

Microbiological Decolorization of Crystal Violet Dye by Indigenous *Bacillus* spp. Isolated from Garden Soil

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Abstract

Background: Synthetic dyes that are routinely used in various industries are becoming a threat to our environment if emitted without treatment. Previous studies were performed to detect textile dye degradation by isolates collected from effluents of textile industries. Here, our study was designed to investigate whether indigenous *Bacillus* spp. collected from garden soil was able to degrade hazardous crystal violet (CV) dye.

Materials and Methods: In this regard, we collected soil samples from a garden, diluted and plated on nutrient agar (NA) media. After incubation, the isolates were subjected to amylase activity to primarily screen *Bacillus* spp. The isolates with amylolytic activity were exposed to physiological, cultural and biochemical examinations to presumptively identify *Bacillus* spp. The latter was screened on NA media containing CV dye and further evaluated for percent dye decolorization in liquid nutrient broth (NB) both time- and concentration-dependently.

Results: We have found four isolates (A2, A4, B4, and C4) that were presumptively identified as *Bacillus* spp. All four *Bacillus* isolates showed decolorization on solid media. Differential dye degradation potential was observed by the isolates in liquid culture. Among the four *Bacillus* isolates, isolate A2 was found to possess the highest decolorization potential of CV dye. Our data showed that isolate A2 can degrade 98.56%, 94.04% and 75.63% CV dye at 0.025, 0.05 and 0.1 mg/mL concentrations following 9 days of incubation. After isolate A2, isolates A4 and B4 showed great potential where isolate C4 showed the lowest competence in decolorizing CV dye.

Conclusion: Therefore, indigenous garden soil *Bacillus* spp. can be utilized in bioremediation of highly toxic textile dyes following detailed studies.

Key words: *Bacillus* spp.; Crystal Violet; Decolorization; Garden soil; Textile dyes.

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I. Introduction

As with the growing population, the revolution in industries is taking part to meet all the basic needs that have a direct influence on our environment¹. Proper management of discharged wastewater coming from various industries is a major concern worldwide although the scenario is even worse for a country like, Bangladesh. The country with a 7.3% gross domestic product (GDP) annual growth becomes the world's seventh-highest growing economy. The readymade garments (RMG) sector took the biggest part among all the industries taking part in that GDP². Garments and textile industries consume the highest amount of water that is when discharged to the nature comprising of deleterious chemicals as coloring dyes, sizing agents and many other recalcitrant chemicals³. It was estimated that around 100,000 commercial dyes are present and roughly 1 million tons of dyes are produced per year but the concern is around 10% dye of the total usage is disposed of as waste (£). Disposal of dyes have crucial consequence in the environment even at very low concentration as they have effect on chemical oxygen demand (COD) and biological oxygen demand (BOD), aesthetic deterioration, an obstacle in penetrating oxygen and sunlight to water bodies and they or their metabolites might have toxic, carcinogenic and mutagenic effect to flora and fauna^{3,4,5}. Although some physical, and chemical treatments and their strategies are available but the situation is worst in developing countries due to partially or completely ignoring the treatment procedure before discharge⁶. Moreover, that treatment procedures are not very effective in terms of cost, activity, and environmental safety as a secondary level of environmental pollution occurs due to huge amounts of sludge are generated that is difficult to dispose of⁷. Therefore, biological agents are gaining more importance in bioremediation purpose owing to their economical scheme and potential to produce less sludge that reduces pollution risk⁸. From last few years, *Bacillus* species are gaining more awareness as they have huge potential in degrading and mineralizing various complex chemicals including environmentally unsafe synthetic commercial dyes^{9,10,11}.

Crystal violet (CV), a triarylmethane dye, used for various purposes including textile and printing

industries as well as in manufacturing ball-point pens, inkjet printers and coloring diverse products like leather, detergent, etc (Wikipedia). The dye is considered a biohazard substance because of its carcinogenic and mutagenic effects along with its recalcitrant property¹². To reduce the havoc to the environment, we made an effort to detect whether *Bacillus* spp. isolated from garden soil can decolorize and biodegrade CV dye.

