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Microbial Decolorization of Crystal Violet Dye by a Native Multi-Metal Tolerant *Aeromonas caviae* MT-1 Isolate from Dye-Contaminated Soil: Optimization and Phytotoxicity Study

Manikant Tripathi[1]*, Neelam Pathak[2], Vinod Kumar Chaudhary[3], Pankaj Singh[1], Pradeep Kumar Singh[2], Banjagere Veerabhadrappa Thirumalesh[4,5], Saroj Bala[6], Avnish Kumar Maurya[2], Naveen Patel[7] and Brijesh Kumar Yadav[3]

[1]Biotechnology Program, Dr. Rammanohar Lohia Avadh University, Ayodhya - 224001, Uttar Pradesh, India; manikant@rmlau.ac.in; manikant.microbio@gmail.com

[2]Department of Biochemistry, Dr. Rammanohar Lohia Avadh University, Ayodhya - 224001, Uttar Pradesh, India

[3]Department of Environmental Sciences, Dr. Rammanohar Lohia Avadh University, Ayodhya - 224001, Uttar Pradesh, India

[4]Microbial Processes and Technology Division, CSIR-National Institute for Interdisciplinary Science and Technology, Thiruvananthapuram - 695019, Kerala, India

[5]Academic of Scientific and Innovation Research (AcSIR), Ghaziabad - 201002, Uttar Pradesh, India

[6]Department of Microbiology, Punjab Agriculture University, Ludhiana - 141001, Punjab, India

[7]Department of Civil Engineering, IET, Dr. Rammanohar Lohia Avadh University, Ayodhya - 224001, Uttar Pradesh, India

Abstract

In the recent past, one of the main environmental issues is the contamination of textile dye wastes. The toxicity of dyes poses adverse effects on the flora and fauna of the ecosystem. The present study aimed to isolate bacteria that decolourize crystal violet dye, optimization of various environmental factors for effective decolourization, and phytotoxicity analysis. Out of 13 isolated bacteria, a single isolate was able to grow at 250 mg/L crystal violet dye concentration in a synthetic medium and identified as *Aeromonas caviae* MT-1 strain (accession number; LC720408) using morphological, biochemical and molecular analyses. Presumably, this is the first report of crystal violet dye decolourization by a native *Aeromonas caviae* isolate. In this study, after a 72-hour incubation period, a maximum of 98.0% dye decolourization was observed at neutral pH and 35°C with 5% v/v bacterial culture under static culture conditions. Dye decolourization was inhibited to a significant degree by the rising of its concentration. UV-Vis spectra analysis of samples before and after decolourization showed the possible degradation of crystal violet dye by *A. caviae*. The strain MT-1 was also tolerant to toxic heavy metals like arsenic, lead, and chromium. Phytotoxicity tests revealed that decolourized dye products inhibited *Vigna radiata* growth less than the un-decolourized dye solution. The findings revealed that a native multi-metal tolerant *A. caviae* MT-1 isolate could decolourize crystal violet dye rapidly, and possibly have the ability for extensive treatment of dye-contaminated waste.

Keywords: *Aeromonas caviae,* Crystal Violet Dye, Decolorization, Heavy Metals, Phytotoxicity, Waste Treatment

**Author for correspondence*

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1. Introduction

Coloured wastewater released from textile industries contaminates soil and aquatic system. The effluent contains various types of dyes and inorganic and organic pollutants which adversely affect the ecosystem. Such toxicants pose health problems to humans and animals. The toxic and carcinogenic nature of various types of dyes has been reported by many researchers[1,2]. Thus, they must be remediated from the effluent before its disposal. Remediation techniques using microbes or enzymatic methods gained attention for the bio-decolourization of dyes because these processes are effective, less costly and offer a green technology. The azo bond(s) are responsible for the dyes' resistance and colour, and the inclusion of reactive groups renders such dyes xenobiotic in nature[2]. The dyes are not only harmful to living things but also mutagenic and carcinogenic. As a result, dye(s) must be eventually degraded to save our ecosystem.

