the malachite green dye was evaluated. The potential bacteria were identified so that they can be effectively utilized commercially and industrially for the degradation of malachite green.

Materials and Methodology

Materials

Nutrient agar, nutrient broth, malachite green dye, Tris HCl, Tris Base, EDTA, Phenol, Chloroform, molecular grade water, and agarose used in this study were purchased from Hi-Media, Mumbai, India. Taq DNA polymerase, Taq buffer, dNTPs, and MgCl2 were purchased from DSS Takara Bio India Pvt. Ltd., New Delhi (India).

Sample collection

Effluent was taken from the drainage canals of New Market, Bhopal (Madhya Pradesh). Approximately 1000 mL of effluent was collected from each site using sterile glass bottles.

Physico-chemical analysis of effluent water

The physico-chemical analysis of the collected water sample was done by testing its Appearance, Odour, Temperature, pH, Electrical Conductivity, Turbidity, Colour, Dissolved oxygen (DO), Chemical oxygen demand (COD), Biological oxygen demand (BOD), Total Solid, Total dissolved solids (TDS), Total Hardness as $CaCO_3$, Phosphate as PO_4, Alkalinity, Chloride, Sulphate as SO_4, Total Suspended Solid.

Isolation of Bacteria, Colony characteristics and microscopic observation

The collected sample was serially diluted to the concentration of 10-9 and plated on a nutrient agar medium. The plates were incubated at 35 ± 2°C for 24 h. Later, the plates were observed for the presence of bacterial colonies which were purified and stored on NAM slants at 4°C. After the isolation of seven dye degrading bacterial isolates, their colony characteristics and microscopic were observed. Fresh cultures of the isolates were used to study colony characteristics, gram reaction and cell morphology. The study of colony characteristics was determined according to Engelkirk and Duben-Engelkirk (2008) and smear preparation by Gram's staining [18].

Primary screening for malachite green dye decolorization in liquid media

Each bacterial isolate was inoculated into 10 mL nutrient broth containing malachite green dye. The test tubes were incubated at 35 ± 2°C on incubator for 72 h after which the culture broth was centrifuged at 2000×g for 30 min and absorbance value of supernatant was measured spectrophotometrically at 620 nm (UV-VIS Double Beam Spectrophotometer 2201, SYSTRONICS). Potential bacterial isolates were selected for further studies. The percentage of dye decolorization was calculated using the following formula:

Genomic DNA extraction and molecular identification of potential isolates

Genomic DNA was extracted using the boiling lysis method. Briefly, freshly grown bacterial cultures were centrifuged at 6000 rpm for 10 minutes, and the pellets were suspended in sterile distilled water. The suspension was heated at 95°C for 10 minutes, followed by rapid cooling on ice. The lysate was centrifuged, and the supernatant containing DNA was collected and stored at –20°C. 16S rRNA regions of the selected potential bacterial isolates were amplified using PCR (BIO-RAD T-100 Thermocycler). The 50 µl reaction mixture contained 2 µl bacterial DNA, 5µl buffer (Takara), 0.25 µl 5U/µl Taq polymerase (Takara), 0.5 µl 10 mM dNTPs (Takara), 0.5 µl (10 pmol) each of the universal 16S primers 25F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) were used for amplification. PCR reaction cycles included primary denaturation at 92ºC for 2 min, followed by 35 cycles of denaturation at 92ºC for 1 min, annealing at 48ºC for 30 sec, and extension at 72ºC for 2 min. The final extension was done at 72ºC for 6

min. The amplified PCR products were analyzed using 1% agarose gel containing ethidium bromide. The amplified 16S rRNA was sequenced through universal 16S rRNA primers from Hi-Media, Mumbai (India).

Sequence and phylogenetic analysis

The most indistinguishable sequences of the strain were identified from the NCBI database of Genbank using the BLAST algorithm for homology were performed using the ClustalW algorithm software. The phylogenetic tree was constructed using the sequence of bacterial isolate with closely related species by MEGA v11 software [19].

