Original Research Article

Biodegradation of synthetic dyes by bacteria isolated from textile industry water effluent

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

Results: In the current study 24 bacterial isolates were isolated from the textile water and screened for their ability to decolourize 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 decolourizing 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 200mg/L respectively after 24 h. 89.3% of 100mg/L malachite green dye was decolourized by isolate R-14 in 72 h. Congo red dye at concentration 100mg/L was decolorized 88.96% by isolate R-8. R-1 and R-6 exhibited 71.1% and 74.6% degradation of methyl red dye respectively at 100mg/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

Keywords: *Synthetic dye; Textile effluent; Physico-chemical analysis; Decolourization; 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 the in fabric colouring process and the wastewater discharged afterward is contaminated with a load of chemical molecules [1]. This water when reaches the river, the pond, or the lake causes toxicity within the aquatic environment. It blocks the sunlight penetration and increases biological oxygen demand (BOD) of water, the water resource causing death and ecological disturbances in the aquatic life.

These colouring agents due to their xenobiotic nature are resistant to Physico-chemical degradation and require a biological approach for their breakdown. All textile dyeing industry uses a large amount of different artificial and natural colours during the textile dyeing process, and in the end, the water is released with a large number of harmful chemical molecules [1]. This is merged with other water sources through the drains. The textile industry and dying induce changes in the chemical, physical and biological characteristics of the aquatic environment which includes changes in temperature, smell, the taste of water which is dangerous for public health, animals, wildlife, fish, and other natural diversity. 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].

The current approach to removing dye from effluent involves Physico-chemical methods. The major drawback of these methods is that they are expensive, and in spite of removing the colours, a problem arises in the settlement of concentrated sludge accumulation. Chemical and physical methods 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 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]. The biological process is cheaper as well as the end products are not poisonous [7]. Microorganisms like bacteria, algae, fungi, and yeasts, can degrade and still totally mineralize a lot of azo dyes under certain environmental conditions [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 Material

Nutrient agar media, nutrient broth, 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 buffer, dNTPs, and $MgCl_2$ were purchased from Invitrogen, USA.

2.2 Sample collection and Physico-chemical analysis of water

The water sample was procured from the effluent site of the textile dye industry located in the industrial area of Mandideep, Raisen (Madhya Pradesh, India). The collected sewage water sample was analyzed for 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 [9].

2.3 Isolation of dye decolorizing bacteria

The collected sample was serially diluted to the concentration of 10^{-9} and plated on a nutrient agar medium (NAM). The incubation was done at 37°C for 24 h. Later, the plates were observed for the presence of bacterial colonies which were later purified and stored on NAM slants at 4°C.

2.4 Primary screening for dye decolorizing bacterial isolates

Bacterial isolates were tested for their ability to degrade textile dyes. The bacterial isolates were point inoculated on NAM plates incorporated with 10mg/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. Efficient bacterial isolates were selected for further studies.

2.5 Secondary screening of dye decolorizing isolates

The potential bacterial isolates selected from primary screening were tested further for their ability to decolorize 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 100mg/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., 2004 [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 different concentrations (25, 50, 100, and 200mg/L) of CR, MR, MG, and MB dyes. The flasks were observed continuously for 72 h for the degradation of dye. Further, the culture broth was centrifuged at 2000×*g* for 30 min. The supernatant was collected and its absorbance value 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 from 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. The final volume was adjusted using 41.75µl MQ water. PCR reaction cycles included primary denaturation at 92 ºC for 2 min, followed by 35 cycles of denaturation at 92 ºC for 1 min, annealing at 48ºC for 30 sec, and extension at 72ºC for 2 min. The final extension was at 72ºC for 6 min. The PCR products were analyzed using 1% agarose gel containing ethidium bromide. The amplified 16S rRNA was sequenced using 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 19 species by MEGA-X software.

3. RESULTS and DISCUSSION

3.1 Sample collection and Physico-Chemical Analysis

The main source of environment toxicity is textile dye industrial waste. This not only affects the quality of drinking water but also affects plants in the soil and the 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 shown in Table 1. Its temperature was found to be 28ºC and pH 7.5. The textile effluent sample was blackish-green colour with an unpleasant odour. The total dissolved solid value was found to be 93mg/L. Dissolved oxygen is a primary requisite for aquatic life [12]. The dissolved oxygen was recorded in the sample was 16mg/L. The chemical oxygen demand was recorded at 0.04mg/L and the Biological oxygen demand was recorded at 37.6mg/L. The total hardness of the water sample was observed at 200mg/L. Hardness is mostly due to the high quantity of calcium and magnesium salts and chloride [13]. Turbidity is the muddiness or lack of clarity of water caused by large numbers of being particles that are normally undetectable by the naked eye. The dimension of turbidity is only a solution test of water quality [14].