Several researchers reported the toxic and adverse effects of chemical pollutants on the ecosystem[3–5]. Crystal violet is a triphenylmethane dye which is used for various biological purposes. Research findings showed that crystal violet dye is mutagenic, carcinogenic, and regarded as a biohazard agent[2,6]. Prolonged exposure to crystal violet dye might result in irreversible blindness, and respiratory and kidney problems in humans[3,7]. The textile industry uses dyes. But, due to improper wastewater treatments, the discharged effluent from dye industries containing various types of toxic organic and inorganic pollutants including crystal violet mixed with water systems, and pose a serious threat to ecosystems. During dye processing, most of the water is discharged from the textile sector as coloured wastewater. The colourful effluents block sunlight from reaching the waterways[2]. Thus, proper treatment of such toxic dyes and other chemicals must be done before the discharge of the effluent into water bodies.

The physicochemical techniques used to decolourize dyes are less effective, more expensive, and produce secondary waste, which poses a disposal issue. It has also been tried to use conventional biological techniques alone or in conjunction with physical and/or chemical techniques. However, because of their effectiveness, low cost, less production of secondary sludge, and environmentally friendly nature, advanced biological methods have drawn more attention[8–10]. Microbial methods have been applied for the remediation of various organic and inorganic environmental pollutants[11,12]. According to reports, the bacterial treatment for decolourization/degradation of dyestuffs is a rapid and effective process[13]. There are several bacteria reported for decolourization of dye under their suitable cultural and nutritional conditions[14–19]. Every bacterial culture displayed distinct nutritional and cultural needs. Thus, for the treatment of coloured pollutants, cultural and nutritional conditions should be optimized for knowing a suitable environmental situation for the best dye decolourization. The microorganisms that are isolated from soil contaminated with dye are useful for bioremediation operations[9,20]. Interest in microorganisms that can decolourize dye at a high pace under anoxic/microaerophilic environments has recently increased[15,19].

A phytotoxicity study is used to assess the toxic impact or damage caused by any chemical like dyes, metals, and other organic and inorganic contaminants present in the soil on the growth of plants. Thus, it is significant to explore the toxic effect of dyes and decolourized metabolites on plant growth. The present investigation was envisaged to optimize environmental conditions for effective discolouration of crystal violet dye and phytotoxicity analysis to assess the impact of decolourized samples on plant growth.

2. Materials and Methods

2.1 Sampling

The dye-contaminated different soil samples were collected near the discharge points, and power looms in Tanda, Ambedkar Nagar, Uttar Pradesh, India. Four different soil samples, approximately 10 cm in depth, from the soil surface were collected using a sterile spatula in sterile plastic bags. All samples were transported to the laboratory and preserved at 4°C in the refrigerator for further analysis.

2.2 Isolation and Screening of Potential Bacterial Isolates

Pour plate technique[21,22], was used to isolate dye-decolourizing bacterial isolates from contaminated soil on solid Minimal Salt Medium (MSM) containing (g/L); K_2HPO_4, 1.0; $CaCl_2$, 0.02; $FeCl_3$, 0.05; $MgSO_4.7H_2O$, 0.02; glucose, 4; ammonium nitrate, 2; agar, 20, supplemented with 50 mg/L crystal violet dye, and incubated at 35°C (REMI CIS-24 PLUS). By repeatedly streaking on the same medium, the isolates were purified. Additionally, the bacterial isolates were tested and screened on MSM agar plates with altered dye concentrations (50-300 mg/L) to identify the most effective bacterial isolate that was used for further research on dye bio-decolourization. The dye used in this study was purchased from Sigma-Aldrich (United States).

2.3 Identification of Potential Crystal Violet Dye-Decolourizing Bacterium

Morphological and biochemical characterization was used to identify the potential bacterial isolate[23]. After 24 h of incubation at 35°C, morphological and biochemical characterization was done by studying bacterial morphology, motility test, presence of spore, Gram's staining, endospore staining, catalase, carbohydrate utilization test, H_2S production and other biochemical examinations. Further 16S rRNA gene sequence amplification was done for bacterial identification at the molecular level. Molecular identification was carried out at Cytogene Research Lab, Lucknow (India). The obtained 16S rRNA partial gene sequence was submitted to the DNA Data Bank of Japan, and bacterial phylogeny was constructed using the neighbour-joining method[24], to know the evolutionary relationship.

2.4 Inoculum Preparation

The bacterial inoculum was prepared for dye-decolourization study in nutrient broth medium in 250 ml Erlenmeyer flasks by transferring a loop full of culture, and incubating at 35°C for 24 h incubation period under shaking (120 rpm) condition.

