Journal of Advances in Biology & Biotechnology

25(6): 46-56, 2022; Article no.JABB.91643
ISSN: 2394-1081

Biodegradation of Synthetic Dyes by Bacteria Isolated from Textile Industry Water Effluent

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Authors' contributions

This work was carried out in collaboration among all authors. Author RPK carried out the experimental work, analyzed the data, prepared the figures and wrote the first draft of the manuscript. Author SSP designed the study and supervised the experimental work. Author TAS checked and corrected the first draft of the manuscript. Author AP also designed the experimental work and finally corrected the entire manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JABB/2022/v25i630289

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/91643

Original Research Article

Received 09 July 2022
Accepted 17 September 2022
Published 20 September 2022

ABSTRACT

Aims: Biodegradation of synthetic dyes by bacteria isolated from textile industry water effluent.
Study Design: Different bacterial strains were isolated from the water effluent generated by textile industry. These bacteria were identified using morphological and molecular characterization and their ability to degrade synthetic dyes was evaluated. The dyes used in this study were methylene blue, malachite green, congo red, and methyl red.
Place and Duration of Study: Samples were collected from textile industrial area of Mandideep, Raisen (Madhya Pradesh, India). All the experiments were conducted in Department of Microbiology, Barkatullah University, Bhopal (Madhya Pradesh, India) between January 2019 to June 2019.
Methodology: Textile dye effluent was collected from industrial area of Mandideep, Raisen (Madhya Pradesh, India). The collected effluent sample was analyzed for its physical characteristics (pH, temperature, colour, odour, electrical conductivity (EC), total dissolved solids (TDS), chemical oxygen demand (COD), biological oxygen demand (BOD), dissolved oxygen (DO), turbidity, and total hardness). Different bacteria were isolated from this effluent using nutrient agar medium. These bacteria were screened for their ability to degrade synthetic dyes like methylene

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blue, malachite green, congo red, and methyl red. The potential bacterial isolates were identified using morphological and molecular methods and its ability to degrade synthetic dyes was estimated.

Results: In the current study 24 bacterial isolates were isolated from the textile water and screened for their ability to degrade methylene blue, malachite green, congo red, and methyl red. The secondary screening revealed five bacterial isolates (R-1, R-2, R-6, R-8, and R-14) possess potential of degrading all four dyes. All the isolates were tested for genetic similarity using BOX-PCR which revealed isolates R-1, and R-2 to be similar, therefore four isolates R-1, R-6, R-8, and R-14 were selected for further study. Isolate R-14 effectively degraded 79.84% of methylene blue and 84.23% of methyl red at 200 mg/L respectively after 24 h. 89.3% of 100 mg/L malachite green dye was degraded by isolate R-14 in 72 h. Congo red dye at concentration 100 mg/L was degraded 88.96% by isolate R-8. R-1 and R-6 exhibited 71.1% and 74.6% degradation of methyl red dye respectively at 100 mg/L concentration. All the four potential isolates were identified using 16S r-RNA sequencing which revealed isolate R-1, R-6, R-8, and R-14 to be *Pseudomonas geniculata, Bacillus altitudinis, Bacillus subtilis, and Citrobacter freundii* respectively. This study reports effective degradation of synthetic dyes using bacterial isolates.

Conclusion: The potential bacteria *Pseudomonas geniculata, Bacillus altitudinis, Bacillus subtilis,* and *Citrobacter freundii* can be industrially employed for the biodegradation of synthetic dyes.

Keywords: Synthetic dye; textile effluent; physico-chemical analysis; biodegradation; BOX-PCR; quantitative assay.

1. INTRODUCTION

Synthetic dyes are widely used in the textile, food, cosmetic, and paper printing industry. The annual production of synthetic dyes is estimated to be 1 million tons worldwide which makes it a commercially important product. Apart from its application in different sectors, the textile industry is the largest consumer of synthetic dyes. The textile industry uses a large amount of synthetic dye in the fabric colouring process and the wastewater discharged afterward is contaminated with a load of chemical molecules [1]. This water when discharged to a water body (river, pond, or lake) will have adverse impact on the aquatic environment. It blocks the sunlight penetration and increases biological oxygen demand (BOD) of water, causing death and ecological disturbances in the aquatic life. The presence of dyes on the surface and subsurface water is responsible for many water-borne diseases like severe irritation, dermatitis, mucous membrane, perforation of the nasal septum, and respiratory tract [2]. These colouring agents due to their xenobiotic nature are resistant to physico-chemical degradation and require a biological approach for their breakdown.

