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Decolourization and Detoxification of Azo Dye, Malachite Green by *Pseudomonas monteilii* Strain RZT1, a Bacterium Isolated from Textile Wastewater

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Authors' contributions

This work was carried out in collaboration among all authors. Authors TN, AKS and MFH designed the study, performed the statistical analysis and wrote the protocol and the first draft of the manuscript. Author TN did the experimental works of the study. Author MJA did works for extraction and amplification of 16s rDNA. Authors MKM and ASC managed the literature searches. Author MFH edited the manuscript and finalized it. All authors read and approved the final manuscript.

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ABSTRACT

Discharge of textile industrial effluent without proper treatment has become a severe hazard for the animal health and environment worldwide. Therefore, this study was designed to isolate azo dye-degrading bacteria from textile wastewater and evaluate their ability to biodegrade reactive dyes into non-toxic products. The potent bacterial strain which was isolated from textile wastewater was identified as *Pseudomonas monteilii strain RZT1* on the basis of 16S rDNA sequence. The isolated bacterial strain exhibited good decolorization ability with yeast extract supplementation as cosubstrate in static conditions for Malachite Green dye. The optimal condition for the decolorization of Malachite Green dye by *P. monteilii strain RZT1* were at pH 7.0 and 28°C. Decolorization rates of Malachite Green dye by *P. monteilii strain RZT1* were varied with initial dye concentration as follow: 84.8%, 75.4%, 63.4% and 45.5% decolorization for 100ppm, 200ppm, 300ppm and 400ppm initial dye concentration respectively. We investigated the effects of dyes used in the textile industry on the seed germination of Five crops - Rice (*Oryza sativa*), Wheat (*Triticum aestivum L.*), Khesari (*Lathyrus sativus*), Mustard (*Brassica nigra*) and Bitter Melon (*Momordica charantia*). It was found that textile dye Malachite Green had negative effect on seed germination and seedling growth in test cultures. The harmful effects of dye on seed germination and early seedling growth parameters were augmented with increase of dye concentration. Interestingly, treatment of the Malachite Green dye with isolated bacteria reduced the adverse effects of that dye on seed germination and seedling growth. Thus, it indicated the potentiality of *P. monteilii strain RZT1* for bioremediation of textile effluents into a non-toxic form for plants.

Keywords: Decolourization; detoxification; azo dye; Pseudomonas monteilii strain RZT1; seed germination.

1. INTRODUCTION

"Environmental pollution due to the release of industrial wastewater containing many types of azo dyes is a big problem worldwide. Azo dyes are used in many industries such as textiles dyestuffs, foodstuffs, cosmetics and printing. Recently, the deterioration of water resources with the rapid growth of industries (sugar, paper, tannery, textile and dyeing industries) in many countries has come into the discussion. It generally disrupts the habitats of the living organisms when discharged into the environment without proper treatment" [1]. "The continuous irrigation of agricultural land with the effluent wastewater causes heavy metal accumulation in the grown crops" [2]. "When this effluent discharged into the water sourses, they restrict the light penetration and inhibit the activities of aquatic lives by decreasing photosynthesis and oxygenation of water reservoirs" [3]. Moreover, the many Azo dyes are reported as xenobiotic, mutagenic and carcinogenic [4-7]. Therefore, wastewater contaminated with azo dyes appears as serious problems due to their negative impacts on water ecosystems and human health.

Effects of wastewater from the textile industry on plants have been studied by some workers [8-10]. However, the harmful effects of textiles wastewater from plants depends on the species, the stage of the plant's life cycle is affected by, and the types and concentrations of harmful substances in the effluent. Over the past three decades, some physical, chemical and biological bleaching methods have been adopted by the paper and textile industries [11-14]. Various physical methods can used to remove Azo dyes of wastewater. Some of these methods are effective but quite expensive because they create a significant amount of chemicals. In such situations, bioremediation can be a real hope. Various types' microorganisms including bacteria, fungi, yeasts, actinomycetes and algae capable of breaking down azo dyes have also been used for biodegradation of textile dyes [11, 15-22]. So, the present study was designed for isolation of potent bacterial strain from local textile wastewater which was able to decolorize as well as detoxify azo dye Malachite Green as evaluated by the effects of the bioremediated Malachite Green dye on seed germination and seed development.

2. MATERIALS AND METHODS

2.1 Collection and Storage of the Sample

Wastewater was collected from different textile industries located in Sathia, Sirajgonj, Gazipur, Madhapdi, Narshingdhi, Bangladesh. Samples collected from textile industries were in the form of liquid wastewater and sludge. All samples were collected in sterile plastic bottles and

polyethylene bags and then stored at 4°C in the refrigerator to avoid changes in their physico-chemical properties.

2.2 Dyes and Culture Media

Malachite Green dye was purchased from DysinChem limited, Dhaka, Bangladesh.

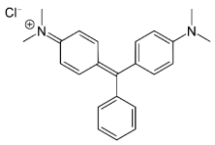

Fig. 1. Malachite green

2.3 Isolation and Screening of Dye Decolorizing Bacteria

The potent bacteria were isolated from collected samples by Luria Bertani (LB) enrichment medium culture technique modified with 20 ppm of Malachite Green dye for microbial adaptation. Bacterial colonies showed a clear discolored area around them on LB agar were collected and cultured for 24 h in MS medium modified with 1 ml/1 TE dissolution. The growth of bacterial colonies was observed after 24 h of incubation at 28°C. Effect of dyes on the growth of bacterial strains was determined in MS medium supplemented with 20 ppm of Malachite Green dye.