Table 1. Physico-chemical characterization of textile dye effluent sample.

S. No.	Name of the Parameters	Dye effluent sample
1)	Temperature	28℃
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 decolorize various textile dyes. All the selected bacterial isolates were named R-1 to R-24. The gram staining and microscopic analysis revealed that 8 are Gram-positive and 16 are Gram-negative bacterial isolates. The pure cultures were preserved on nutrient agar slants at 4ºC.

3.3 Primary screening of bacterial isolates

All the 24 isolates were tested in presence of different dyes (10mg/L) to estimate their decolorization ability. A clear halo around the bacterial colony was conclusive of the ability of bacteria to decolorize dye. All bacterial isolates included in our study exhibited dye decolorization. 25% isolates decolorized four dyes (CR, MR, MB, and MG), 33% isolate decolorize three dyes, 12% isolate decolorize two dyes and 25% isolate decolorize a single dye. Therefore, 10 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 10mg/L concentration of different dyes.

Bacterial isolates	Dye Degradation (10mg/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 decolorize all four dyes. Isolate R-6 and R-8 were able to degrade malachite green effectively with a zone diameter

of 6.5mm which was the highest among all the isolates. Similarly, isolate R-5 decolorized methylene blue and congo red with maximum efficiency and yielded a 4mm and 5mm halo respectively. All the isolates except R-5 were able to decolorize 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)			
	100mg/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	-	-

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. After observing the Gel image and seeing the results of UPGMA: Jaccard's DisSimilarity coefficient; model [15]. It was revealed that Isolate no. "R-1, R-2", "R-14, R-15", "R-16, R-17", and "R-18, R-19" were shown as a similar band. similar band pattern, that confirmed 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.

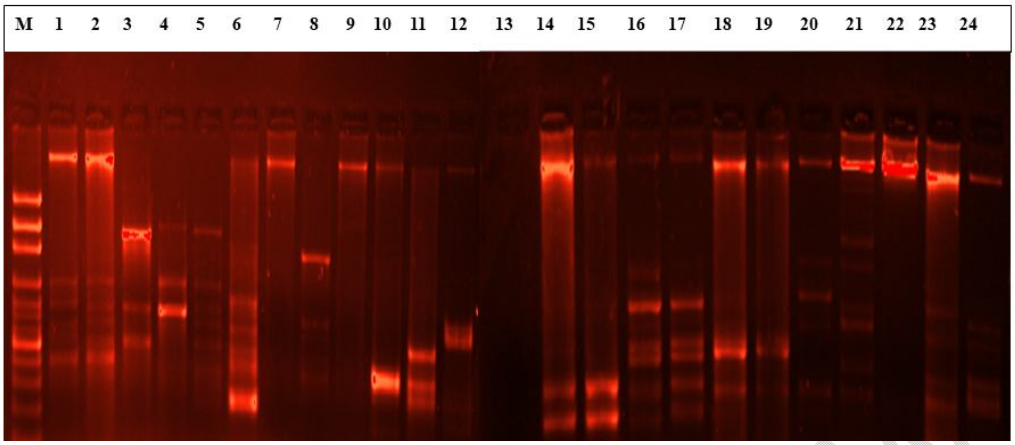

Figure 1. BOX PCR profile of the 24 selected bacterial isolates.

Lane 1-M, Lane 2 -R1, Lane 2 -R3, 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, Lane19-R18, Lane 20-R19, Lane21-R20, Lane 22-R21, Lane 23-R22, Lane 24-R23 and Lane 25-R24.