II. Materials and Methods

Sample collection and processing:

Soil samples were collected in a sterile beaker using a sterile spatula at a depth of 2-3 cm from a garden soil of Stamford University Bangladesh, Shiddeswari campus, Dhaka, Bangladesh. One gram of soil sample was dispensed into 99 mL of sterile distilled water and homogenized. One ml of homogenized soil sample was transferred into 9 mL sterile normal saline and a serial dilution was carried out up to 10⁻⁶ dilution.

Screening of amylase producing bacteria:

Serially diluted bacterial cultures (100 µL) were spread on nutrient agar media and incubated at 37°C for 24 h. Their colony morphology was noted and subsequently, isolated colonies were streaked on starch agar media containing starch as the only carbon source for starch hydrolysis test to detect their amylolytic activity. The plates were incubated at 37°C for 24-48 h. Following incubation, plates were flooded with Gram's iodine (Gram's iodine-250 mg iodine crystals added to 2.5 g potassium iodide solution, and 125 mL of water, kept at room temperature) to identify the zone of clearance around the colony. Deep blue color around the growth indicates a negative result that is no amylolytic activity where the zone of clearance produced by amylase producers. The pure cultures showing clear zones were subcultured at regular intervals and maintained on to nutrient agar slants at 4°C.

Identification of *Bacillus* spp.:

Isolated amylase producing bacteria were presumptively identified as *Bacillus* spp. by morphological, cultural and biochemical characteristics. The biochemical tests included methyl red, Voges-Proskauer, citrate utilization, indole production, H₂S production, motility, gelatine hydrolysis, sugar hydrolysis, oxidase, and catalase.

Screening for dye decolorization:

CV dye was collected from a textile industry located in Savar. Stock concentration was prepared using distilled water in a vial and kept at 4°C until used. The isolates were cultured on CV dye containing NA media at a concentration of 0.025 mg/mL concentration for 24-72 h. The culture plates were checked every day for any decolorization of media surrounding the colony.

Dye decolorization assay:

Decolorization experiment of CV dye was performed by using CV dye in a 100 mL conical flask containing 50 mL of nutrient broth. A 50 µL of 24 h old *Bacillus* culture corresponding to Mcfarland standard 0.5 was used as inocula to inoculate the 0.025, 0.05 and 0.1 mg/mL CV dye supplemented broth. The inoculated conical flasks were incubated at room temperature for 3, 4, 7 and 9 days to detect dye decolorization. Following incubation, decolorization of dyes by selected isolates was observed at each time period and only 5 mL cultures were centrifuged at 4,000 rpm for 20 min and the supernatants were subjected to UV-spectrometry at 620 nm and the absorbance was recorded. The uninoculated media with CV dye was served as respective blank for the dye decolorization assay. The percentage of dye decolorization was calculated as stated before (Alalewi and Jiang, 2012).

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

III. Results

Isolation and characterization of *Bacillus* spp.:

Among a good number of bacterial isolates, only 4 bacterial isolates namely 2A, 4A, 4B and 4C were presumptively found to belong to the genus *Bacillus* spp. following characterization by morphological (Table 1), cultural (Table 2) and biochemical examinations (Table 3). Figure 1 showed the microscopic images of the presumptively identified *Bacillus* isolates.

Figure 1: Microscopic observation of the *Bacillus* isolates.

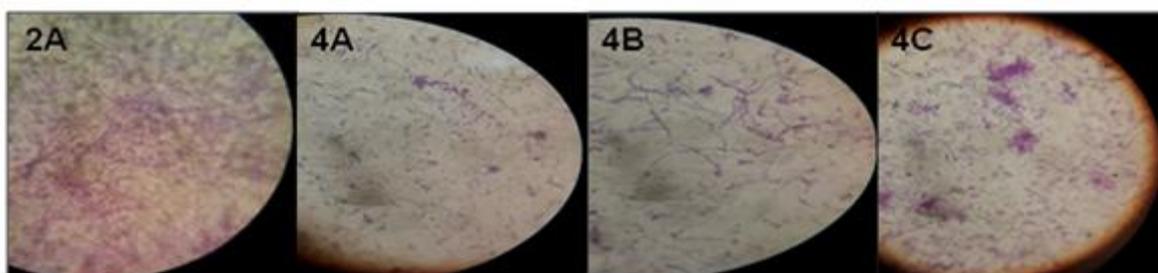


Table 1: Microscopic features of the *Bacillus* isolates.