Optimization of malachite green dye decolorization by bacterial isolates

To enhance the Malachite green decolorization efficiency of the selected bacterial isolates. Different parameters such as pH, temperature and malachite green dye concentration were optimized for maximum decolorization of malachite green. To observe the effect of different pH, temperature and malachite green dye concentration on malachite green dye decolorization by bacterial culture pH range was from 3, 5, 7, 9 and 11 were adjusted in each test tubes of nutrient broth with the help of 1 N HCl and 1N NaOH. Different temperatures 25°C, 30°C, 35°C, and 40°C were selected for observation of dye decolorization by bacterial isolates. Bacterial isolates were grown for different dye concentration 50, 100, 150, 200 and 250 mg/L in nutrient broth medium for the optimization of dye decolorization.

Quantitative assay of malachite dye decolorization

The potential isolates were introduced in nutrient broth containing of malachite green dye. The tubes were incubated at 35ºC and observed regularly for dye decolorization till 96 h after which the culture broth was centrifuged at 2000×g for 30 min and absorbance value of supernatant was measured spectrophotometrically at 620 nm.

Results and Discussion

Sample collection and physico-chemical analysis

Drainage effluent sample were taken from the drainage canals of New Market, Bhopal, and placed in 1000 ml sterilized plastic bottle screen for bacteria capable of dye degrading, we collected one sample from a drainage canal with stagnant effluent. The samples were immediately transported to the laboratory under cooled conditions and processed within 24 hours to maintain microbial integrity. Each sample was labelled with sample code and date of collection. The collected water sample was analyzed for physico-chemical characteristics (Table 1) [20].

Table 1: Physico-chemical analysis of drainage effluent.

S. No.	Physicochemical Parameter	Effluent Sample	Bureau of Indian Standard (IS 10500: 2012) for drinking water	
			Acceptable	Cause of rejection
1.	Appearance	Blackish Brown	Clear	Not Clear
2.	Odour	Not agreeable	Agreeable	Not Agreeable
3.	Temperature (°C)	30.4	-	-
4.	Potential of Hydrogen (pH)	6.9	6.5- 8.5	Not acceptable
5.	Electrical Conductivity (mS/cm)	4.9	-	-
6.	Turbidity (NTU)	8	1	5
7.	Colour (Hazen)	698	5	15
8.	Dissolve Oxygen (mg/L)	1.2	6-8	Below 6
9.	Chemical Oxygen Demand (mg/L)	201	Not acceptable	Not acceptable
10.	Biological Oxygen Demand (mg/L)	19	Not acceptable	Not acceptable
11.	Total Suspended Solid (mg/L)	4100	30	Above 30
12.	Total Solid (mg/L)	856	-	-
13.	Total Dissolved Solid (mg/L)	3415	500	2000
14.	Total Hardness as $CaCO_3$ (mg/L)	145	200	600
15.	Phosphate as PO_4 (mg/L)	65	Not acceptable	Not acceptable
16.	Alkalinity (mg/L)	674	200	600
17.	Chloride (mg/L)	1781	250	1000
18.	Sulphate as SO_4 (mg/L)	145	200	400

Isolation of Bacteria, Colony characteristics and microscopic morphology observation

Seven bacterial isolates were isolated from a textile effluent sample. All the bacterial isolates were tested for their ability to degrade malachite green dyes. All the selected bacterial isolates were named BS1 to BS7. The gram staining and microscopic analysis revealed 4 isolates to be gram-negative and 3 isolates gram-negative bacteria (Table 2).

Table 2: Morphological Characteristics of Bacterial Isolates.

Isolate Code	Margin	Shape	Colour	Elevation	Size	Texture	Opacity
BS1	Filamentous	Rhizoid	Creamy	Raised	Large	Shiny	Opaque
BS2	Entire	Round	Creamy	Flat	Moderate	Shiny	Opaque
BS3	Filamentous	Filamentous	Creamy	Flat	Large	Mucoid	Opaque
BS4	Undulate	Irregular	Creamy	Umbonate	Large	Shiny	Opaque
BS5	Undulate	Irregular	Creamy	Convex	Large	Mucoid	Opaque
BS6	Undulate	Irregular	White	Convex	Moderate	Shiny	Opaque
BS7	Undulate	Irregular	Creamy	Flat	Large	Shiny	Opaque

Primary screening for malachite green dye decolorization in liquid media

Bacterial isolates were tested for their ability to degrade malachite green dye. All seven bacterial isolates (BS1, BS2, BS3, BS4, BS5, BS6 and BS7) were inoculated on nutrient broth medium containing 100 mg/L malachite green dye. The test tubes were incubated at 35 ± 2°C for 24 h (Figure 1a and 1b).