2.4 Genomic DNA Extraction & 16S rDNA Gene Amplification

Genomic DNA was extracted from dye decolourizing bacteria using CTAB method [23]. The PCR primers used to amplify 16S rDNA fragments were the bacteria-specific primers a forward primer 8F (5'-AGA GTT TGA TCC TGG CTC AG-3'; Tm: 61°C); and a reverse primer 806R (5'-GGA CTA CVS GGG TAT CTA AT-3'; Tm: 67.4°C). A total of 25 µl of reaction mixture consisted of – water 15µl, MgCl2 2.5µl, buffer 2.5, dNTPs 0.5µl, template 1µl, primer (forward 2 µl and reverse 2 µl). The PCR amplification was performed by Swift™ Minipro Thermal Cycler (Model: SWT-MIP-0.2-2, Singapore) using the following program: Denaturing at 95°C for 5 minutes, followed by 40 cycles of 40 seconds of denaturing at 95°C, 60 seconds of annealing at 65°C and 2 minutes of elongation at 72°C with a final extension at 72°C for 10 minutes. Then, the PCR products were subjected to 1% agarose gel electrophoresis, stained with ethidium bromide and visualized on a UV transilluminator for the presence of about 1500 bp PCR products.

The amplified PCR product was purified using AccuPrep® Gel Purification Kit (Bioneer Company, Korea) according to the manufacturer's protocol. PCR amplified 16S rDNA of the isolates screened as submitted for automated sequencing (Applied Biosystems 3130) at the Center for Advanced Scientific Research (CARS) of the University of Dhaka, Bangladesh. the sequence generated from the automatic sequence of PCR amplified DNA analyzed by NCBI BLAST Program (http://www.ncbi.nlm.nih.gov) for discover a similar organism possible through association of similar sequences. Finally, the isolates were determined based on partial sequence alignment of 16S rDNA with sequences available in database.

2.5 Sequencing of 16S rDNA and BLAST Analysis

The nucleotide sequence of the 16S rDNA was sequenced on both sides through the BigDye chain termination cycle sequencer (ABI) and the sequence was decoded on Dideoxy Sanger 3130XL String Genetic Analyzer (ABI). The final method was then assembled by the Cap3 program for genetic sequencing. Gene sequence was determined by looking for similarities in the database via BLASTn for 16S rDNA.

2.6 Effect of Different Parameters on Process of Dye Decolorization

Effect of initial dye concentration on discoloration of Malachite Green dye by isolated bacteria was tested after 96 h incubation as described previously [17]. In short, to examine the effect of different dye concentrations on color change, MS medium supplemented with 100, 200, 300 and 400 ppm Malachite Green dye was adjusted to pH 7. Then, the medium was inoculated with bacterial strains incubated at 28°C for 192 h.

2.7 Measurement of Decolorization Efficiency

The decrease in absorbance at absorption maxima (λmax) was monitored using a UV-visible spectrophotometer to evaluate decolorization activity in terms of % decolorization. Uninoculated MS medium supplemented with corresponding dyes were used as a reference. At different time interval, 2 ml of sample was taken from reaction mixture and centrifuged at 10000 rpm for 10 min for biomass separation. The concentration of dye in the supernatant was determined by monitoring

the absorbance at maximum absorption wavelength (λmax) at 660 nm. Decolorization percentage was calculated according to the following formula:

$$Dye\ Decolourization\ (\%) = \frac{Initial\ absorbance - Final\ absorbance}{Initial\ absorbance} \times 100$$

2.8 Seed Germination Test

In this experiment, the effect of four concentrations of Malachite Green dye were assessed on the seed germination of five crops viz. Rice (*Oryza sativa*), Wheat (*Triticum aestivum L.*), Khesari (*Lathyrus sativus*), Mustard (*Brassica nigra*) and Bitter Melon (*Momordica charantia*). Healthy and uniform seeds were washed with distilled water. The seeds were treated with 0.2% mercuric chloride for 2 minutes, followed by rinsing with autoclaved distilled water to remove mercuric acid [24]. Then, seeds of each crop were soaked with solutions of different dye concentrations (50, 100, 200 and 400 ppm) for 12 hours. Then 10 seeds of each crop were placed on Whattman filter paper in sterilized Petri plate. The filter papers were moistened with 5 ml distilled water (control) or at different concentrations of dye in distilled water every 12 hours. All dishes were stored at room temperature (28±2°C). In another independent experiment, 200 ppm dyes were treated with bacterial isolates in 96 hours. After 96 hours, the treated dye was used for seed germination experiments as described above.

Number of seeds responded for germination was observed on 8th day and growth was also observed. The shoot length of the seedlings was recorded at every 48 hours for 15 days. Fresh mass and root length of the seedlings were determined after 15 days. The seedlings were uprooted and washed thoroughly with distilled water and lengths of root were measured.

The germination percentage was measured using the following formula [25].

$$Germination(\%) = \frac{Number\ of\ seed\ germinated}{Total\ Number\ of\ seeds\ set\ for\ test} \times 100$$

Phytotoxicity of the dye effluent on seedling growth was calculated by using the following formula [26].

$$Phytotoxicity(\%) = \frac{Radical\ length\ of\ contol - Radical\ length\ of\ test}{Radical\ length\ of\ contol} \times 100$$

Seedling vigor index was calculated using the following formula [27].

$$Vigor\ Index = Germination\ Percentage \times Length\ of\ Seedling$$

$$Germination\ Index = \frac{No.\ of\ seeds\ germinated\ at\ first\ count + \cdots No.pf\ seeds\ germinated\ in\ last\ count}{Days\ of\ First\ Count + \cdots Days\ of\ Final\ Count}$$

3. RESULTS

3.1 Physico-chemical Characteristics of Textile Effluent

In the present study, physic-chemical characteristics of the collected textile effluent was analyzed and the results were showed in Table 1.

3.2 Isolation and Identification of Dye Decolorizing Bacteria

Isolated bacterial strains identified by morphological and biochemical tests were subjected to 16S rRNA gene sequence analysis. Analysis of 16S rRNA gene sequence revealed that the isolate was *Pseudomonas monteilii* strain RZT1 (Accession Number: OM095453) (Fig. 2).