2.5 Culture Conditions

The dye-containing medium was inoculated with selected potential bacterial isolate at 4.0% (v/v; OD A_{620} 1.07) having colony-forming units $2.9x10^6$ per ml and incubated at 35°C for 96 h. The experiments were performed in triplicates. Periodically, media samples were taken out and their dye content and cell density were examined at 24 h intervals for measurement of dye- decolourization and bacterial growth, spectrophotometrically.

2.6 Effect of Shaking and Static Culture Conditions

The dye decolourization was studied under shaking (120 rpm) and static culture conditions in an Erlenmeyer flask and screw-capped tubes, respectively containing broth medium and which was processed as above.

2.7 Effect of Dye Concentration

A varied concentration of crystal violet dye from 50 to 250 mg/L was added in medium broth and processed as above under no shaking conditions to see how different initial dye concentrations affected the decolourization of the chosen bacterial isolate.

2.8 Effect of Carbon and Nitrogen Sources

To determine the effect of carbon (viz., glucose, sucrose and starch) at 1.0% (w/v) and nitrogen (viz., ammonium sulfate, ammonium chloride and peptone) sources at 0.2% (w/v) on the extent of dye-decolourization were evaluated to find the appropriate source of carbon and nitrogen for effective and maximum dye-decolourization.

2.9 Effect of Inoculum Size

An appropriate dose of bacterial culture is important for efficient bioremediation. In this study, the nutritionally optimized medium was inoculated with varied inoculum sizes of 1.0-6.0% (v/v). The MSM (pH 7.0) containing optimal dye concentration (100 mg/L) was incubated under static conditions for 72 h incubation.

2.10 Multi-metal Tolerance Test and Minimum Inhibitory Concentration Determination

The selected MT-1 isolate was also tested for tolerance to heavy metals like arsenic, chromium, lead and mercury. The bacterium was inoculated on the varied concentration of metals (25-200 mg/L) in a medium (nutrient agar), for 24 h at 35°C. The experiments were performed in triplicates.

2.11 Phytotoxicity Study

This experiment was carried out to assess the impact of dye before and after decolourization on the plant's (*Vigna radiata*) growth under laboratory conditions at room temperature. To initiate the experiment, the air-dried soil was artificially polluted with 100 mg/kg crystal violet dye solution along with control air-dried soil which is not contaminated with dye. Also, the decolourized sample was uniformly mixed with air-dried soil for the stabilization of dye-decolourized metabolites with soil. The experiments were performed in three combinations; SET-I (Soil without dye), SET-II (Soil with dye) and SET-III (soil with decolourized sample). The growth parameters were measured on the 10^{th} day of the plant growth in all the treatments. The shoot and root length were measured using a standard scale and values were recorded in centimetres. The dried weight of the shoot and root parts of the plant were determined with the help of electronic balance. The experiments were carried out in triplicates.

3. Analytical Determinations

3.1 Dye-Decolourization Assay

To determine, the extent of per cent colour removal before and after dye-decolourization, the supernatant samples drawn after centrifuged at 10,000 rpm in a cooling centrifuge at 4°C (REMI CPR-24 PLUS) for 10 min were analyzed at 588 nm, spectrophotometrically (LABTRONICS Model LT-2910). Per cent decolourization was calculated using the following formula[8,15]:

$$\text{Decolorization extent (\%)} = \frac{\text{Initial absorbance - Final absorbance}}{\text{Initial absorbance}} \times 100$$

3.2 Bacterial Growth

During decolourization, the growth of the bacterium was measured spectrophotometrically at 620 nm at every 24 h interval as per the method described by Garg *et al*[15]. For measuring growth, 1.0 ml broth samples were centrifuged, the supernatant decanted, washed the pellets that were resuspended in distilled water for the growth study.

3.3 UV-Vis Spectral Analyses

The bacterial discolouration of crystal violet dye was investigated by UV-Vis spectra before and after dye-decolourization between 450 to 700 nm. This experiment was performed to ensure colour removal by selected and identified bacterial isolates.

3.4 Statistical Analyses

A decolourization study was performed in triplicates. Using the Microsoft Excel application, the standard deviation was calculated, and the findings are shown as mean standard deviation values (of three observations).