Chemical and physical methods that are currently used for the treatment of dye-containing effluent include coagulation, flocculation, membrane filtration, adsorption using activated carbon, electro-chemical destruction, precipitation by photochemical reaction, chemical oxidation method, and UV light radiation is used as another physical method [3]. The major drawback of utilizing these physico-chemical methods is that they are expensive and leads to accumulation of sludge. The biological process has gained much attention in recent times due to its cost-effectiveness and less sludge formation [4,5]. Certain studies have found that microbes can biodegrade and biosorb dyes present in wastewater [6]. Microorganisms like bacteria, algae, fungi, and yeasts, can degrade and still totally mineralize a lot of azo dyes under certain environmental conditions [7,8].

In the present study, different bacteria were isolated from the textile dye effluent, and their potential to degrade the dye was evaluated. The potential bacteria were identified so that they can be effectively utilized commercially and industrially for the degradation of synthetic dyes.

2. MATERIALS AND METHODS

2.1 Materials

Nutrient agar, malachite green, methylene blue, methyl red, congo red, 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 DNA polymerase, Taq buffer, dNTPs, and $MgCl_2$ were purchased from GeNei Laboratories Pvt Ltd, Bengaluru (India).

2.2 Sample Collection and Physico-chemical Analysis of Water

The sample was procured from effluent site of the textile dye industry located at industrial area of Mandideep, Raisen (Madhya Pradesh, India). The physico-chemical analysis of the collected sewage water sample was done by testing its pH, temperature, color, odor, electrical conductivity (EC), total dissolved solids (TDS), chemical oxygen demand (COD), biological oxygen demand (BOD), dissolved oxygen (DO), turbidity and total hardness as suggested by Sriram and Reetha, 2015 [9].

2.3 Isolation of Dye Degrading Bacteria

The collected sample was serially diluted to the concentration of 10^{-9} and plated on a nutrient agar medium (NAM). The plates were incubated at 37°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.

2.4 Primary Screening for Dye Degrading Bacterial Isolates

Bacterial isolates were tested for their ability to degrade textile dyes. The isolates were point inoculated on NAM plates which were incorporated with 10 mg/L dye (Methyl red, Congo red, Methylene blue, and Malachite green). The plates were incubated at 37°C for 24 h. After incubation, plates were observed for the clear zones. Potential bacterial isolates were selected for further studies.

2.5 Secondary Screening of Dye Degrading Isolates

The potential bacterial isolates selected from primary screening were tested further for their ability to degrade synthetic dyes at higher concentrations. The dyes included in secondary screening were congo red (CR), methyl red (MR), malachite green (MG), and methylene blue (MB) which were added to NAM at 100 mg/L concentration. The selected bacterial cultures were point inoculated and incubated at 37ºC for 48 h and observed for the zone formation.

2.6 Molecular Differentiation of Isolated Bacterial Species

The phenol-chloroform method was used to obtain genomic DNA as suggested by Moore et al. [10]. The molecular differentiation of isolated bacterial species was done using the BOX PCR method using a specific primer (CTACGGCAAGGCGACGCTGACG) [11].

2.7 Quantitative Assay of Decolorization of Dye

The potential isolates were introduced in nutrient broth containing different concentrations (25, 50, 100, and 200 mg/L) of CR, MR, MG, and MB dyes. The flasks were incubated at 37ºC and observed regularly for dye degradation till 72h after which the culture broth was centrifuged at 2000×g for 30 min and absorbance value of supernatant was measured spectrophotometrically at 490nm (UV-1601 UV-VISIBLE SPECTROPHOTOMETER, SHIMADZU). The percentage of decolorization was calculated by applying the absorbance value to the following formula.

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

2.8 Identification of Bacterial Isolate by 16S rRNA Sequencing

16S regions of the selected potential bacterial strains were amplified using PCR (BIO-RAD T-100 Thermocycler). The 50µl reaction mixture contained 2µl bacterial DNA, 5µl buffer (GeNei), 0.25µl 5U/µl Taq polymerase (GeNei), 0.5µl 10mM dNTPs (GeNei), 0.5µl (10pmol) each of the universal 16S primers F (5'-AGAGTTTGATCCTGGCTCAG-3') and R (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 using ABI 3100 Genetic Analyser (Applied Biosystems, USA) at BioInnovations, Mumbai India. The most indistinguishable sequences of the strain were identified from the NCBI database of Genbank using the BLAST algorithm and multiple alignments for homology were performed using the Clustal*W* algorithm software. The phylogenetic tree was constructed using the sequence of our bacterial isolate with closely related 14 species by MEGA-X software.

3. RESULTS AND DISCUSSION

3.1 Sample Collection and Physico-Chemical Analysis

The waste generated from textile dyeing industries is a major cause of environmental toxicity. These textile water effluents not only affect the quality of drinking water but also affect the plants and aquatic ecosystem. The sample was collected from the waste discharge of the dyeing industry in Mandideep, Raisen (Madhya Pradesh), India. These industries discharge the Blackish-green colored effluents into the environment causing water and soil pollution. The collected water sample was analyzed for physico-chemical characteristics.