3.3 PH and Temperature

In this study, the bacteria having potential to decolorize textile dyes were isolated from collected wastewater of fibers industrial sector. 3 morphologically different bacteria were separated from the wastewater and 1 of them was able to decolorize the Malachite Green dye. This isolates grew optimally at 28°C and pH 7 (Figs. 3 and 4).

3.4 Effect of Textile Dye Concentration on Decolorization

Effect of initial dye concentration on dye discoloration capability of the bacterial isolate was measured. 0.5% yeast extract was used as a co-substrate. The dye decolourization rate increased with the increase of incubation period. However, dye decolourization percentage decreased with the increase of initial dye concentration (Fig. 5). 84.8%, 75.4%, 63.4% and 45.5% decolourization occured after 192 hours of incubation period at 100, 200, 300 and 400 ppm dye concentration.

3.5 Effect of Textile Dye on Seed Germination

We investigated the effect of the dye Malachite Green on the seed germination of five crops viz

Rice (*Oryza sativa*), Wheat (*Triticum aestivum L.*), Khesari (*Lathyrus sativus*), Mustard (*Brassica nigra)* and Bitter Melon (*Momordica charantia*). It was found that the germination rate of seeds decreased with increasing dye concentration (Figs. 6, 7, 8, 9, 10 and 16), indicating that the germination rate was dependent on the concentration. The highest seed germination rate was found in control group (irrigated with water) while the lowest germination rate was observed in seeds irrigated with 400 ppm dye. The germination rate of Rice, Wheat, Mustard, Khesari, and Bitter Melon at 400 ppm dye concentration was 20%, 20%, 20%, 30% and 10% respectively. In this experiment, the seed germination effect of a 200 ppm Malachite Green dye treated (bleached) or untreated with the isolated bacteria was investigated to determine the feasibility of isolated bacteria for crop release. The germination rate of Rice, Wheat, Mustard, Khesari, and Bitter Melon was 40%, 40%, 40%, 40% and 20% respectively when irrigated with 200 ppm dye, while those rates were 68%, 65, 55%, 57% and 35% respectively when irrigated with 200 ppm dye treated with bacterial isolate *Pseudomonas monteilii* strain RZT1. The germination rate of seeds soaked with untreated dye was significantly lower than that of seeds soaked with treated dye and distilled water (control) indicating that the isolate *Pseudomonas monteilii* strain RZT1 was able to reduce the toxic effects of the dye Malachite Green (Figs. 6, 7, 8, 9, 10 and 18).