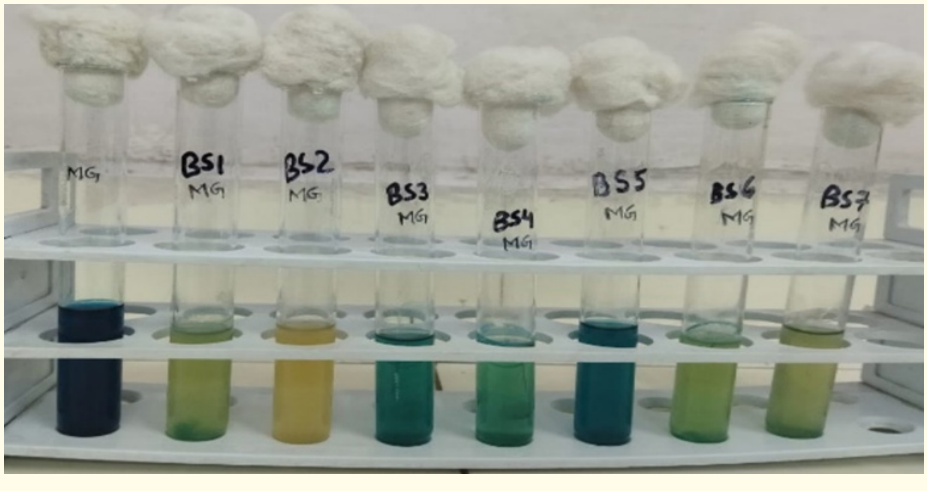

Figure 1a: Primary Screening of bacterial isolates.

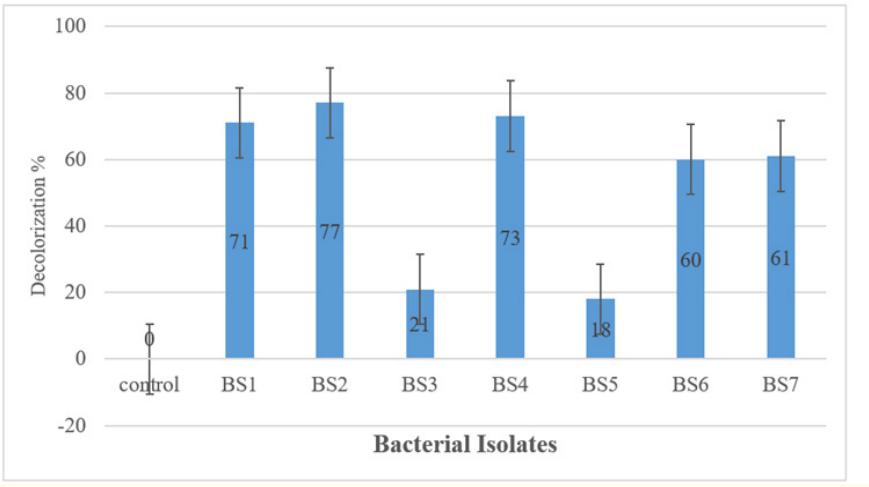

Figure 1b: Different bacterial isolates showing decolorization of malachite green.

Identification of Bacterial Isolate by 16S rRNA Sequencing and phylogenetic analysis

The potential bacterial isolates were identified based on the partial sequences of the 16S rRNA gene. The bacterial isolates BS2 and BS4 were identified as *Pseudomonas otitidis* and *Aeromonas hydrophila* respectively based on BLASTn. The 16S rRNA partial sequences of the isolate were submitted to the NCBI GenBank under accession no. PX275544 (BS2), and PX275546 (BS4). The phylogenetic tree of potential isolates was constructed with closely related sequences obtained from BLASTn (Figure 2).