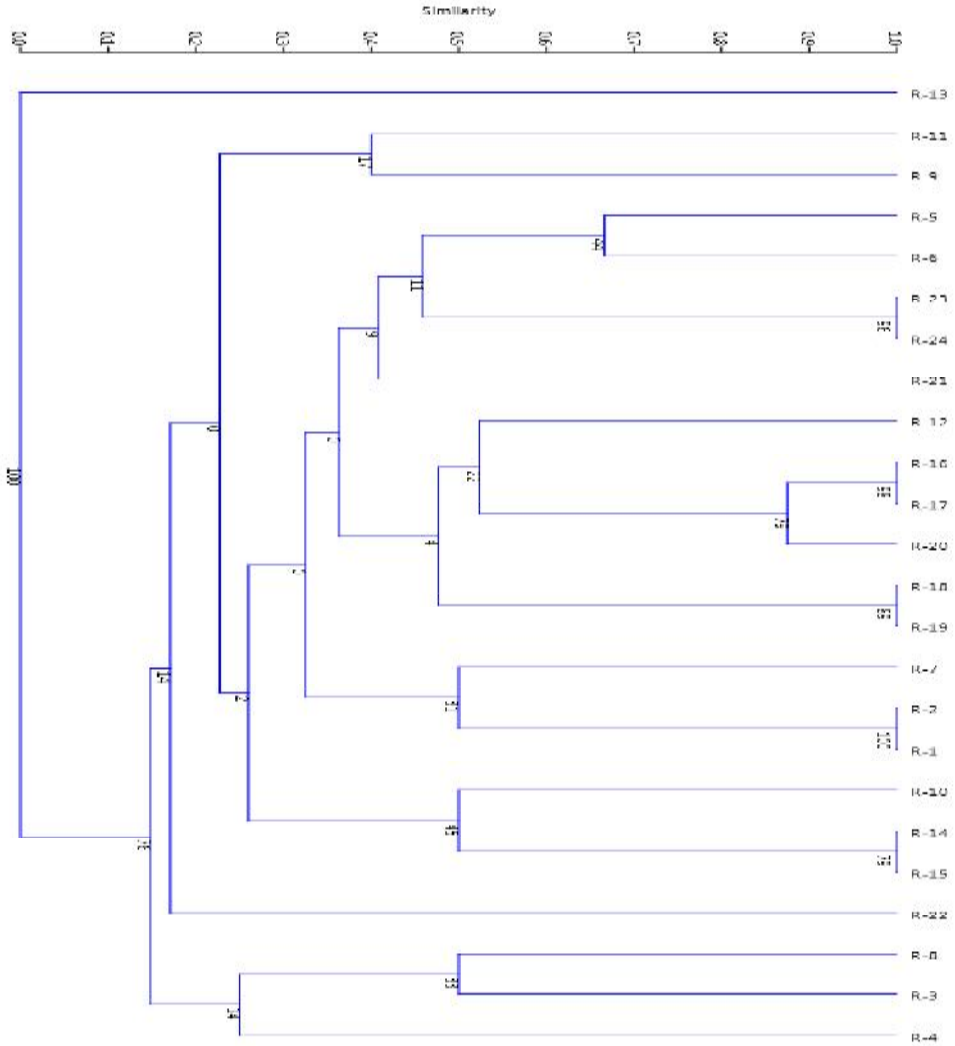

Figure 2. Clustering of the 24 selected isolates based on the BOX PCR profile.

3.6 Quantitative assay of dye decolourization

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

3.6.1 Congo red dye decolourization

The maximum decolorization of (80.01%) congo red; which is an azo dye, was observed at the concentration of 25mg/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 decolourized only 63.32% of

congo red. All the bacterial isolates exhibited high degradation of congo red dye at 72 h. This implicates that the bacteria while attaining the log phase of its growth cycle utilized the dye as its source of nutrition. At 50mg/L and 100mg/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 200mg/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 decolorize congo red effectively even at the high concentration of 200mg/L and exhibited degradation of 54.46%.