Table 1. Physico-chemical characterization of textile dye effluent sample [12-14]

S. no.	Name of the parameters	Dye effluent sample
1)	Temperature	28°C
2)	pH	7.5
3)	Colour	Blackish-green
4)	Odour	Unpleasant
5)	Total dissolved solid (mg/L)	93
6)	Dissolve oxygen (mg/L)	16
7)	Chemical oxygen demand (mg/L)	0.04
8)	Biological oxygen demand (mg/L)	37.6
9)	Total hardness (mg/L)	200
10)	Turbidity(NTU)	89

3.2 Isolation of Bacteria by Textile Water Effluent

Twenty-four bacterial strains were isolated from a textile effluent sample collected from the dye contaminated region of Mandideep, Raisen (M.P.). All the bacterial isolates were tested for their ability to degrade various textile dyes. All the selected bacterial isolates were named R-1 to R-24. The gram staining and microscopic analysis revealed 8 isolates to be gram-positive and 16 to be gram-negative bacteria.

3.3 Primary Screening of Bacterial Isolates

All 24 isolates were tested in the presence of different dyes (10 mg/L) to estimate their degradation ability. A clear halo zone around the bacterial colony was conclusive of the ability of bacteria to degrade dye. All the bacterial isolates included in our study exhibited degradation of dye. 25% isolates degraded four dyes (CR, MR, MB, and MG), 33% isolate degrade three dyes, 12% isolate degrade two dyes and 25% isolate degrade a single dye. Potential dye degrading ten isolates, R-1, R-2, R-5, R-6, R-8, R-9, R-14, R-16, R-20 and R-24 were selected for secondary screening.

Table 2. Primary screening of isolated bacteria at 10 mg/L concentration of different dyes

Bacterial isolates	Dye degradation (10 mg/L)			
	MR	MB	MG	CR
R-1	+	+	++	+
R-2	+	+	+	+
R-3	-	++	+	+
R-4	-	+	+	++
R-5	+	+	-	++
R-6	+	+	+	++
R-7	-	+	+	-
R-8	+	+	+	+
R-9	+	-	+	-
R-10	-	+	-	+
R-11	+	+	-	+
R-12	+	+	+	-
R-13	-	++	-	++
R-14	+	++	+	+
R-15	+	++	+	++
R-16	-	+	-	-
R-17	+	-	-	+
R-18	+	-	-	-
R-19	-	++	-	-
R-20	-	+	+	+
R-21	+	-	+	-
R-22	++	-	-	-
R-23	+	-	-	+
R-24	++	+	++	+

3.4 Secondary Screening of Bacterial Isolates

In secondary screening five bacterial isolates R-1, R-2, R-6, R-8, and R-14 were able to degrade all four dyes. Isolate R-6 and R-8 were able to degrade malachite green effectively with a halo zone diameter of 6.5mm which was the highest among all the isolates. Similarly, isolate R-5 degraded methylene blue and congo red with maximum efficiency and yielded a 4mm and 5mm halo zone respectively.

All the isolates except R-5 were able to degrade malachite green dye. The isolates that we're able to degrade all CR, MR, MB, and MG dyes were selected for further study.

Table 3. Secondary screening of the selected 10 isolates at 100 mg/L concentration of all the four dyes

Isolates	Zone formation after 48 h (In mm)			
	100 mg/L			
	Congo red	Malachite green	Methylene blue	Methyl red
R-1	2.0	4.0	3.0	4.0
R-2	2.0	3.0	2.0	3.5
R-5	5.0	-	4.0	-
R-6	2.0	6.5	3.0	4.5
R-8	3.0	6.5	2.0	2.5
R-9	-	2.0	-	-
R-14	2.0	5.0	1.0	2.0
R-16	-	1.5	-	-
R-20	-	1.5	1.0	-
R-24	1.0	3.0	-	-