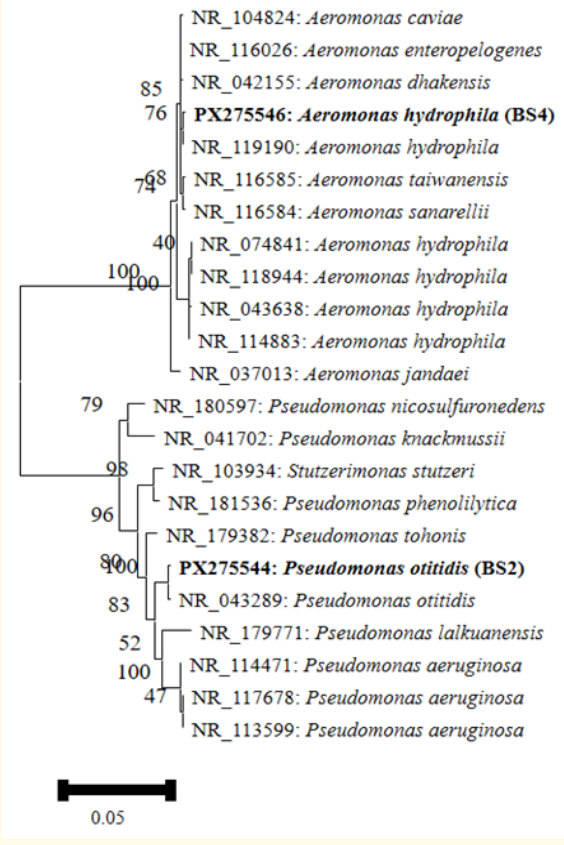

Figure 2: The Neighbor-Joining tree for bacterial strains was constructed from partial 16S rRNA gene sequences, with genetic distances estimated using the Kimura 2-parameter model in MEGA v11 software.

Optimization of malachite green dye decolorization by bacterial isolates

Optimization experiments were conducted to determine the most effective conditions for malachite green dye decolorization by the selected bacterial isolates *Pseudomonas otitidis* and *Aeromonas hydrophila*. Three key parameters were tested: pH, temperature and dye concentration. The results showed that decolorization efficiency was influenced by all the tested factors.

Effect of pH on malachite green dye decolorization

In this study, Figure 3 shows the maximum decolorization activity was noted at pH 3–11. Bacterial isolates BS2 and BS4 showed the maximum decolorization at pH 7 (77% and 72% decolorization respectively after 72 h). However, at pH 5, 7 and pH 9 a valuable MG decolorization was obtained (BS2 69%, 77% and 59% and BS4 58, 48, 69 and 59 respectively after 72 h).

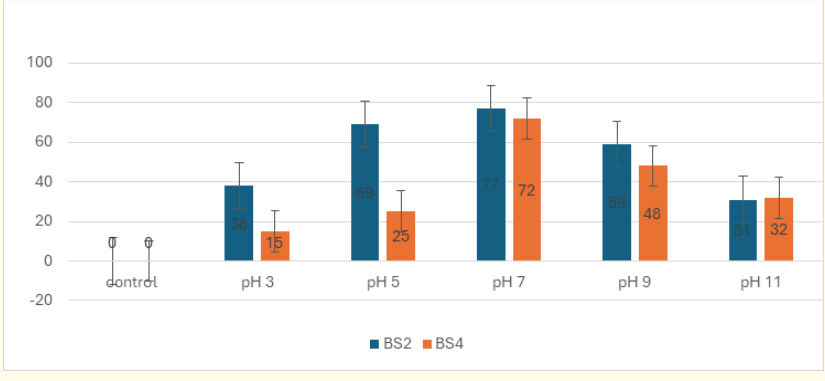

Figure 3: Effect of pH dependent decolorization of malachite green.

Although specific pH data was not detailed in the original results, based on standard optimization practices, pH would typically be assessed in the range of 5.0 to 9.0. Maximum decolorization is generally expected around neutral to slightly alkaline pH [21]. Vijayalakshmidevi and Muthukumar (2014) observed that *Ochrobactrum* sp. JN214485 showed maximum MG decolorization at pH 6 [13]. Similarly, Du., *et al*. (2011) reported efficient MG decolorization by *Pseudomonas* sp. strain DY1 at pH 6.6 [11]. Song., *et al*. (2020) found that *Pseudomonas veronii* JW3-6 showed the highest decolorization activity within the pH range of 5–7, with an optimum at pH 7 [16]. Consistent with these findings, the best MG decolorization by *Stenotrophomonas maltophilia* was observed at pH 6–7 [17]. However, Tao., *et al*. (2017) reported that MG decolorization by *Pseudomonas* sp. YB2 was independent of pH, showing complete decolorization across a broad pH range of 5–9 [14]. El-Bendary., *et al.,* (2023) observed that *Pseudomonas plecoglossicide* MG2 showed the maximum decolorization at pH 6–7 [4].