4. Results

4.1 Isolation and Screening of Crystal Violet Decolorizing Bacteria

The microbial analysis of dye-contaminated soil revealed the presence of bacteria which were able to decolourize crystal violet dye. A total of 13 bacterial isolates were grown on minimal salt agar medium at 50 mg/L crystal violet dye, while a single isolate was grown at 250 mg/L concentration of dye, with glucose as a co-substrate (Table 1). Whereas no bacterial isolates were grown above 250 mg/L crystal violet dye concentration.

Table 1. Screening of crystal violet dye-decolourizing bacteria

Dye concentration (mg/L)	Number of bacterial isolates grown
50	13
100	5
150	2
200	1
250	1
300	-

4.2 Identification of potential bacterial isolate

In our laboratory, the crystal violet dye-decolourizing bacterium was identified by examining its morphological and biochemical features by Bergey's Manual of Determinative Bacteriology (Table 2).

Table 2. Morphological and biochemical characteristics of bacterial isolate

Bacterial characteristics	Observation
Shape	Small rods
Motility	+
Spore	-
Gram's staining	Gram-negative
Catalase activity	+
Citrate	+
Gas production	-
H_2S production	-
Voges-Proskauer	-
Carbohydrate utilization Glucose Sucrose Starch	 + + +

- : for negative reactions

+ : for positive reactions

The 16S rRNA partial gene sequence of the isolate, 1340 bp, was deposited to the DNA Databank of Japan (DDBJ) with the accession number LC720408. The culture showed the most resemblance to *Aeromonas caviae* when the acquired sequence was subjected to a BLAST search. Figure 1 shows the phylogenetic tree created using the Neighbour Joining (NJ) method.

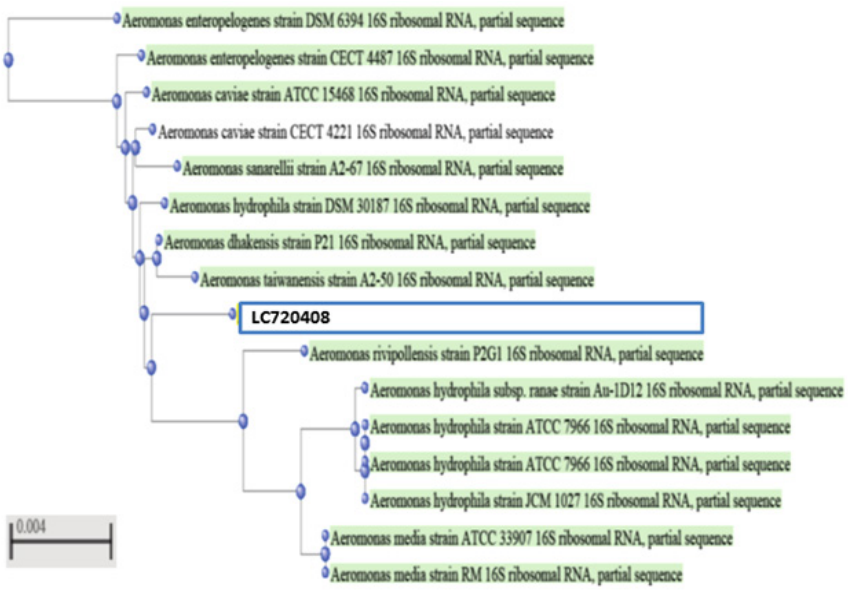


Figure 1. Phylogenetic tree of MT-1 strain (accession number LC720408).

4.3 Effect of aeration

The results reveal better decolourization efficiency of bacterial isolate under static conditions compared to shaking culture conditions (Figure 2). Under the static condition, a maximum of 98.2% dye-decolourization was reported at 72 h incubation. However, a maximum of 47% decolourization was reported under shaking culture conditions (at 48 h incubation), whereas no increase in decolourization was observed at 72 h under shaking. Thus, further experiments were performed with no shaking.

4.4 Effect of Dye Concentration

Figure 3 showed per cent crystal violet dye-discolouration at different time intervals. The findings revealed that with rising dye levels, discolouration of crystal violet was decreased which reveals the inhibitory effect of higher dye concentration. Maximum 86.2% crystal violet dye decolourization was observed at 100 mg/L concentration at 72 h incubation, whereas, the extent of dye-decolourization was slightly better at 50 mg/L concentration. Figure 3 also reveals that there was a marginal increase in per cent decolourization from 72 to 96 h. Thus, further study was performed at a higher dye concentration (100 mg/L) for 72 h incubation.