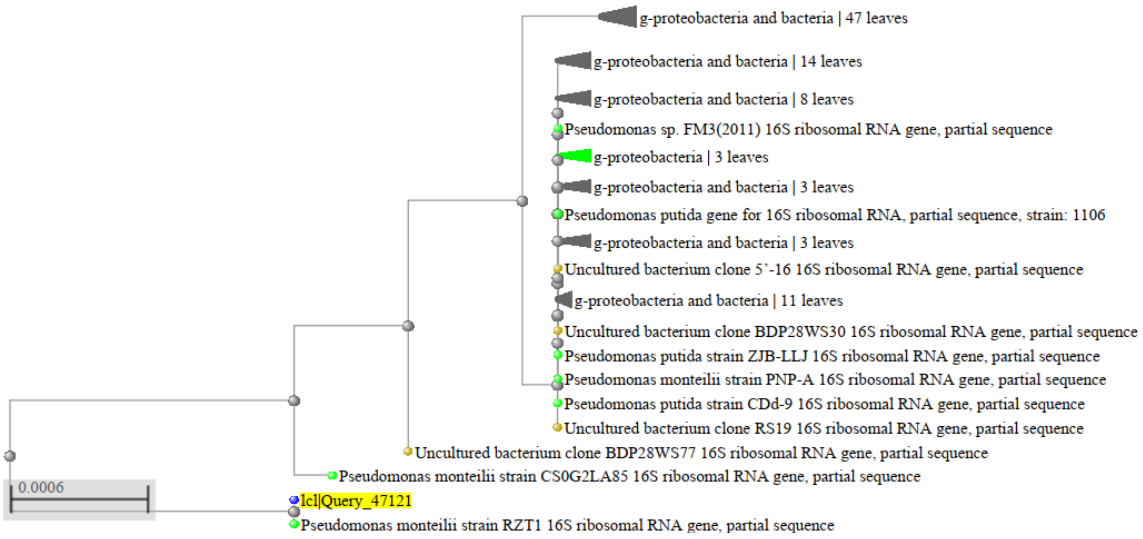

Fig. 2. Phylogenetic tree of the *Pseudomonas monteilii* strain RZT1

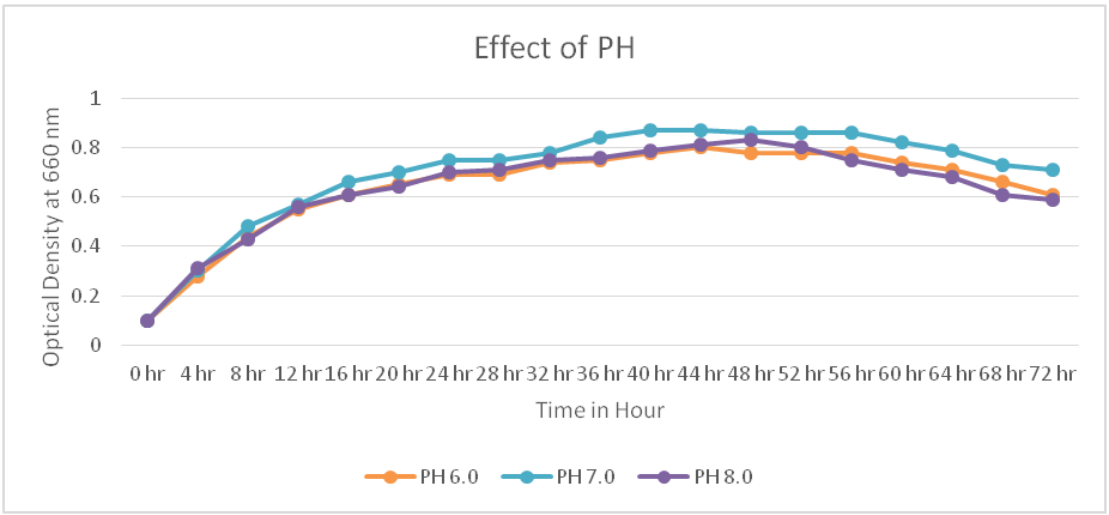

Fig. 3. Effect of PH on bacterial growth

Table 1. Physico-chemical characteristics of textile effluent

Sr. No	Source of sample	TS (mg/l)	TDS (mg/l)	pH	COD (mg/l)	BOD (mg/l)	Temp (℃)	Color	Odor
1	Water	3700	2300	6.3	710	270	35	Black	Foul
2	Sludge	4800	2100	6.8	750	280	35	Black	Foul
3	Water	4300	2500	7.4	620	250	35	Black	Foul
4	Sludge	4100	2700	6.0	785	310	35	Black	Foul

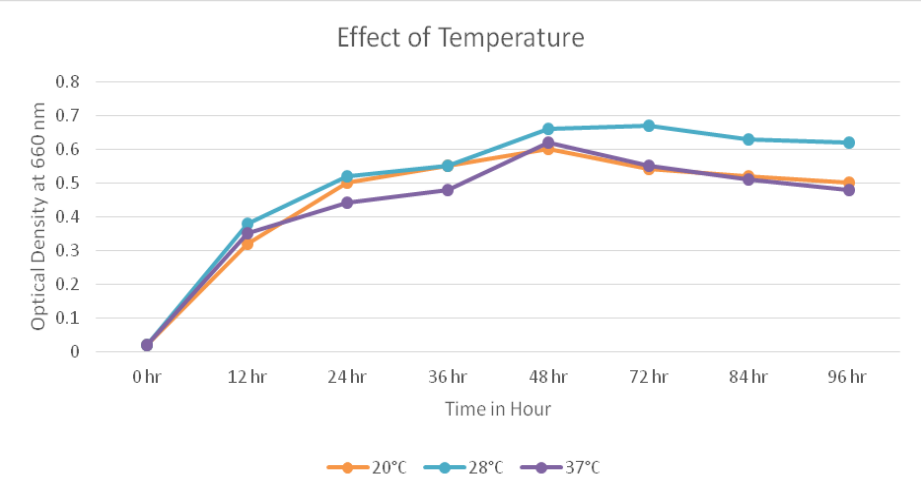

Fig. 4. Effect of temperature on bacterial growth

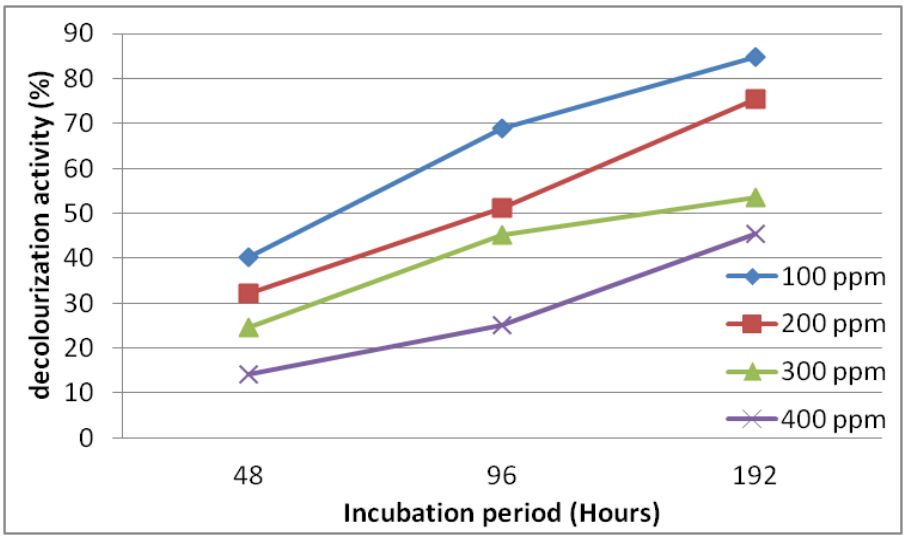

Fig. 5. Effect of Textile Dye Concentration on Decolorization Activity of malachite green

3.6 Effect of Textile Dye on Root, Shoot, Seedling and Radical Length of 5 Crops

Inhibition of plant growth by toxic pollutants is a global agricultural problem. The result of this study revealed that the root, shoot and radical lengths of the 5 studied crops gradually decreased with increase of dye concentration, demonstrating the toxic effect of the Malachite Green dye on these crops growth (Figs. 11, 12, 13, 14, 15 and 17). The highest and the lowest growth of these crops were observed at control and 400 ppm dye concentrations respectively. Maximum root length was observed on control; 10.18, 12.11, 11.30, 11.8 and 13.1 cm for Rice, Wheat, Mustard, Khesari, and Bitter Melon respectively. While minimum root length was

obtained at 400 ppm dye concentration; 1.5, 2.1, 3.1, 2.5 and 1.5 cm for Rice, Wheat, Mustard, Khesari, and Bitter Melon respectively. Similarly, maximum shoot length was recorded on control; 15.01, 17.5, 15.2, 14.2 and 15.9 cm for Rice, Wheat, Mustard, Khesari, and Bitter Melon respectively, while minimum shoot length was found for 400 ppm dye; 3.1, 2.8, 4.2, 3.1 and 2.8 cm for Rice, Wheat, Mustard, Khesari, and Bitter Melon respectively. Likewise, maximum radical length was observed on control. There was no significant difference between seedling growth parameters for the control and the lower dye concentration (50 ppm) treatment. But at higher concentration of dye (200 ppm), the growth parameters of seedling was affected negatively which was recovered by the treatment of dye with the bacterial isolate (Figs. 11, 12, 13, 14, 15 and 17).