Microscopic observation	2A	4A	4B	4C
Shape	Long rod	Short rod	Long rod	Short rod
Arrangement	Chain, Single	Single	Chain, Single	Single
Gram Reaction	Gram positive	Gram positive	Gram positive	Gram positive

Table 2: Colony characteristics of the *Bacillus* spp. on nutrient agar media.

Characteristics	2A	4A	4B	4C
Size	Medium	Small	Large	Small
Form	Irregular	Irregular	Irregular	Irregular
Margin	Undulate	Undulate	Undulate	Undulate
Elevation	Flat	Flat	Flat	Flat
Consistency	Moist	Moist	Butyrous	Moist
Opacity	Opaque	Opaque	Opaque	Opaque
Pigmentation	Off white	Off white	Off white	Off white

Table 3: Biochemical characteristics of the *Bacillus* isolates.

Tests	Media	Observation			
		2A	4A	4B	4C
Starch hydrolysis	Starch agar plate	+	+	+	+
Methyl red test	GPB broth	+	+	+	+
Voges-Proskauer test	GPB broth	+	+	+	+
Indole production	1% peptone	-	-	-	-
H ₂ S production	2% peptone	-	-	-	-
Sugar hydrolysis	Triple sugar iron agar slant	A/A	A/A	A/A	A/A
Motility test	MIU media	+	+	+	+
Gelatin hydrolysis	Gelatin media	-	-	-	-
Citrate utilization	Simmons citrate agar slant	-	-	-	-
Oxidase	Nutrient agar	+	+	+	+
Catalase	Nutrient agar	+	+	+	+

Screening of CV dye decolorization by *Bacillus* spp.:

Following inoculation of the *Bacillus* isolates onto CV containing nutrient agar plates showed a clearing appearance of the CV dye surrounding the cultures. The clearance of CV dye was observed with all the four isolates in both NA and NB media. Representative pictures of CV dye decolorization by isolate 4B on NA media following 24 h of incubation and on NB media by 2A, 4A, 4B and 4C isolates at 0.025, 0.05 and 0.1 mg/mL concentrations after 4 days of incubation were presented in Figure 2 and Figure 3, respectively. Moreover, the dye decolorization potential of CV dye following incubation at 3, 4, 7 and 9 days with the isolates 2A, 4A, 4B and 4C were summarized by bar graphs in Figure 4, 5, 6 and 7, respectively. Among the four *Bacillus* isolates, isolate 2A was found to possess the highest decolorization potential of CV dye. Our data showed that isolate 2A can degrade 98.56%, 94.04% and 75.63% CV dye at 0.025, 0.05 and 0.1 mg/mL concentrations following 9 days of incubation. After isolate 2A, isolates 4A and 4B also showed great potential where isolate 4C showed the lowest competence in decolorizing CV dye. However, the highest decolorization was observed after 3 days of incubation at 0.025 and 0.1 mg/mL concentration by isolate 2A, but at 0.05 mg/mL concentration by isolate 4B. A time- and concentration-dependent increase of dye decolorization was observed in all the cases. Furthermore, a gradual increase of dye decolorization was detected in most of the cases but a sharp increase of decolorization was observed after 7 and 9 days of incubation by isolate 4C with 0.025 mg/mL concentrations.

Figure 2: A representative picture of CV dye decolorization by 4B isolate on CV dye containing NA media after 24 h of incubation.

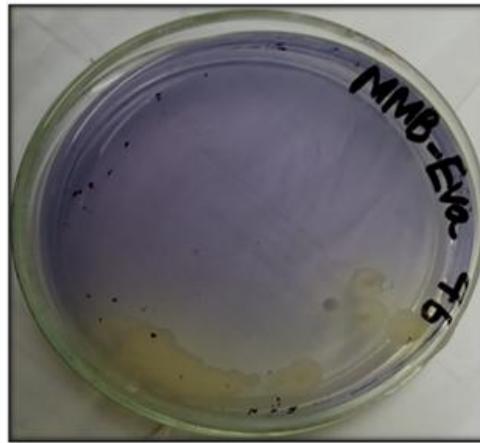


Figure 3: Decolorization assay on 0.025, 0.05 and 0.1 mg/mL CV dye containing NB media by the isolates at 4th day of incubation.