4.5 Effect of Carbon and Nitrogen Sources

Figures 4 and 5 depict the influence of different carbon and

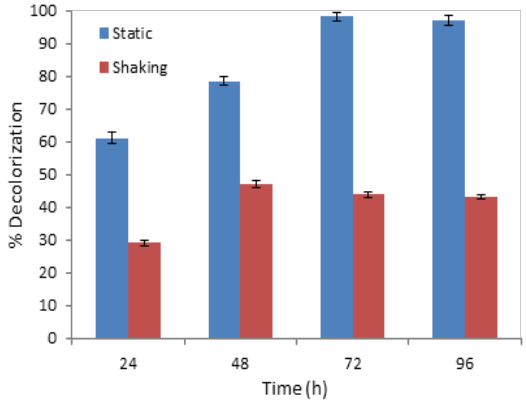


Figure 2. Effect of static and shaking on crystal violet discolouration at initial 50 mg/L concentration.

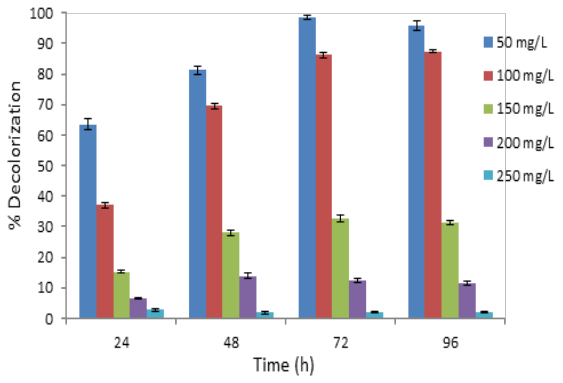


Figure 3. Influence of crystal violet dye concentration on the extent of decolourization.

nitrogen sources to the extent of dye- discolouration under optimized 100 mg/L crystal violet concentration, 4.0% v/v bacterial culture at pH 7.0 and 35°C under no shaking conditions during 72 h incubation. Maximum decolourization (86.5%) was evident with glucose as a co-substrate at 1.0% (w/v) level during 72 h incubation following sucrose and starch, respectively (Figure 4). Whereas among different nitrogen sources tested, ammonium sulfate was found to be the best nitrogen source for maximum decolourization (94.5%) of dye (Figure 5).

4.6 Effect of Inoculum Size

The results shown in Figure 6 revealed that during the incubation period of 24-72 h, the dye- decolourization increased with time at each dose of bacterial culture (1.0–6.0%, v/v) under study. However, the per cent discolouration increased synchronously at each time up to 72 h as the inoculum size was raised from 1.0 to 5.0% (v/v). Maximum dye-decolourization was achieved with a 5% v/v inoculum

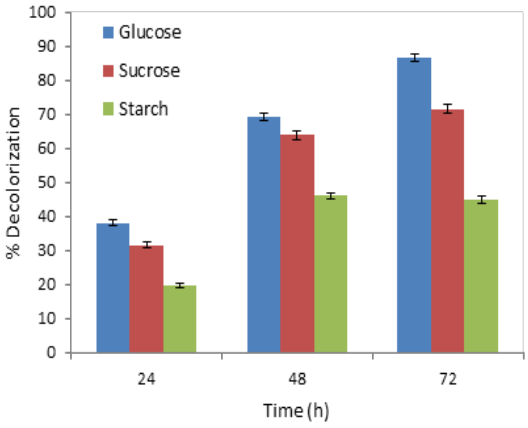


Figure 4. Effect of carbon sources on dye-decolourization.

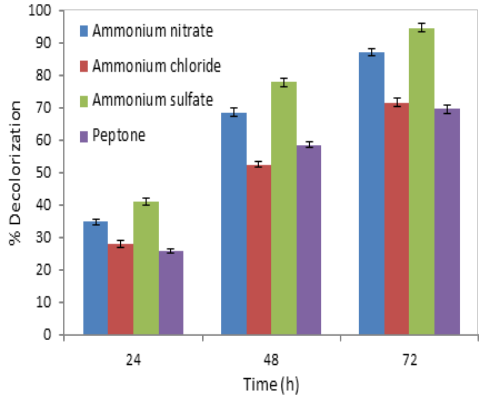


Figure 5. Effect of nitrogen sources on dye-decolourization.