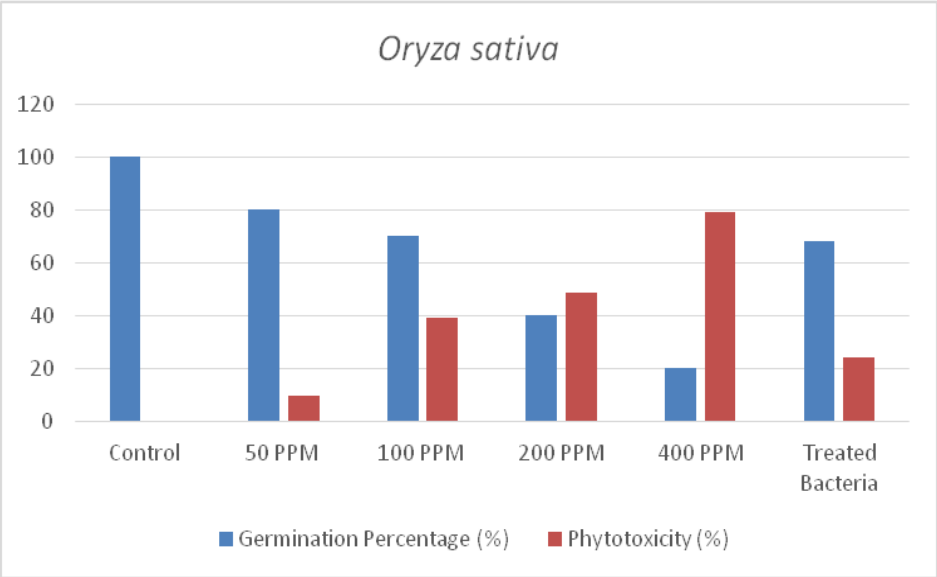

Fig. 6. Germination percentage and phytotoxicity of *Oryza sativa*

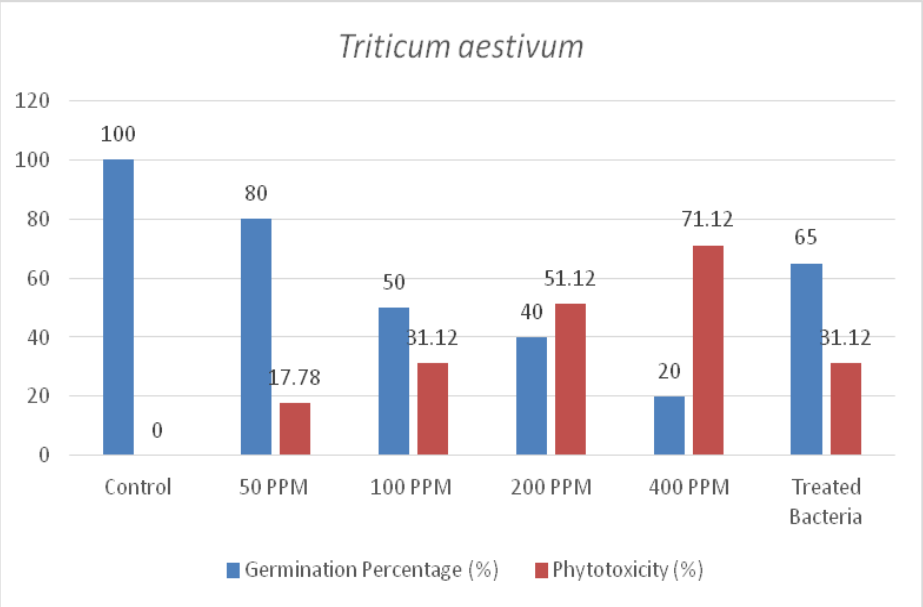

Fig. 7. Germination percentage and phytotoxicity of *Triticum aestivum*

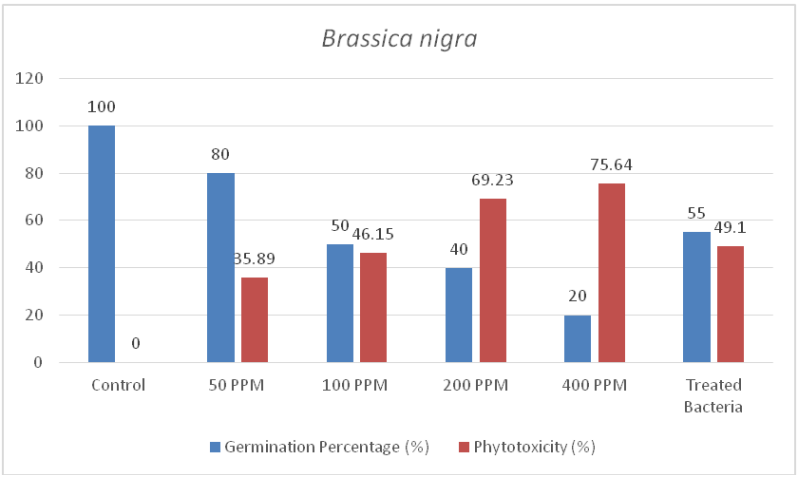

Fig. 8. Germination percentage and phytotoxicity of *Brassica nigra*

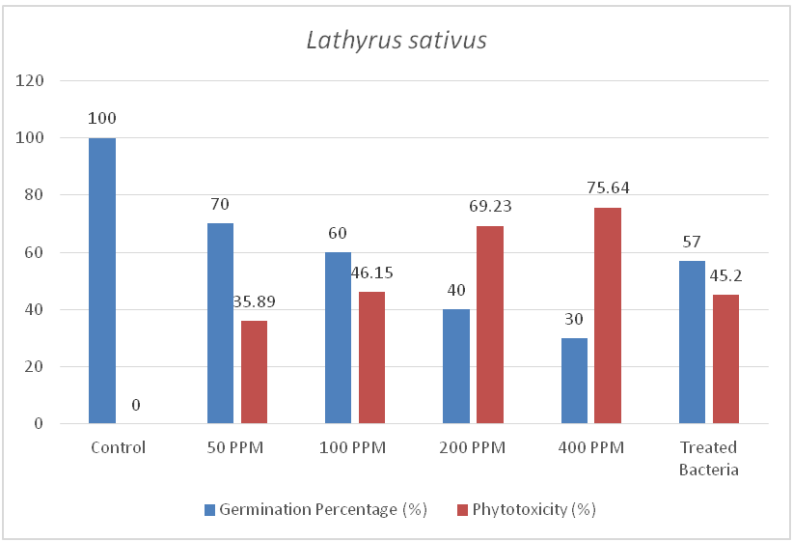

Fig. 9. Germination percentage and phytotoxicity of *Lathyrus sativus*

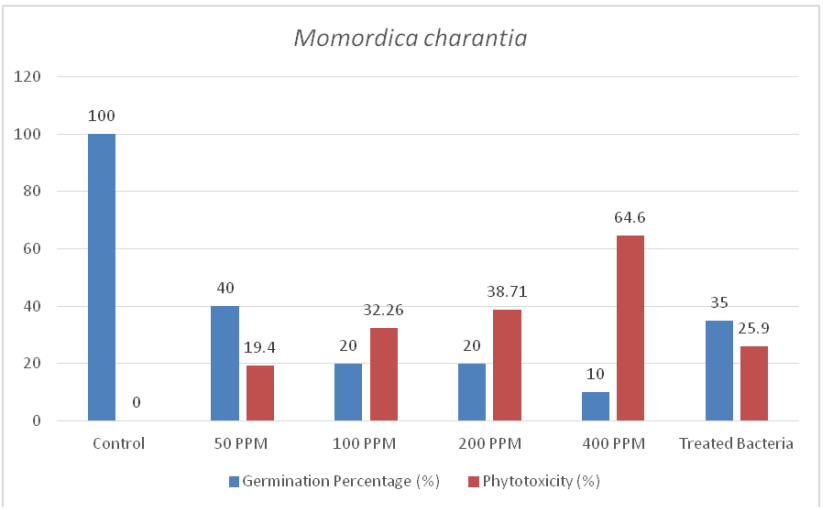

Fig. 10. Germination percentage and phytotoxicity of *Momordica charantia*

3.7 Effects of Textile Dye on Seedling Fresh Weight

The raw weight of seedlings decreased with the increase of dye concentration (Table 2). The best fresh weight of seedlings of control Rice, Wheat, Mustard, Khesari, and Bitter Melon was 1.1, 1.2, 1.1, 1.2 and 1.4 gm respectively. Contrary, the lowest fresh weight of seedling of Rice, Wheat, Mustard, Khesari, and Bitter Melon was recorded at 400 ppm dye concentration which was 0.40, 0.31, 0.31, 0.45, 0.51 gm respectively (Table 2).

3.8 Phytotoxicity of Textile Effluent on Seedling Growth

The increase of dye concentration significantly impaired the growth of seedlings (Table 2). The application of dye at a higher concentration suppressed the total dry matter production and root length of seedlings. There are no phytotoxicity observed in control but maximum phytotoxicity was observed at 400 ppm dye which were 79.26% 71.12%, 75.6%, 63.2% and 64.6% for Rice, Wheat, Mustard, Khesari and Bitter Melon respectively. In rice 48.78% phytotoxicity was observed at 200 ppm dye concentration while that was 24.39% at 200 ppm treated dye.

3.9 Seedling Vigor Index and Germination Index

The highest seedling vigor index was observed in control which was 2519, 2961, 2650, 2600 and 2900 for Rice, Wheat, Mustard, Khesari, and Bitter Melon respectively. Contrary, the lowest seedling vigor was observed at 400 ppm dye which was 92, 98, 548, 608 and 126 for Rice, Wheat, Mustard, Khesari, and Bitter Melon respectively. In rice, the vigor index was recorded 492 at 200 ppm dye concentration, but the vigor index was increased up to 1150 after treatment of 200 ppm dye with the bacterial isolate. For the germination index, similar characteristics was recorded as shown in Table 2.