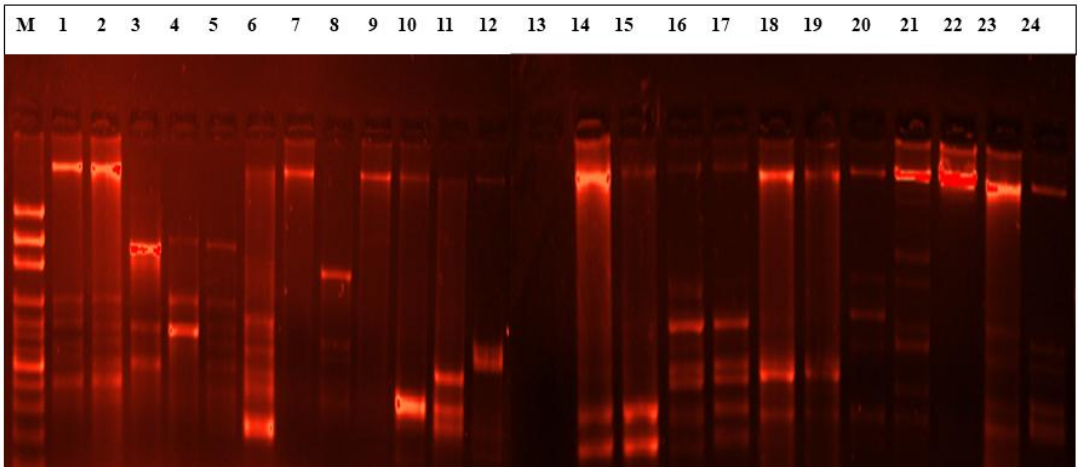

Fig. 1. BOX PCR profile of the 24 selected bacterial isolates

Lane 1 -M, Lane 2 -R1, Lane 3 -R2, Lane 4 - R3, Lane 5 -R4, Lane 6 -R5, Lane 7 -R6, Lane 8 -R7, Lane 9 -R8, Lane 10 -R9, Lane 11 -R10, Lane 12 -R11, Lane 13 -R12, Lane 14 -R13, Lane 15 -R14, Lane 16 -R15, Lane 17 -R16, Lane 18 -R17, Lane 19 -R18, Lane 20 -R19, Lane 21 -R20, Lane 22 -R21, Lane 23 -R22, Lane 24 -R23, and Lane 25 -R24

3.5 BOX PCR Result

Among the 24 bacteria isolates many were morphologically similar. The genetic similarity was observed using the BOX-PCR method. BOX PCR method was strongly differentiated on the molecular level. The bands of the gel were analysed using UPGMA: Jaccard's DisSimilarity coefficient; model [15] which revealed that isolate “R-1, R-2”, “R-14, R-15”, “R-16, R-17”, and “R-18, R-19” were exhibiting similar band pattern, confirming that those species were identical to each other. Since R-1 and R-2 exhibited similarity in BOX PCR, isolate R-1 was chosen for further studies as it exhibited better results in secondary screening as compared with R-2. The isolates R-1, R-6, R-8, and R-14 were selected for further investigation.

3.6 Quantitative Assay of Dye Degradation

After the secondary screening, the selected isolates R-1, R-6, R-8, and R-14 were analyzed for their ability to degrade four dyes; congo red, methyl red, malachite green, and methylene blue at different concentrations.

3.6.1 Congo red dye degradation

The maximum degradation of (80.01%) congo red, which is an azo dye, was observed at the concentration of 25 mg/L by isolating R-14 after 72 h of incubation. Isolate R-1 and R-8 were able to degrade 72.76% and 72.68% of the dye respectively, whereas R-6 degraded only 63.32% of congo red. All the bacterial isolates exhibited

high degradation of congo red dye at 72 h. This implies that the bacteria while attaining the log phase of its growth cycle utilized the dye as its source of nutrition. At 50 mg/L and 100 mg/L dye concentration, R-8 exhibited an appreciable degradation of 89.51%, and 88.96% respectively. Abu Talha et al., 2018 reported 77% degradation of congo red using *Brevibacillus parabrevis* [16]. Enhancing the dye concentration further to 200 mg/L, isolate R-8 exhibited only 18.79% degradation which suggested that this high level of dye was toxic for the isolate. Isolate R-14 was able to degrade congo red effectively even at the high concentration of 200 mg/L and exhibited degradation of 54.46%.

3.6.2 Methyl red dye degradation

Potential bacterial isolates were tested for another azo dye, methyl red for degradation. R-14 exhibited a good potential for degrading methyl red and degrading 80.5% of the dye (25 mg/L) after 72 h of incubation. Isolates R-1, R-6 and R-8 showed dye 64.54%, 73.92% and 67.14% degradation of dye in 72 h at 25 mg/L concentration respectively. Among isolate R-14 showed its excellency and degraded methyl red even at higher concentrations of 50 mg/L, 100 mg/L, and 200 mg/L by 92.38%, 90.18%, and 84.23% respectively only after 24 h of incubation. This shows that isolate R-14 utilized methyl red effectively as the source of nutrition. Even at higher concentrations, R-14 thrived well in the presence of the dye which explicates that it was able to tolerate dye toxicity efficiently. Maniyam et al., (2018) reported 65% degradation of methyl red dye using *Rhodococcus sp.* UCC 0008 in 72 h [17]. Other isolates also exhibited degradation of 70.7% (R-8), 74.6% (R-6) and 71.1% (R-1) of methyl red in 72 h at 100 mg/L concentration.