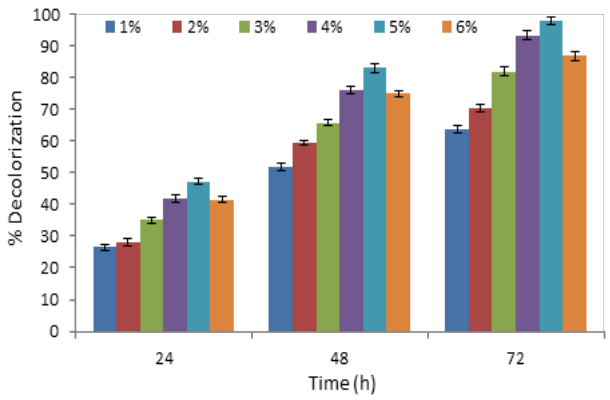


Figure 6. Effect of inoculum dose on crystal violet decolourization under optimized conditions.

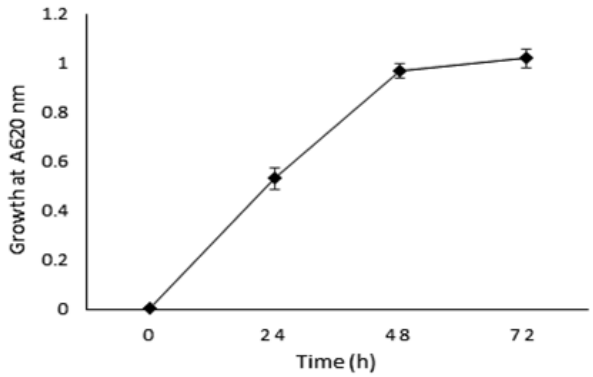


Figure 7. Growth study of crystal violet dye-decolourizing *A. caviae* isolates under optimized cultural conditions.

dose. But, the amount of dye-decolourization was lessened by increasing the inoculum dose further, from 5.0 to 6.0%.

Bacterial growth was also determined using an optimized 5% v/v inoculum dose, and it was found that the degree of dye-decolourization was directly correlated with bacterial growth. *A. caviae* growth was sharply increased from the 24 to 48 h incubation period. However, the maximum growth of the bacterium was achieved at 72 h (Figure 7).

4.7 Multi Heavy Metal Tolerance and MIC

The bacterium was found tolerant to different heavy metals. However, increasing levels of each heavy metal were inhibitory for the growth of the MT-1 bacterial isolate. The isolate was tolerant to arsenic, chromium and lead; but, unable to grow on an NA medium amended with mercury (Table 3). The results reveal that this dye-decolourizing bacterium can also be applied to detoxify such heavy metal-contaminated sites.

Table 3. Multi-heavy metal tolerance and minimum inhibitory concentration (MIC) determination of *Aeromonas caviae* isolate

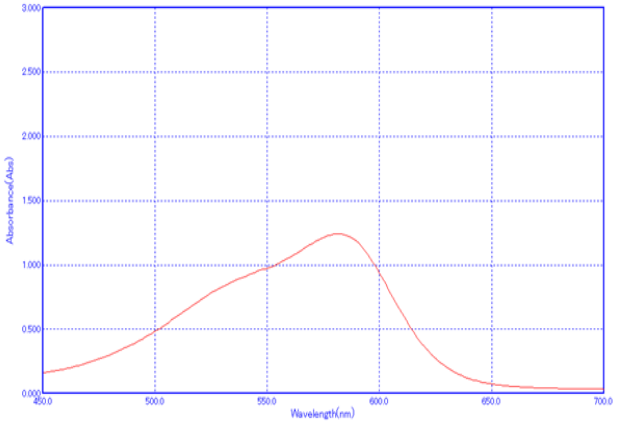
Concentration (mg/L)	Bacterial growth			
	Heavy metal salts			
	Sodium arsenate (As)	Potassium dichromate (Cr)	Lead acetate (Pb)	Mercuric chloride (Hg)
25	+	+	+	-
50	+	+	+	-
75	+	+	+	-
100	+	+	+	-
125	+	-	+	-
150	+	-	+	-
175	-	-	+	-
200	-	-	-	-