4. DISCUSSION

“In this study, azo dye decolourizing bacteria *Pseudomonas monteilii* strain RZT1 was isolated and characterized. This bacterial strain was selected after being grown in an enrichment medium supplemented with dye as the sole carbon source as well as in mineral salt medium which confirm the ability of the isolated bacterial species to survive in the presence of the dye. Biodegradation without any extra carbon sources is very difficult. So, optimization experiments were initiated by supplementing the mineral salt medium containing dyes with 0.5% of yeast extract. Metabolism of yeast extract is considered essential for the regeneration of NADH, which is the electron donor for the azo bond reduction” [28]. Azo reductase is reported to be the key enzyme for azo dye degradation.

“At lower dye concentration, bacteria showed maximum decolourization activity. 84.8%, 75.4%, 63.4% and 45.5% decolourization occurred after 192 hours of incubation period in 100, 200, 300 and 400ppm dye concentration. Decrease in decolourization ability at high dye concentration might be due to the toxicity of the dye which was supported by other studies” [16]. “Azo dyes generally contain one or more sulphonic-acid groups on aromatic rings, which might act as detergents to inhibit the growth of microorganisms” [16]. “Another reason of the toxicity at higher concentration may be due to the presence of heavy metals (metal-complex dyes) and the presence of non-hydrolyzed reactive groups which may retard the bacterial growth (reactive dyes)” [29]. Our results revealed that a substantial decrease of different parameters of plants *i.e* germination percentage, radical length, various attributes of root development, fresh weight, phytotoxicity, vigor Index, germination index at higher concentrations of textile dyes. Our results are in agreement with some earlier reports which have also demonstrated a same response of plants when irrigated with effluent [30-34]. Suppression of germination can be caused by reduced water intake by seeds with high concentrations of wastewater, which ultimately affects energy-forming compounds [35] total solids and heavy metals [36,37].