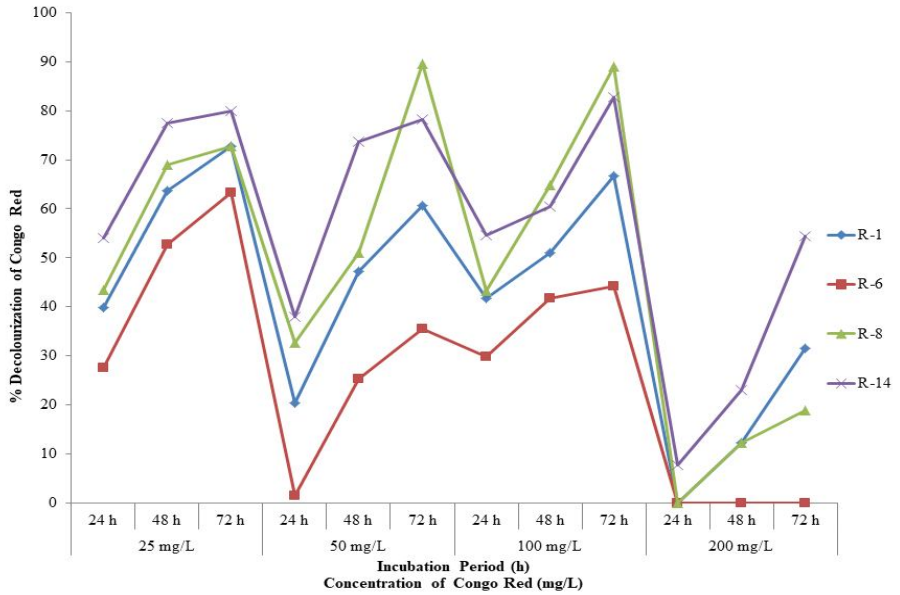

Figure 3. Percentage degradation of Congo red dye.

3.6.2 Methyl red dye decolorization

Potential bacterial isolates were tested for another azo dye, methyl red for decolourization. R-14 exhibited a good potential for degrading methyl red and decolourizing 80.5% of the dye (25mg/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 25mg/L concentration respectively. Among Isolate R-14 showed its excellency and decolorized methyl red even at higher concentrations of 50mg/L, 100mg/L and 200mg/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% decolourization 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 100mg/L concentration.

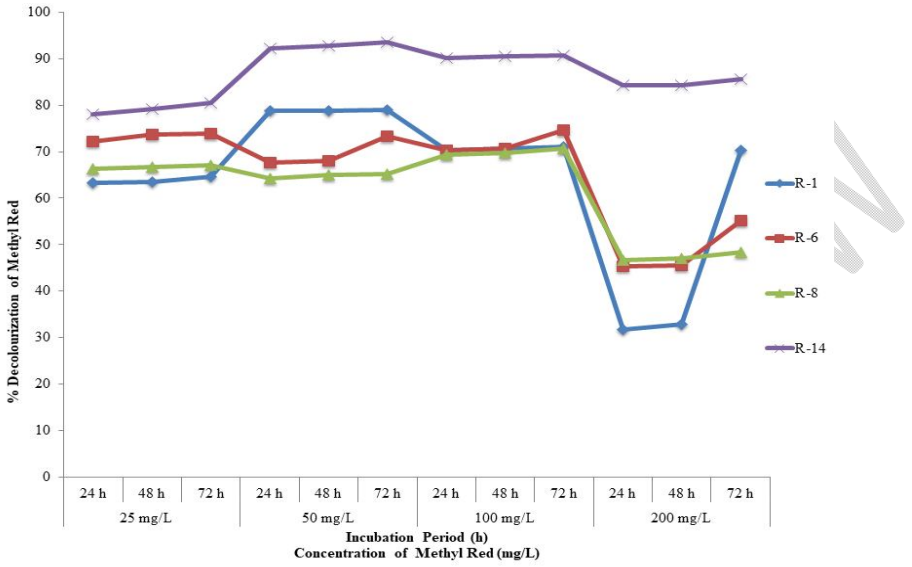

Figure 4. Percentage degradation of Methyl red dye.

3.6.3 Methylene blue dye decolourization

The thiazine dye methylene blue dye (Hi-media) was evaluated for its decolourization 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 concentration of 100mg/L and 200mg/L respectively. The decolourization efficiency of isolate R-14 was enhanced with the increasing concentration of methylene blue dye from 25mg/l to 200mg/l which suggests that it utilized this dye as the carbon source [18]. Isolate R-6 also decolourized 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.