4.8 UV-Vis Spectra Study Of Crystal Violet Dye and its Decolourization

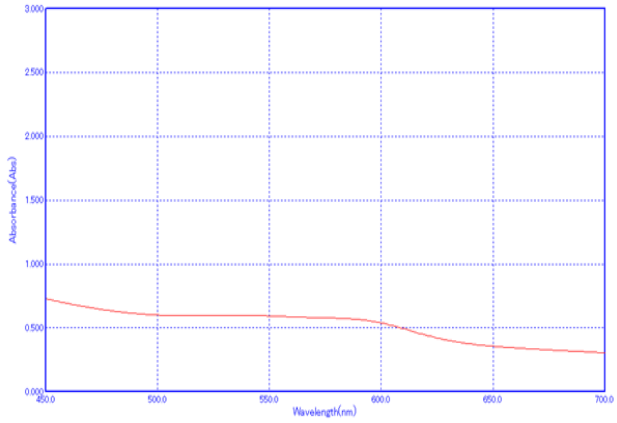
Dye industry wastewater and azo dyes undergo degradation to form harmless intermediate and colourless products. Dye and its decolourization were shown in Figure 8 which revealed the colour removal of crystal violet. In spectral analysis, the absorption maximum of the control (uninoculated dye containing supernatant) was observed at 588 nm and showed a peak at this wavelength (Figure 9 (a), (b)). However, the peak almost disappeared in inoculated decolourized samples. The peak height and area in Figure 9



Figure 8. Visual colour disappearance in decolourized crystal violet dye under optimized conditions.



(a) UV-Vis spectra of control.



(b) UV-Vis spectra of the decolourized sample.

Figure 9. UV-Vis spectral analyses of; **(a)** Crystal violet dye, and **(b)** Decolorized sample

Table 4. Phytotoxicity analysis of dye and its decolourized metabolites on *Vigna radiata* growth

SETS	Shoot length (cm)	Root length (cm)	Weight of shoot (g)	Weight of root (g)
Soil	18.5±0.2	2.0±0.15	0.1507±0.0008	0.0112±0.0003
Soil + dye	9.5±0.3	0.9±0.06	0.0319±0.0004	0.0025±0.0002
Soil mixed with decolourized dye	16.3±0.4	1.7±0.20	0.0795±0.0005	0.0085±0.0004

± Standard deviation

showed the decolourization of crystal violet dye. Presumably, the results revealed that crystal violet dye was degraded during its decolourization by *A. caviae*.

4.9 Phytotoxicity Study

This study was performed to assess the effect of decolourized dye sample, and crystal violet dye on plant growth at ambient temperature. The results are presented in Table 4. *Vigna radiata* growth was lesser in the presence of dye which reveals the toxic effect of dye on plant growth. However, the growth of the plant was better in soil mixed with the decolourized sample. Whereas, the growth of the plant in SET-I was comparable with detoxified samples. The experiments were performed in three combinations; SET-I (Soil without dye), SET-II (Soil with dye) and SET-III (soil with decolourized sample). The plant growth was measured at 10 days.

5. Discussion

Discharge of any wastewater without proper treatment in aquatic ecosystems poses serious threats to the environment and health of humans, animals and plants. The coloured wastewater discharged from textile industries is one of the major problems and its proper treatment is required for environmental protection[2,25]. There are different components found in textile effluent, but colours play the most influence on its toxicity. Natural dye-decolourizing microbial isolate can be better for on-site treatment as that bacterium might be acclimatised to natural conditions. In this study, an indigenous bacterial isolate *Aeromonas caviae* was identified through morphological, biochemical and molecular (16S rDNA analysis) characterization. However, the 16S rRNA gene sequence analysis is the most accepted method employed for bacterial classification and identification[22]. The results showed that increasing the concentration of dye was inhibitory for bacterial growth. Other researchers also reported similar types of findings[15–17].

Numerous physicochemical operational aspects, including oxygen levels, dye concentrations, inoculum sizes, the addition of various carbon and nitrogen sources, and others, have a direct impact on how well azo dyes are decoloured by bacteria in biological treatment procedures[16]. Therefore, it is crucial to understand each aspect that affects the bacterial decolourization of azo dyes to make the procedure more effective, quicker, and realistically applicable. Phytotoxicity of wastewater is also one of the important problems.

