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Isolation and Characterization of Dye Decolorizing Bacteria from The Textile Dye Effluents

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Article History	Abstract
   	The most significant challenge confronted by the textile industries is the discharge of dye effluents which contains toxic chemicals posing a considerable threat to environmental pollution. Biological method of treating effluents using bacteria is one of the renewed and accepted method in effluent treatment. The present study aims to isolate and characterise the dye decolorizing bacteria from the effluent samples of a dye industry from Chinnalapatti, Tamilnadu. The dye effluents were collected from the effluent plant of a leading dye industry and the Physiochemical parameters were analysed. The samples were plated in a Nutrient Agar and bacterial isolation were performed according to the standard microbiological procedures. The bacterial isolates identified by biochemical identification were tested for its efficiency to decolorize Methyl red and crystal violet dyes. Among the three isolates S1showed 98% decolorizing efficiency in reducing Methyl red dye and 92% efficiency in decolorizing crystal violet dye. Sample S2 showed 85% activity in decolorizing methyl red dye and 86% activity in decolorising Crystal violet dye. S3 showed a low decolorizing activity compared to S1 and S2. Both of these phenotypically identified isolates are subjected to molecular identification by 16s gene amplification and gene sequencing. The obtained FASTA sequence were analysed for the phylogenetic relationship and submitted to NCBI. The potential isolate that has the capacity to decolorise the dyes were identified as Bacillus Tropicus and Pseudomonas mosselli. This study proves that these bacterial strains can be used as a potential bioremediation agent in decolorizing the dye effluents. **Keywords:** *Dye Degradation, Dye degrading bacteria, Crystal violet dye, Methyl Red dye*

1. Introduction

In today's scenario synthetic dyes are extensively used in all dye industry because of its cost effectiveness in synthesis, firmness, high stability to light, temperature and durability. (1). These kinds of synthetic dyes are very hard to degrade and leave behind residue materials that are extremely difficult to decompose and have a negative impact on the ecosystem. The soil microflora and aquatic environment may potentially be impacted by this dye pollution (2). Due to high toxicity and carcinogenicity of these effluents' bioaccumulation occurs and this affects the biological food chain. The release of dyes and their metabolites into the environment lowers the ground water level and contaminate the quality of the ground water.

These synthetic dyes can be removed Jond decolorised by different physical and chemical methods. The most common method employed is the use of fentons reagent to oxidize and precipitate the micro molecules in the dyes which is followed by bleaching with chloride or ozone. Photodegradation and membrane filtering are some of the techniques adapted in effluent treatment. (3). Though the physical and chemical methods are advantageous, they are not cost effective and the resultant produce large amount of sludge which leads to secondary level land contamination (4).

Biological approach using microorganisms to decolorise the dye effluents are gaining interest as they do not affect the environment and eco system. Due to its affordability, reduced sludge generation, and environmental friendliness, biological methods using a variety of microorganisms, including bacteria, fungus, yeast, and algae, have gained attention as a viable substitute (5). In the recent years decolourisation of the effluents dyes with a microbial consortium has a greater advantage. more cost-effective and environmentally benign than physiochemical techniques (6). Based on these findings the present investigation aims to isolate and characterise the common dye degrading bacteria from the dye effluents collected from the dye industry in Chinnalapatti, Tamilnadu and to check their decolourisation efficiency in commonly used laboratory dyes.

2. Materials And Methods

Sample collection: Three effluent samples were procured from the stagnant effluent canal from a dyeing industry in Chinnalapatti, Dindigul district, Tamilnadu, India using sterile containers. All aseptic procedures were followed during the sample collection to avoid contamination. The samples were transported to laboratory and preserved in 4° C for further processing. The samples were checked for its pH, temperature, Colour and odour.

Isolation of the bacterial strains: The collected effluent samples were observed for the physiochemical properties and further processed in the laboratory. 1ml of sample were serially diluted with bacteriological saline and 0.1 ml of dilutions were plated separately on a nutrient agar medium and incubated for 48hrs at 37º C. After incubation the individual colonies with identical colony morphology were chosen and purified on a fresh nutrient agar medium.

Identification of the bacterial isolates: Three morphologically identified colonies were further purified in a fresh nutrient agar plate. The three isolates named S1, S2 and S3 were identified using morphological and biochemical Characters following the standard protocol of Bergey's Manual (7).