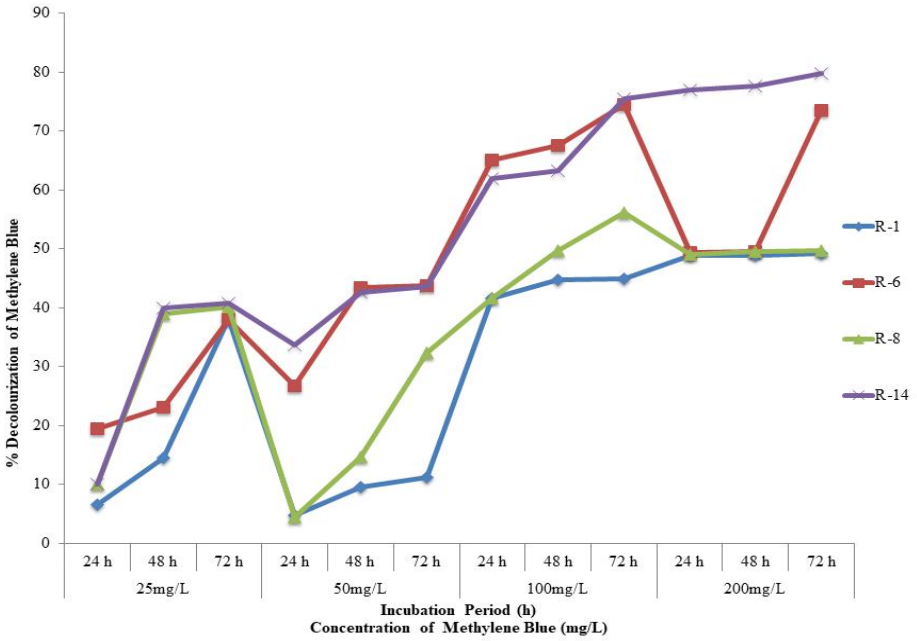

Figure 5. Percentage degradation of Methylene blue dye.

3.6.4 Malachite green dye decolourization

The maximum decolourization of 96.02% of triphenylmethane dye (malachite green) was observed by the isolate R-14 at the concentration of 50mg/L. This isolate degraded 92.23% of dye at 25mg/L concentration in 72 h. Kumar and Saravan (2015) reported 90.1% decolourization of malachite green at 10mg/L concentration in 72 h using bacteria DD4 [19]. In the present study, it was observed that at higher concentrations of 100mg/L and 200mg/L a decolourization 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 (200mg/L) whereas isolate R-6 showed degradation of 57.53% in 72 h. At lower concentrations of 25mg/L and 50mg/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.

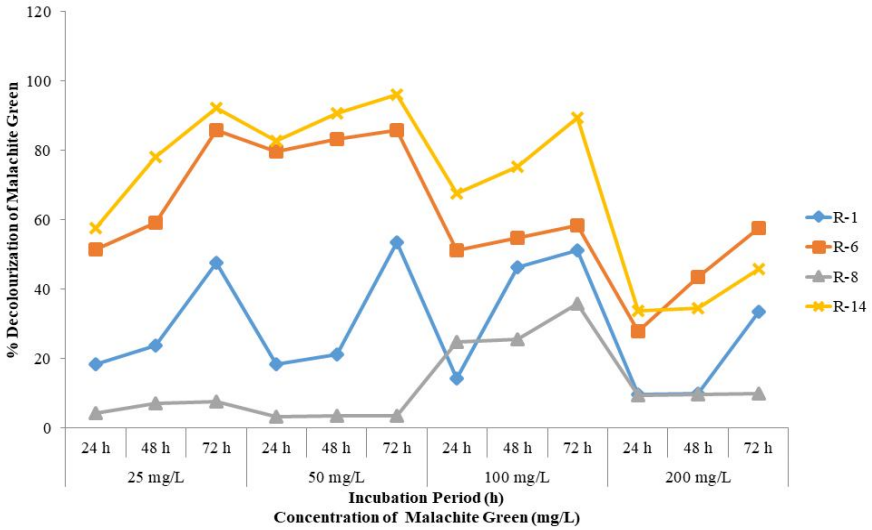

Figure 6. Percentage degradation of Malachite green dye.

3.7 Identification of bacterial isolate by 16S rRNA sequencing

The potential isolates were identified based on the 16S rRNA gene. The complete 16S rRNA sequence of the isolate R-1 (1419 bp), R-6 (1440 bp), R-8 (1435bp), and R-14 (914 bp). The bacterial strain R-1, R-6, R-8, and R-16 was identified as *Pseudomonas geniculata, Bacillus altitudinis, Bacillus subtilis,* and *Citrobacter freundii* respectively based on BLASTn. The 16S rRNA 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 the four potential isolates was constructed with 14 closely related sequences obtained from BLAST.