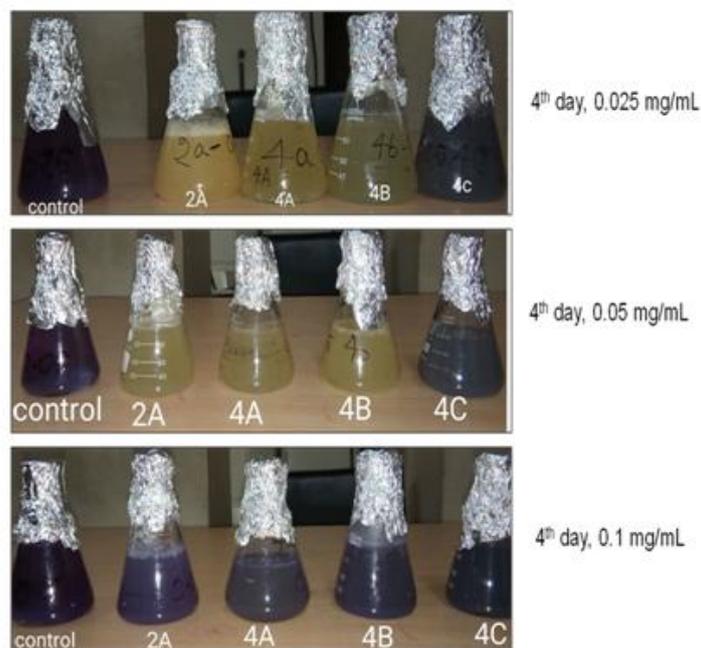


Figure 6: Decolorization of CV dye by the isolate 2A following 3, 4, 7 and 9 days of incubation.

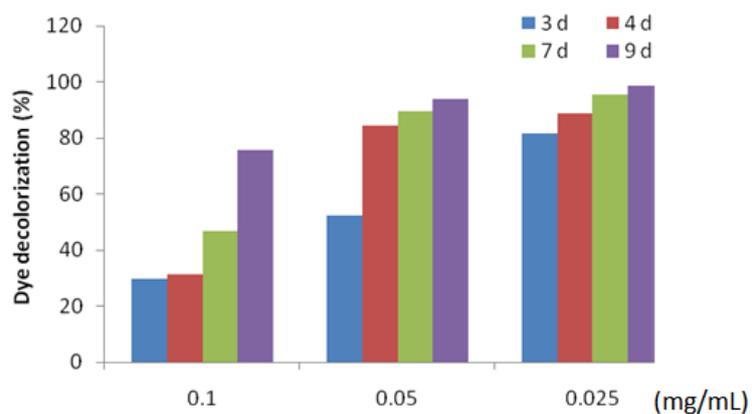


Figure 7: Decolorization of CV dye by isolate 4A following 3, 4, 7 and 9 days of incubation.

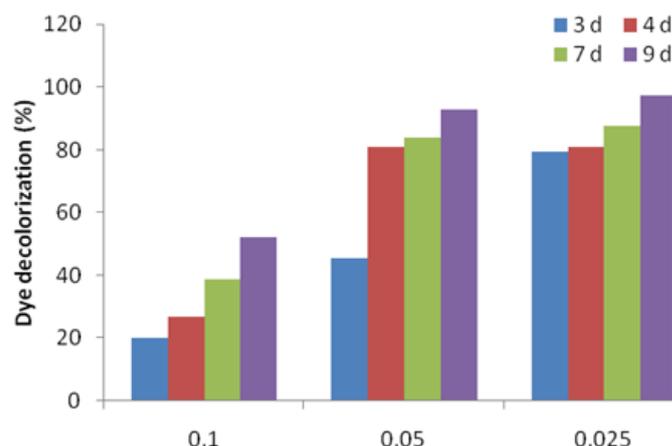


Figure 8: Decolorization of CV dye by the isolate 4B following 3, 4, 7 and 9 days of incubation.