The bacterium was unable to decolourize dye in the absence of glucose as a co-substrate. Tripathi *et al.*,[22] also performed a bioremediation study in the presence of glucose as an additional carbon and energy source. Many researchers observed the impact of different nitrogen and carbon sources on bioremediation efficiency[1,9,26,27]. However, the decline in decolourization efficiency was observed at higher dye concentrations and showed an inhibitory effect on the growth of the MT-1 strain. Similar findings are being reported during bacterial decolourization of various dyes[16,28,29]. However, the inhibitory effect of increasing dye concentration can be reduced when bacterial co-culture was used instead of pure culture, and this might be due to the synergistic effect of both the microorganisms[1,16]. Several researchers reported that increasing dye concentration decreases the decolourization rate[16,30–32].

In the present study, crystal violet dye was effectively decolourized with a 5% v/v inoculum dose. Remediation of crystal violet dye using bacterial cultures was studied by several researchers[33,34]. In a study, Maniyam *et al.*,[35] reported the biological decolourization of crystal violet by *Rhodococcus pyridinivorans* under optimized static cultural conditions with maltose as a carbon source and 12% inoculum size. They also reported the toxic effect of crystal violet at higher levels to decrease the growth of *R. pyridinivorans* as well as the decolourization efficiency. Similarly, Roy *et al.*,[31] studied the degradation of crystal violet dye by *Enterobacter* sp. isolated from textile wastewater. Compared to our findings,

they used 10% v/v inoculum, for the decolourization of the dye. However, Garg *et al.*,[15] used 4% v/v of *Pseudomonas putida* for the decolourization of the dye. Additionally, under optimized conditions, it was observed that bacterial growth directly affects the extent of dye-decolourization which is might be due to its enzyme activity and better nutrient uptake. During dye-decolourization, biodegradation of dye may occur. Many researchers reported biodegradation of azo dyes using microorganisms[14,15,36–38]. Bharagava *et al.*,[36] reported degradation and decolourization of crystal violet dye by *Aeromonas hydrophila* through enzymatic activity. In another investigation, Gao *et al.*,[39] decolourize 34% decolourization of crystal violet dye after 14 days incubation period by *B. adusta* strain. Whereas, in the present study, maximum decolourization was achieved at 72 h by *A. caviae* MT-1 isolate. In a study, Eva *et al.*,[40] reported crystal violet dye-decolourization by a native bacterial isolate from the soil.

In another study, Sani and Ibrahim[30] reported the crystal violet dye decolourization potential by *Bacillus* sp. and fungi. They concluded that dye-decolourization occurred through the biosorption process. Whereas, dye-decolourization might be both metabolisms dependent (through microbial enzymes) and metabolism independent (through the adsorption process). Pinheiro *et al.*,[41] reviewed the potential of bacteria for the bioremediation of dyes. They reported that bacterial degradation of dyes occurs through pure cultures or in the consortium. However, in the present study bacterial culture *A. caviae* was efficiently decolourized 98% of crystal violet dye. The environment is polluted by many other emerging pollutants along with dyes and toxic metals[10]. In the present study, the isolated dye-decolourizing bacterium was resistant to multi-metals which is an added advantage. Toxic effects of dyes on the growth of plants were reported by several researchers[37,42]. *Vigna radiata* is one of the most important crops and its seeds are rich in nutrients. However, the productivity of any crop can be adversely affected by the presence of any toxic chemical in the soil. Thus, such organic and inorganic contaminants must be detoxified for green and sustainable agriculture. The application of the MT-1 isolate can be helpful in the remediation of dye from contaminated soil fields.

6. Conclusions

A potential crystal violet decolorizing native bacterial isolate *Aeromonas caviae* showed effective dye bio-decolourization (98.0%) at 100 mg/L under optimized nutritional and culture within 72 h incubation. This bacterium was also tolerant to toxic heavy metals like arsenic, lead, and chromium at their higher initial concentration, indicating the possible application of this bacterium in such toxicants contaminated sites. Phytotoxicity analyses revealed that decolourization of dye by *A. caviae* MT-1 isolate can be helpful for plant growth promotion and detoxification of crystal violet dye-contaminated sites. Therefore, this study showed that *A. caviae* can be applied for the treatment of dye-contaminated areas and also for sustainable agriculture. Further research is underway to use this bacterium for on-site bioremediation.

7. Acknowledgement

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