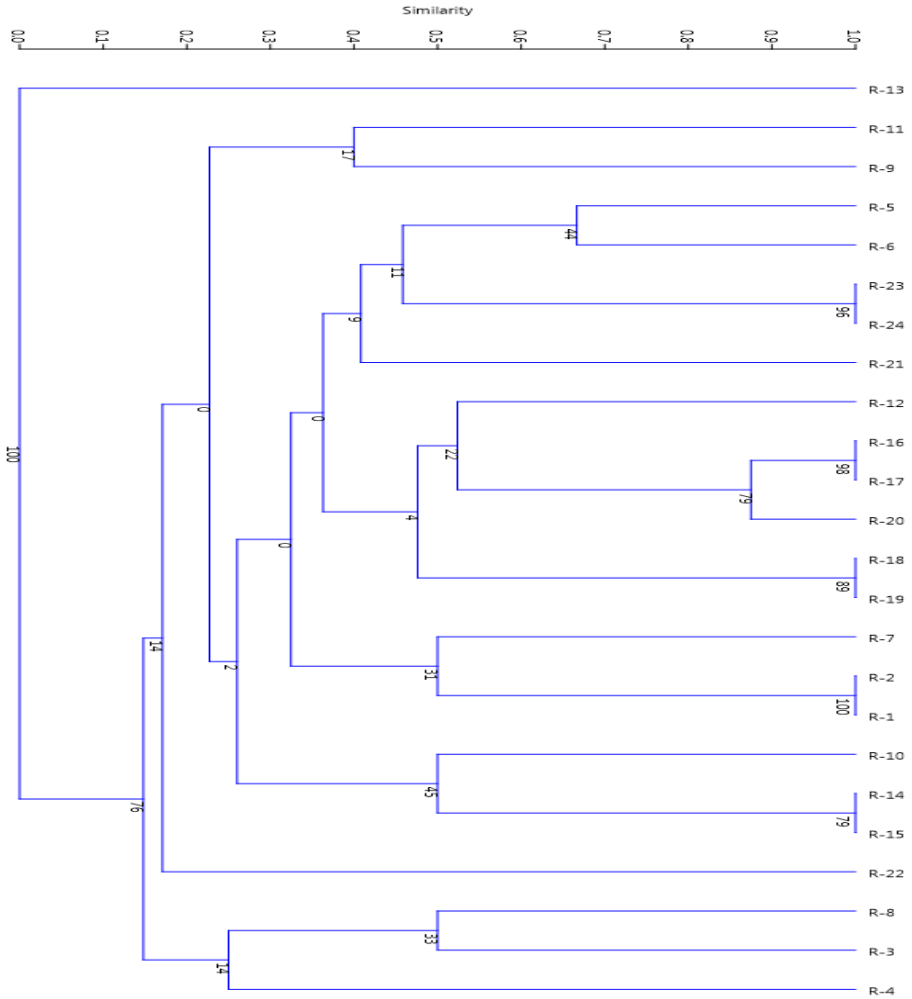

Fig. 2. Clustering of the 24 selected isolates based on the BOX PCR profile

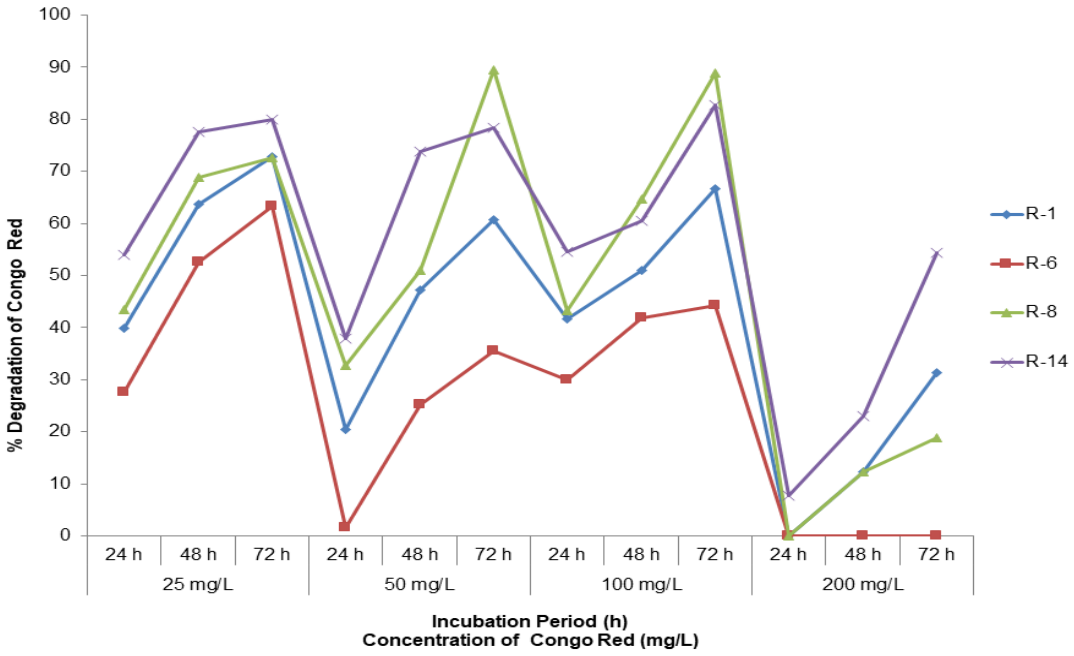

Fig. 3. Percentage degradation of Congo red dye

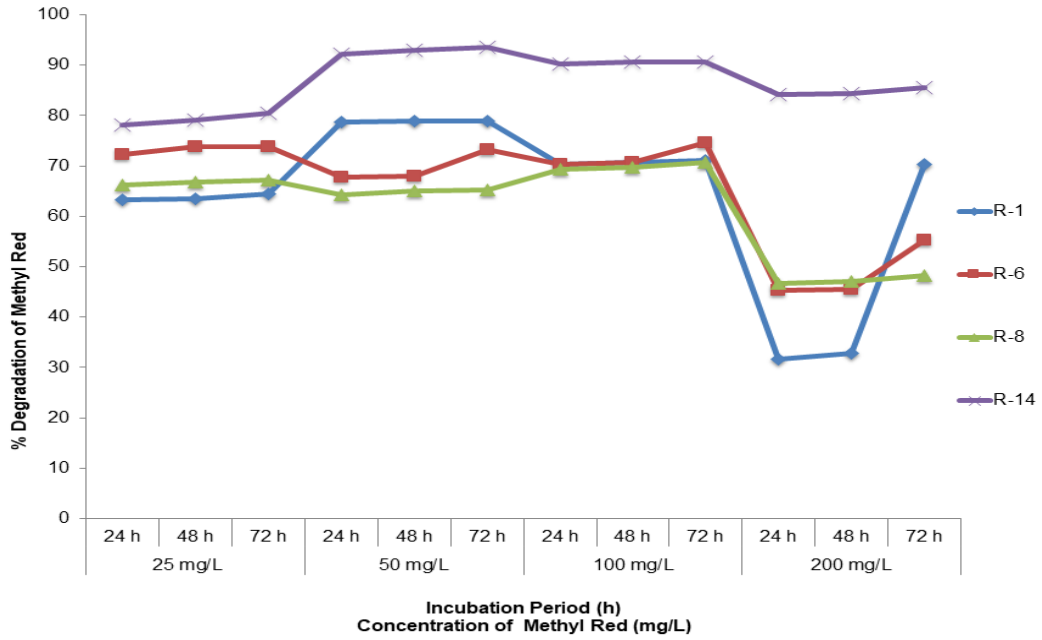

Fig. 4. Percentage degradation of Methyl red dye

3.6.3 Methylene blue dye degradation

The methylene blue dye was evaluated for its degradation by the selected potential bacterial isolates. The bacterial strain R-14 degraded methylene blue effectively by 75.4% and 79.84% in 72 h at high concentrations of 100 mg/L and 200 mg/L respectively. The degradation efficiency of isolate R-14 was enhanced with the increasing concentration of methylene blue dye from 25 mg/L to 200 mg/L which suggests that it utilized this dye as the carbon source [18].

Isolate R-6 also degraded 74.4% and 73.51% of this dye at a similar concentration respectively when incubated for 72 h. This explicated that our isolates were able to tolerate a high concentration of methylene blue and efficiently degraded this dye.