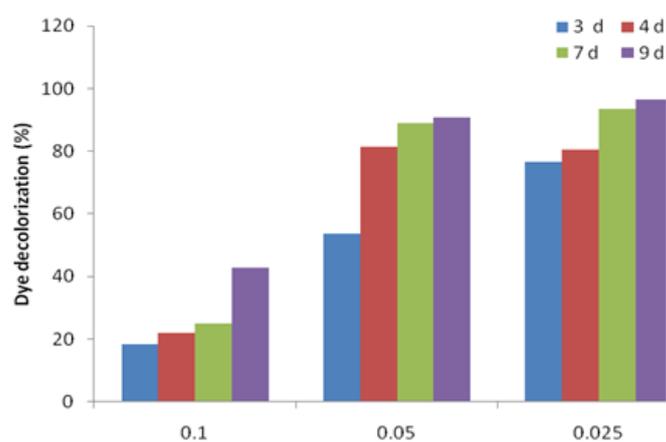
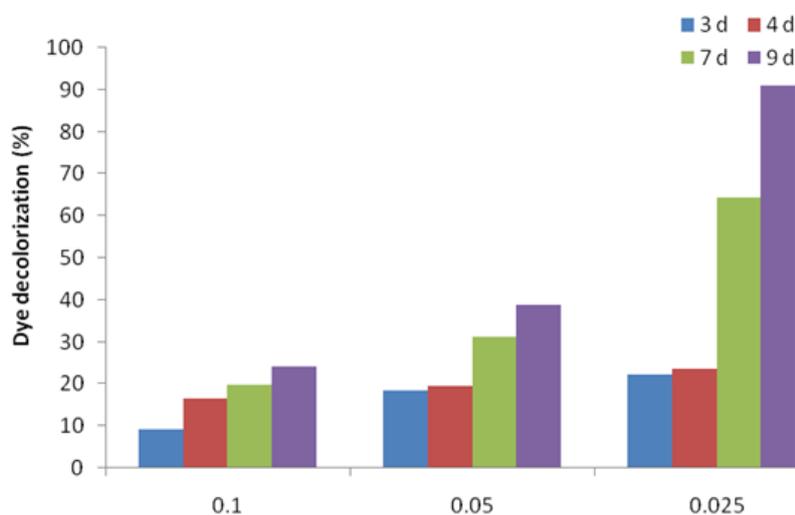


Figure 9: Decolorization of CV dye by the isolate, 4C following 3, 4, 7 and 9 days of incubation.



IV. Discussion

A huge amount of disposal of environmentally unfriendly hazardous and recalcitrant pollutants from various industries is creating an immense threat to the environment as well as to the plants, animals and ultimately, humans. Due to the cost-effectiveness and environmentally caring approaches, microbial processes are the choices in reducing the havoc to mankind. As *Bacillus* spp. is a remarkable bacterium with high potential

to produce diverse metabolites of different usage, we become interested whether they can potentially degrade CV dye at different concentrations and time periods. Few previous studies reported that *Bacillus* species collected from textile industry effluents is capable to degrade CV dye that are used in those industries^{14, 15}. However, we were interested to distinguish whether indigenous *Bacillus* spp. isolated from garden soil can degrade the highly toxic CV dye. Among the four *Bacillus* isolates, isolate 2A was found to possess the highest decolorization potential of CV dye. A previous report showed that CV dye decolorization was 81.25% by *Enterobacter* spp. where our *Bacillus* isolates, 2A, 4A, 4B and 4C showed 81.48%, 79.39%, 76.49% and 22.23% following 72 h of incubation, respectively¹⁶. In another study, the highest decolorization (95%) was observed at 35°C, whereas the decolorization reached 85% at 37°C by the isolate *Bacillus subtilis* ETL-2211 after 24 h of incubation¹⁵. Very prominent dye decolorization was observed in another study where at 35°C *Bacillus subtilis* was able to degrade 100 mg/mL CV dye effectively (90%)¹⁵. In our case, we observed very significant dye decolorization after 72 h of incubation. But, the rate of dye decolorization might be improved by optimizing the conditions such as pH, temperature, inoculum concentrations, and carbon and nitrogen sources as well and their concentrations.

V. Conclusion

Day by day, the increasing growth of industries is creating high risk to the environment as the toxic and hazardous chemicals coming from the effluents. Therefore, microbial approaches are well suited to address this issue. In this regard, our study may enlighten to degrade highly toxic CV dye by using indigenous garden *Bacillus* isolates. We are taking consideration to thoroughly investigate to augment the capacity of those indigenous isolates in dye degradation by optimizing various parameters as well as improve their dye degradation by slowly acclimatize them in increasing dye concentrations.

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