Effect of temperature on malachite green dye decolorization

Incubation temperature influences microbial growth and enzyme activity, thereby impacting the rate of dye decolorization [22]. The MG degradation efficiency of bacterial isolates BS2 and BS4 was evaluated at different temperatures, as shown in Figure 4. The results indicated that the highest degradation activity occurred between 25 to 35 °C, with maximum efficiency at 35 °C (78% for isolate BS2 and 73% for BS4 after 72 h). At this temperature, a significant percentage of decolorization was achieved. In contrast, reduced decolorization was observed at 45 °C, with 42% for BS2 and 45% for BS4.

Du., *et al*. (2011) reported that *Pseudomonas* sp. strain DY1 efficiently decolorized MG (90–97%) at 28–30 °C [11]. Similarly, *Pseudomonas veronii* JW3-6 showed the highest decolorization activity (92.9%) at 30 °C, which decreased to 70.1% at 40 °C [16]. Alaya., *et al*. (2021) also found that *Stenotrophomonas maltophilia* achieved

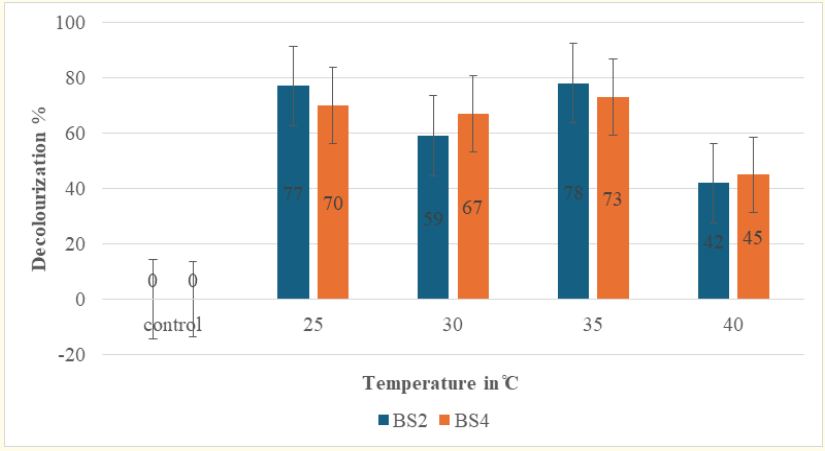

Figure 4: Effect of temperature on dye decolorization efficiency.

effective MG decolorization within 25–30 °C, while efficiency declined above 35 °C [17]. Moreover, the optimum decolorization of MG coincided with the maximum growth of *Pseudomonas* sp. YB2 [14] (Tao., *et al*., 2017) and *Ochrobactrum* sp. JN214485 [13] at 30 °C. El-Bendary., *et al*. (2023) reported that *Pseudomonas plecoglossicida* MG2 exhibited decolorization activity between 30 and 35 °C, with maximum efficiency (91.5%) at 35 °C after 96 h [4].

Effect of dye concentration on malachite green dye decolorization

MG decolorization was evaluated at different initial concentrations of dye (50–250 mg/L). As shown in Figure 5, isolates BS2 and BS4 demonstrated maximum decolorization efficiency at 50–150 mg/L, with nearly complete removal after 72 h at 50 mg/L (81% by BS2 and 62% by BS4). At 100 mg/L, decolorization reached 78%