“The study showed clear inhibition of different parameters of seedlings. The germinated seeds did not get enough oxygen for the toxicity of effluent solution and the radical continuously remained in direct contact with the effluent might be responsible for affecting cell multiplication or the growth” [35]. “The lower effluent concentration might promote the growth because of containing plant nutrients” [38]. “This result was in line with the findings of the other who obtained a decreasing length of radical (23.43–0.90 cm) and plumule (16.40–2.20 cm) of five paddy cultivars with increase of effluent concentration” [39,40]. “Many researchers observed inhibitory effects of various effluents on radical and plumule length of different plant species” [27,41-44].

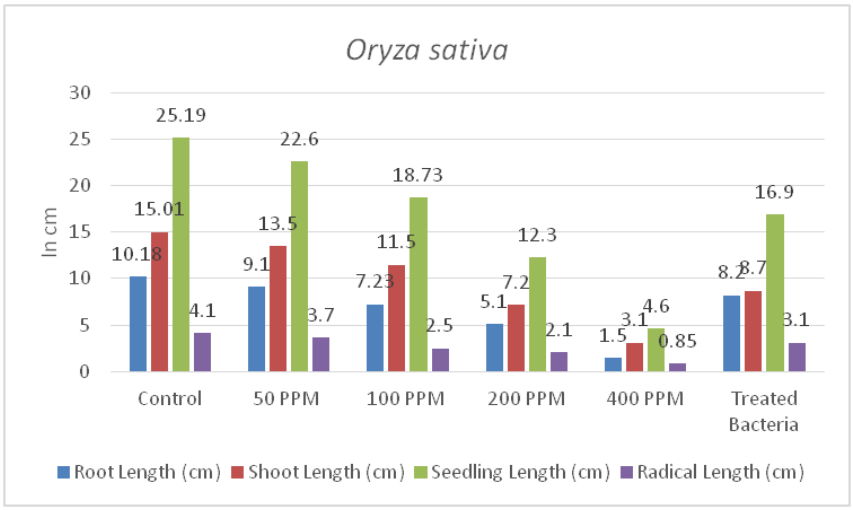

Fig. 11. Root, Shoot, Seedling and Radical length of *Oryza sativa*

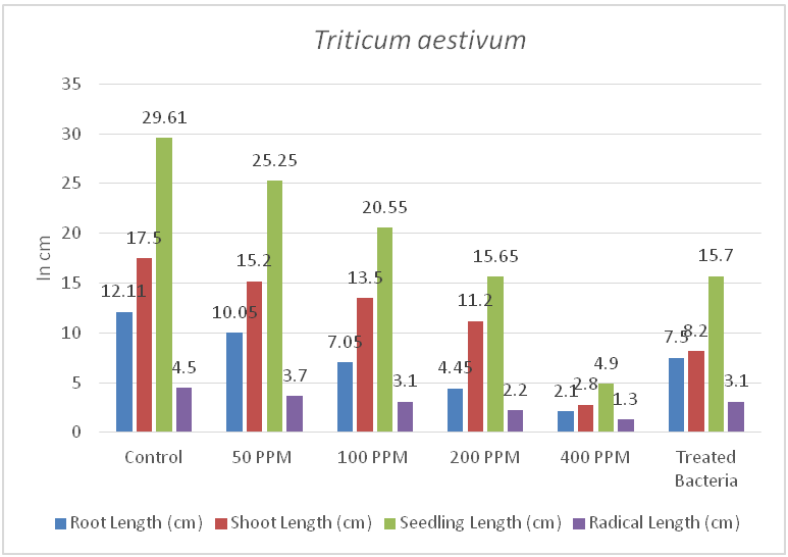

Fig. 12. Root, Shoot, Seedling and Radical length of *Triticum aestivum*

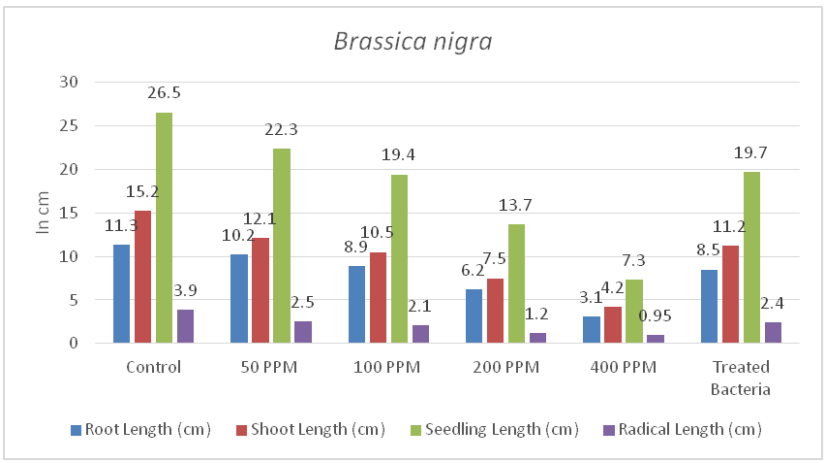

Fig. 13. Root, Shoot, Seedling and Radical length of *Brassica nigra*

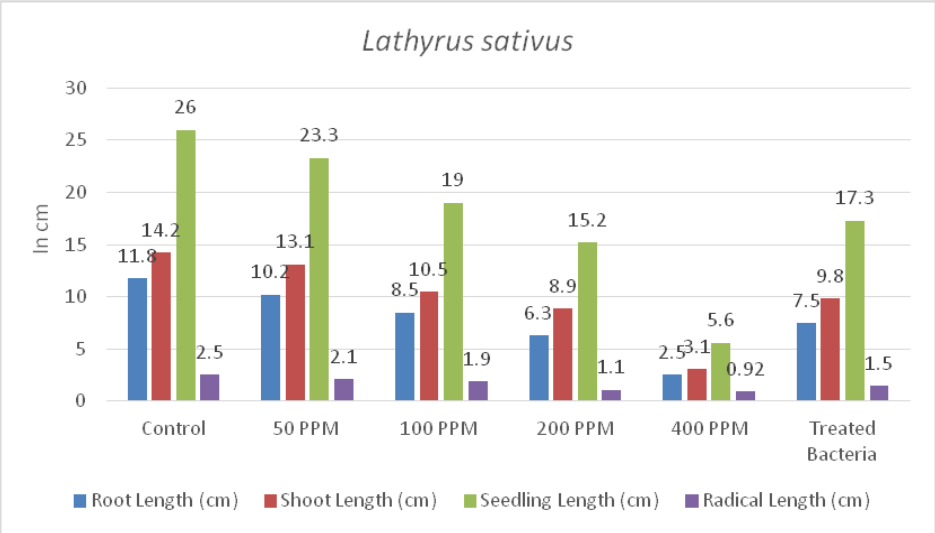

Fig. 14. Root, Shoot, Seedling and Radical length of *Lathyrus sativus*

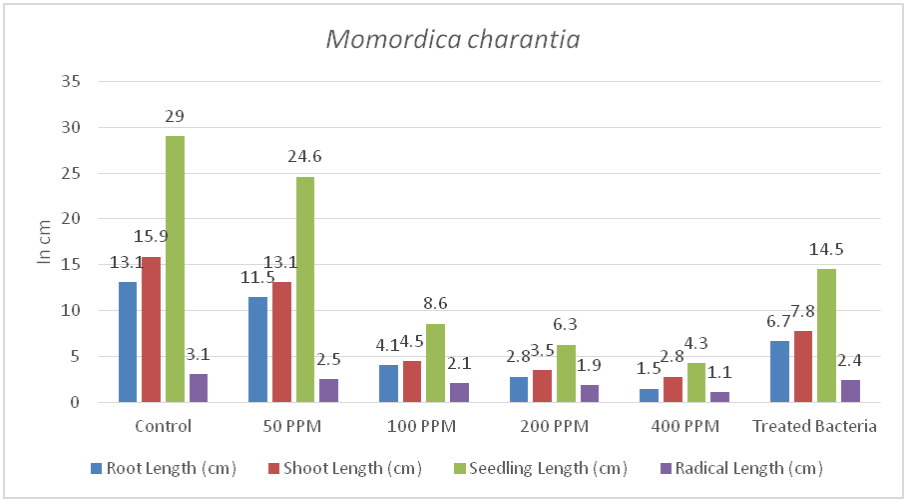

Fig. 15. Root, Shoot, Seedling and Radical length of *Momordica charantia*

Table 2. Fresh weight, vigor index and germination index of different plants at different dye concentration

Name of the plant	Concentration of the dye	Fresh weight	Vigor index	Germination index
Oryza sativa	Control	1.1	2519	1.34
	50 PPM	0.89	1808	0.89
	100 PPM	0.56	1311	0.78
	200 PPM	0.49	492	0.45
	400 PPM	0.40	92	0.23
	Treated Dye	0.75	1150	0.72
Triticum aestivum	Control	1.2	2961	1.23
	50 PPM	0.75	2020	0.78
	100 PPM	0.62	1027	0.56
	200 PPM	0.41	626	0.45
	400 PPM	0.31	98	0.23
	Treated Dye	0.69	1021	0.68

Name of the plant	Concentration of the dye	Fresh weight	Vigor index	Germination index
Brassica nigra	Control	1.1	2650	1.23
	50 PPM	0.98	1784	1.00
	100 PPM	0.75	970	0.67
	200 PPM	0.45	548	0.45
	400 PPM	0.31	146	0.23
	Treated Dye	0.88	715	0.69
Lathyrus sativus	Control	1.2	2600	1.45
	50 PPM	1.0	1631	0.89
	100 PPM	0.85	1140	0.78
	200 PPM	0.55	608	0.45
	400 PPM	0.45	168	0.34