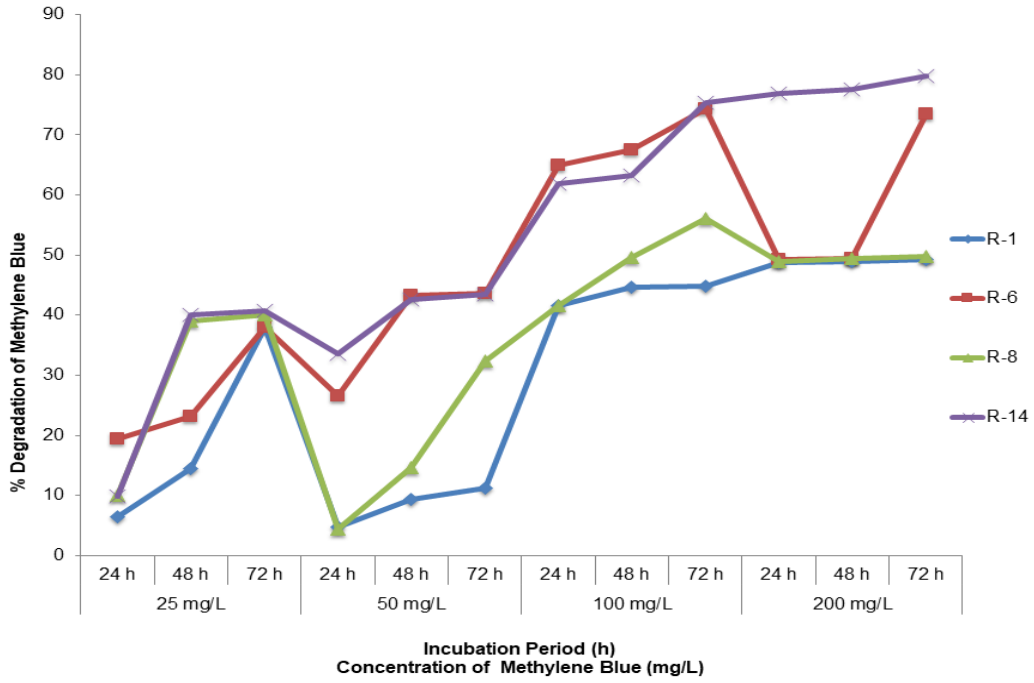

Fig. 5. Percentage degradation of Methylene blue dye

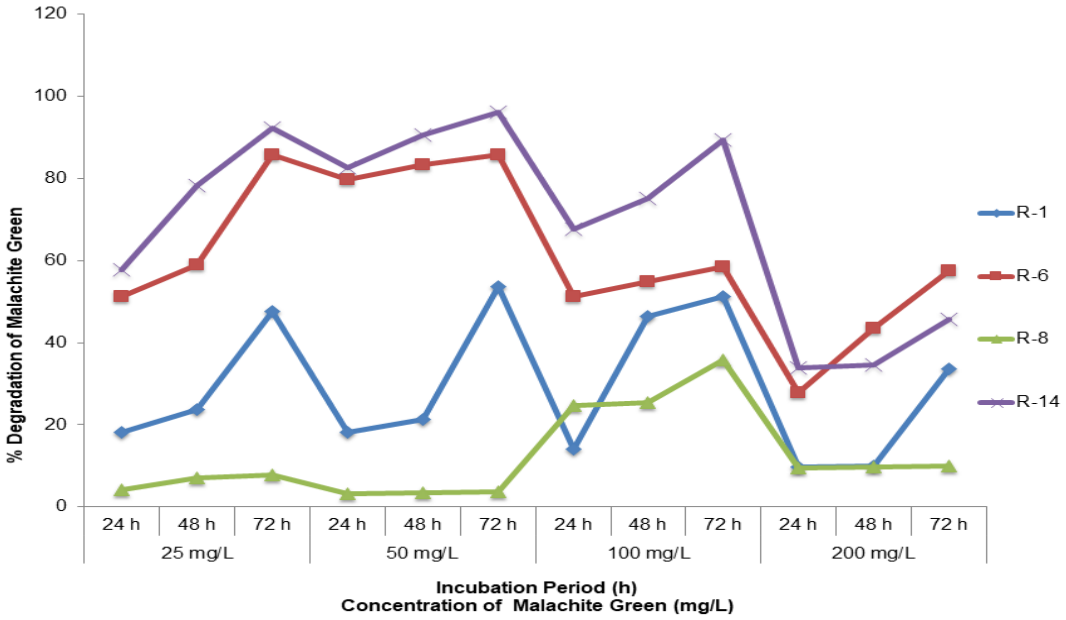

Fig. 6. Percentage degradation of Malachite green dye

3.6.4 Malachite green dye degradation

The maximum degradation of 96.02% of triphenylmethane dye (malachite green) was observed by the isolate R-14 at the concentration of 50 mg/L. This isolate degraded 92.23% of dye at 25 mg/L concentration in 72 h. Kumar and Saravan [19] reported 90.1% degradation of malachite green at 10 mg/L concentration in 72 h using bacteria DD4 [19]. In the present study, it was observed that at higher concentrations of 100 mg/L and 200 mg/L a degradation of 89.30% and 45.78% was achieved respectively after 72 h. Isolate R-14 exhibited good potential for degrading malachite green dye also at higher concentration (200 mg/L) whereas isolate R-6 showed degradation of 57.53% in 72 h. At lower concentrations of 25 mg/L and 50 mg/L, R-6 degraded 85.78% and 85.72% of the dye respectively in 72 h of incubation. This shows that though malachite green dye is toxic and is hard to bioremediate can be broken down in the majority using our potential isolates.