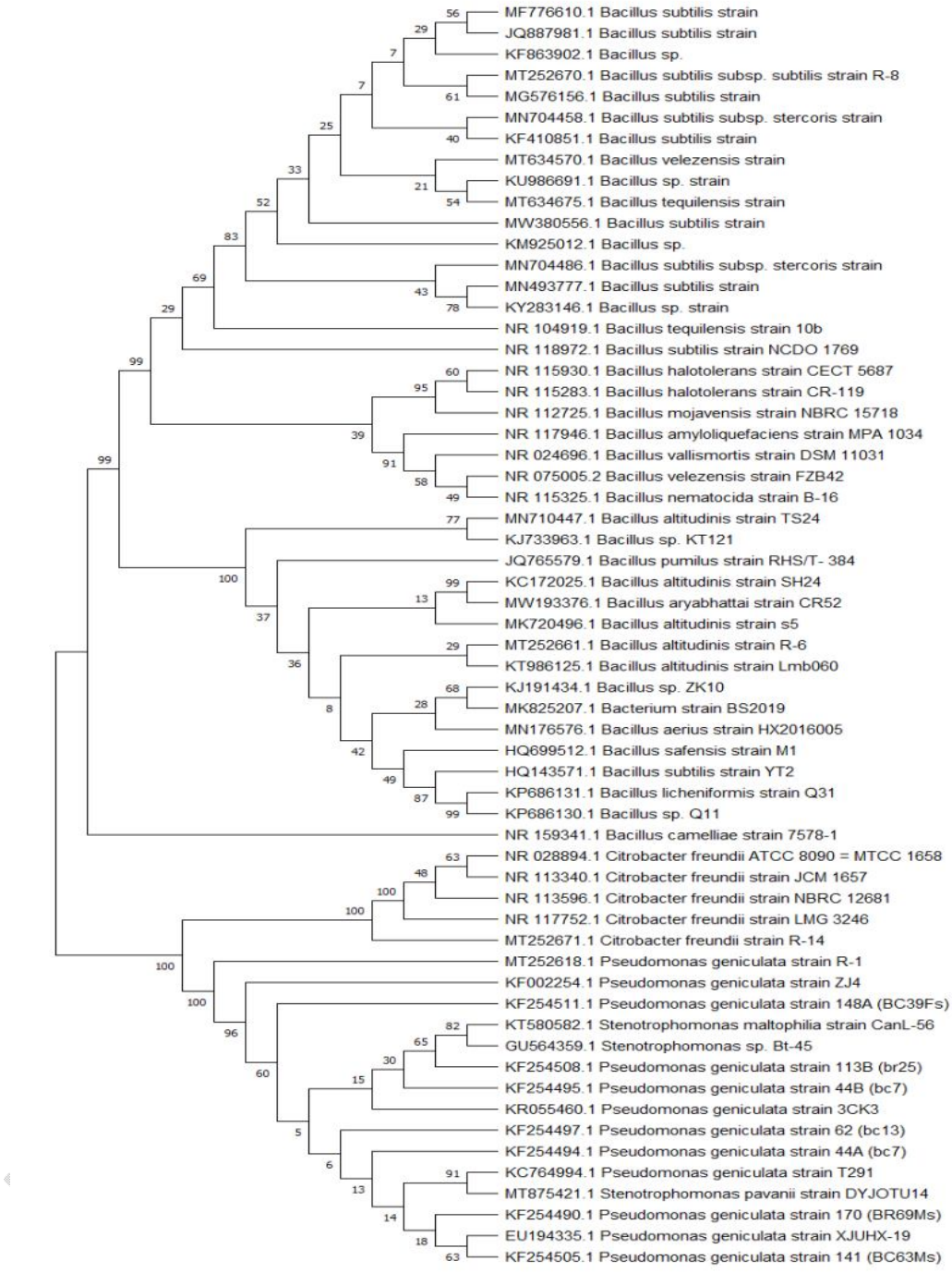

Figure 7. Neighbour Joining tree based on 16S rRNA Gene Sequences Showing the Phylogenetic Relationship between selected Potential Bacterial Isolates R-1, R-6, R-8, and R-14 against NCBI Data Base Sequenced.

4. CONCLUSION

The discharge of synthetic dye in the water resources has become a serious concern and requires essential steps for the treatment of this effluent. The current study inclines 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 their source of nutrition for growth they can be effectively utilized for economic degradation of the dye at a larger scale.

7. REFERENCES

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