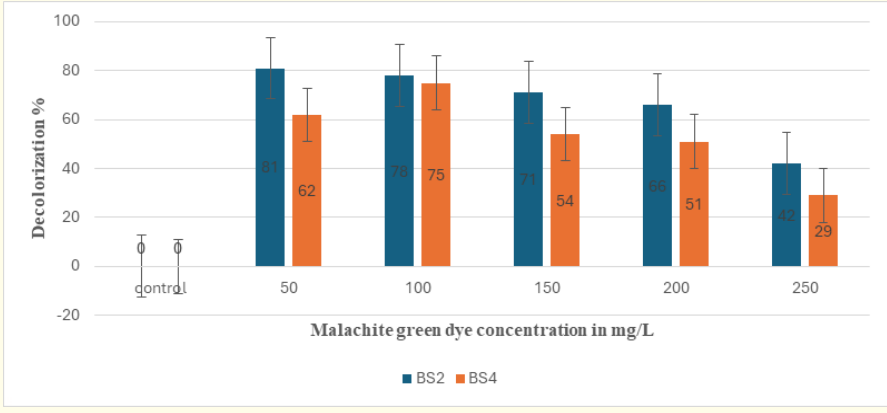

Figure 5: Effect of malachite green dye concentration dependent decolorization.

(BS2) and 75% (BS4), while at 150 mg/L, it was 71% (BS2) and 54% (BS4). A further decline was observed at 200 mg/L, with 66% (BS2) and 51% (BS4). At the highest concentration (250 mg/L), decolorization efficiency decreased markedly to 42% (BS2) and 29% (BS4) after 72 h.

Tony., *et al*. (2009) reported that the decolorization rate declined progressively with increasing dye concentration, likely due to the toxic effects of the dye on bacterial cells or improper binding of dye molecules to enzyme active sites [23]. Consistent with this observation, higher concentrations of MG were found to inhibit

the growth of *Kocuria rosea* MTCC 1532 [10], *Pseudomonas veronii* JW3-6 [16], *Bacillus vietnamensis* MSB17 [15], and *Pseudomonas plecoglossicida* MG2 [4].

Biodecolorization assay of malachite green dye by bacterial isolates

As pH 7 and temperature 35°C were optimized for MG dye decolorization and at these defined pH, temperature and 100 mg/L dye concentration was used for biodegradation assay. The dye decolorization curves (Figure 6a and 6b) revealed that the two potential bacterial isolates *Pseudomonas otitidis* (81% at 96 h) and Aeromonas hydrophila (78% at 96 h).

Joshi and Mhatre (2015) reported maximum degradation of MG dye by *Enterobacter* sp. at pH 7 and 37 °C, which was close to the current study's conditions [24]. Similarly, Du., *et al*. (2011) found

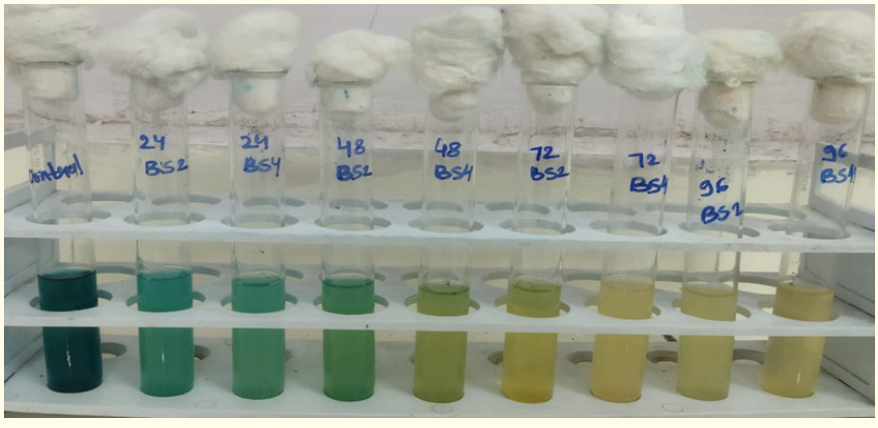

Figure 6a: Biodecolorization assay of malachite green dye by potential bacterial isolates.