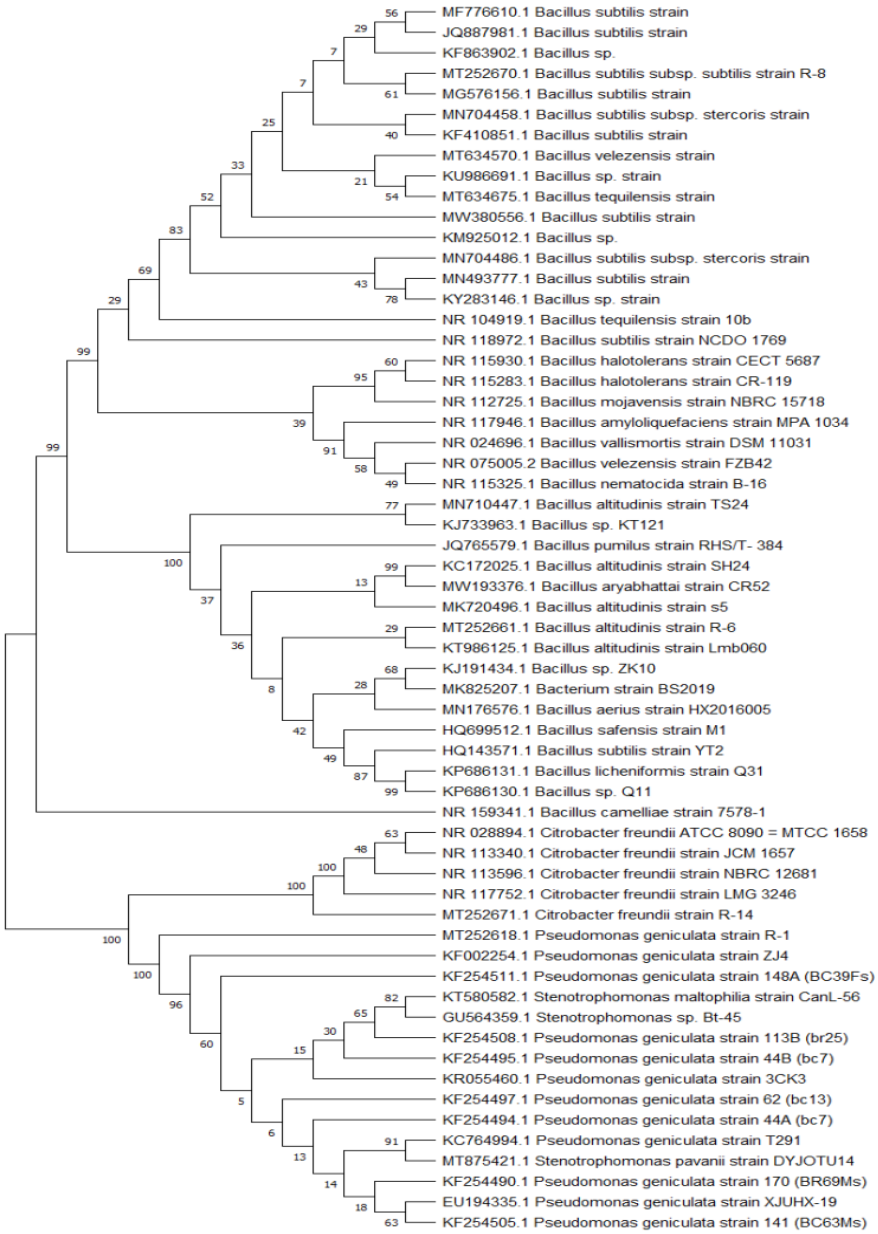

Fig. 7. Neighbour Joining tree for bacterial strains based on partial sequences of the 16S rRNA gene distances was calculated using the Kimura 2 parameter in MEGA-X software [20]

3.7 Identification of Bacterial Isolate by 16S rRNA Sequencing

The potential isolates were identified based on the partial sequences of the 16S rRNA gene. The bacterial strain R-1, R-6, R-8, and R-14 was identified as *Pseudomonas geniculata, Bacillus altitudinis, Bacillus subtilis,* and *Citrobacter freundii* respectively based on BLASTn. The 16S rRNA partial sequences of the isolate were submitted to the NCBI gene bank under accession no. MT252618 (R-1), MT252661 (R-6), MT252670 (R-8) and MT252671 (R-14). The phylogenetic tree of all four potential isolates was constructed with 14 closely related sequences obtained from BLASTn.

4. CONCLUSION

The discharge of synthetic dye in the water resources has become a serious concern and requires essential steps for the pre-treatment of this effluent before discharging. The current study emphasise on the degradation of methylene blue, malachite green, Congo red, and methyl red dye using bacteria isolated from dye effluents. The results of the investigation explicated that the isolated bacteria were able to effectively degrade all four synthetic dyes. The potential bacteria *Pseudomonas geniculata*, *Bacillus altitudinis*, *Bacillus subtilis,* and *Citrobacter freundii* can be industrially employed for the degradation of synthetic dyes. Since these bacteria utilize dyes as the source of nutrition for their growth. They can be effectively utilized for the economic degradation of the dye on a larger scale.

ACKNOWLEDGEMENT

This work was supported by DBT-Barkatullah University Interdisciplinary Life Science Programme for Advanced Research and Education, Delhi, India [102/IFT/SAN/5392/2012-2013 Dated March 20th, 2013].

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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