	Treated Dye	0.78	912	0.75
Momordica charantia	Control	1.4	2900	1.23
	50 PPM	1.1	984	0.56
	100 PPM	0.93	172	0.34
	200 PPM	0.75	126	0.23
	400 PPM	0.51	43	0.12
	Treated Dye	0.83	168	0.35

Fig. 16. Effect of textile dye on seed germination on *Momordica charantia*

Fig. 17. Effect of textile dye on seedling length on *Lathyrus sativus*

Fig. 18. Effect of treated and untreated dye on seed germination of *Triticum aestivum*

Fig. 19. Effect of treated and untreated dye on seedling growth of *Momordica charantia*

The germination percentage of this study was supported by the findings of another study [39] who found a decreasing rate of rice seed germination ranging between 100.00–41.50% among five cultivars. Previous researchers also found the same trend of germination percentage with the increasing levels of effluent [39,45]. This result on fresh weight was in conformity with previous reports [44,46,47]. "The promotion of sapling growth by lower concentration of effluents might be due to creating a favorable environmental condition for the germination utilizing the nutrients present in the effluent" [48,49]. "Due to the high pH, EC, TDS and metallic contents of loom-dye effluent, high level of phytotoxicity might be occurred. Similar toxic effects of industrial effluent were observed by others" [1,44]. "Previous reports also showed higher vigor index with a lower effluent concentration" [44,45].

5. CONCLUSION

It was found that physio-chemical characteristics of the textile dye had negative impact on germination and seedling growth of economical crops of Bangladesh. But, decolourization and neutralization of textile dye by the treatment of the bacterial isolate *Pseudomonas monteilii* strain RZT1 caused significant reduction of the deleterious effects of textile dye on the germination percentage and seedling growth parameters. Taken together, it can be concluded that the isolate *Pseudomonas monteilii* strain RZT1 could be used as novel bacteria for decolourization and detoxification of textile effluents in industrial treatment plant to ensure ecofriendly environment and sustainable development.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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