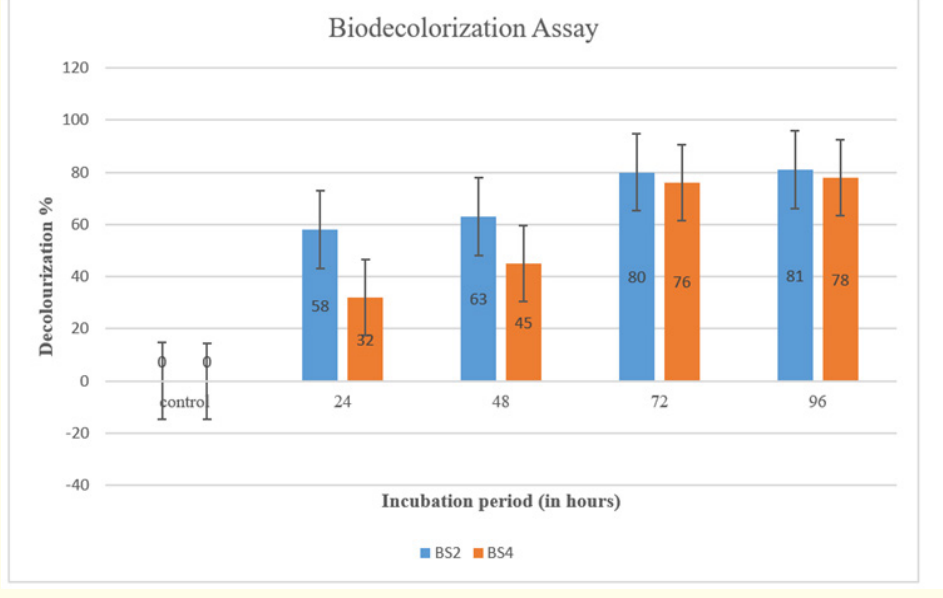

Figure 6b: Malachite green dye decolorization percentage by potential bacterial isolates.

that *Pseudomonas* sp. strain DY1 exhibited optimal MG dye degradation at pH 6.6 and 28–30 °C [11]. The effect of different dye concentrations (15, 30, and 50 mg/L) was also investigated, revealing that higher concentrations led to reduced degradation rates (Figure), a trend consistent with the findings of Wanyonyi., *et al*. (2017) [25]. Furthermore, El-Bendary., *et al*. (2023) reported that *Pseudomonas plecoglossicide* MG2 achieved 90–93% degradation of MG dye under static aerobic conditions at pH 6–7, inoculum size 4–6%, and incubation temperature 30–35 °C [4].

Conclusion

The study successfully isolated seven distinct bacterial strains from a drainage effluent using serial dilution and spread plating techniques. Primary screening revealed that five out of seven isolates are capable to degrade MG dye. But particularly *Pseudomonas otitidis* and *Aeromonas hydrophila*, demonstrated significant potential for malachite green dye decolorization. Notably, isolate BS2 exhibited the highest decolorization efficiency, reaching 80%, followed closely by BS4 with 76%, both within a 72-hour incubation period at 35°C, as confirmed by decolorization assay which shows that both isolates highlight their promise for future use in eco-friendly bioremediation strategies. Gram staining revealed that both selected isolates, *Pseudomonas otitidis* and Aeromonas hydrophila, are Gram-Negative. BLASTn analysis confirmed ≥ 99.864 and 100 Percentage sequence similarity with known bacterial strains. The identified species belonged to the genera *Pseudomonas_otitidis* and, *Aeromonas_hydrophila*. The optimization results showed that both isolates exhibited maximum decolorization at 35°C with a dye concentration of 100 mg/L, where *Pseudomonas otitidis* achieved 80% decolorization and Aeromonas hydrophila reached 76%. Maximum decolorization is generally expected around neutral to slightly alkaline that is (7-9) pH to 100 mg/L. *Pseudomonas otitidis* bacteria shown highest decolorization in 50 mg/L concentration up to 81% of decolorization, a sharp decline in degradation was observed, with only 42% removal at 250 mg/L. Aeromonas hydrophila maintained high decolorization efficiency up to 50 mg/L with 62% decolourization of MG dye. Thus we can conclude that these bacteria (*Pseudomonas_otitidis* and, *Aeromonas_hydrophila*) are more useful to manage wastewater.

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Conflict of Interests

The authors declare they have no conflict of interest.

ORCID ID

- Sheetal Sahare: https://orcid.org/0009-0000-8864-4318
- Dr. Ram Prasad Kushvaha: https://orcid.org/0000-0003-4194-3972
- Dr. Renu Mishra: https://orcid.org/0009-0000-8864-4318
- Dr. Sunil Kumar Snehi: https://orcid.org/0000-0002-0624-1943

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