Screening for Dye Decolourisation: The three bacterial isolates were checked for their efficiency in decolorizing the commonly used dyes Methylene Red, and Crystal violet. The isolates were cultured on 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 experiments were also carried out in three 250 ml flask for three bacterial isolates. each flask containing 100 ml of Nutrient Broth with 15 ml of dye effluents. The pH was adjusted to 7 ± 0.2. Then, the flasks were autoclaved at 121°C at 15 lbs pressure for 15 minutes. The autoclaved flasks were inoculated with 5 ml of bacterial inoculum of each isolate. The flasks were kept in mechanical shaker and incubated at 37 ℃ for 4 days. Samples were drawn at every 24 hours intervals for observation. About 10 ml of the dye solution was filtered and centrifuged at 5000 rpm for 20 minutes. After 72 hrs of incubation Decolourization was assessed by measuring the absorbance at 620 nm of the supernatant with the help of spectrophotometer at wavelength maxima (λm) of respective dye. (8)

Decolourization assay was measured in the terms of percentage decolourization using spectrophotometer. The percentage decolourization was calculated from the following formula,

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

Molecular identification of isolates: Phenotypically identified isolates of bacteria were cultured and the single colony from the pure culture plate was picked for the extraction of total genomic DNA using Phenol Chloroform method. (9). Amplification of 16S rRNAgene was carried out for the sample using universal forward & reverse primers. (PCR condition: 7 minutes for initial denaturation at 95ºC, followed by 40 cycles of 30sec at 95ºC,30sec at 52ºC, and5 minutes at 72ºC) Expected band was amplified in all the samples PCR-generated amplicon was confirmed and purified using genejet PCR purification kit (Thermo Scientific, eulithuania) to remove the primer dimer and other carryover contaminations. The quality of the product was assessed using 1.7% agarose gel along with 100bp DNA ladder as size standard and the product was found to be good for sequencing.

Primers used	Sequence (5' to 3')
Universal 27 (Forward)	AGAGTTTGATCMTGGCTCAG
Universal 1492 (Reverse)	TACGGYTACCTTGTTACGACTT

Amplified PCR products were purified and prepared for Cycle sequencing using the Big Dye® Terminator 3.1 sequence kit (Applied Biosystems, Foster City, California, USA). After cycle sequencing, the products were purified using Ethanol-EDTA purification protocol to remove the un-

incorporated dntp's, ddntp's and primer dimer. The purified sequencing products were dissolved in 12µl Hi-Di formammide and the samples were subjected for denaturation at 950C for 5mins. Denatured products were loaded in for sequencing in forward and reverse direction using Genetic Analyzer 3500 (Life Technologies Corporation, Applied Biosystems®,California 94404, USA).Sequences were aligned and edited using Mega software version 11to confirm the species.(10)

Evolutionary relationships of taxa

The evolutionary history was inferred using the Neighbor-Joining method [11]. The bootstrap

consensus tree inferred from 100 replicates [12] is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches The evolutionary distances were computed using the p-distance method [13] and on the units of the number of base differences per site. This analysis involved 18 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1220 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

3. Results and Discussion
Isolation, screening and identification of bacterial isolates from the effluent samples:

The collected samples S1, S2, and S3 were individually studied for their identification and isolation. After incubation the individual colonies were identified and isolated based on their colony morphology. The three strains isolated were further confirmed by biochemical characterisation specified in the Bergeys Manual of Determinative Bacteriology. The Biochemical characteristics of the isolated colonies are listed in Table 1

CHARACTERISTICS	S1	S2	S3
Gram staining	+	+	+
Shape	Rod	Rod	Rod
Motility	Motile	Motile	Motile
Indole test	+	+	+
Methyl red	+	+	+
Citrate test	-	-	-
Voges Proskauer	-	-	-
Urease test	-	-	-

Table 1: Identification of the three bacterial isolates based on their biochemical and morphological characteristics. indications: + and – indicates positive and negative results.

Identification of a potential dye degrading bacteria: The three isolates which were confirmed physiologically were evaluated for their ability to degrade the commonly used laboratory dyes methyl red (Fig 1) and crystal violet.(Fig 2) Among the three isolates S1 shows greater decolorizing activity when compared to S2 and S3, after 24 hrs of incubation the plates showed a very good decolorizing potential which proves that the bacteria residing in the textile effluent has the indigenous ability in degrading dyes, this property has gained by the bacteria due to high exposure to dyes for a longer period of time. The percentage of decolorising efficiency proves that S1 isolate shows 98% decolorising efficiency in reducing methyl red dye after 72 hrs of incubation and S2 shows 85% decolorising efficiency and S3 shows 78% decolorizing efficiency. Similarly, S1 isolate has 92% efficiency in reducing Crystal violet dye when compared with S2 which has 86% and S3 82%. The percentage of decolorizing efficiency is shown in Table 2 and 3

BLANK	SAMPLE	Absorbance of 620nm		% Of Decolourization
		Initial Absorbance	Final Absorbance	
0.00	S1	0.811	0.805	98%
0.00	S2	0.811	0.879	85%
0.00	S3	0.811	0.62	75%

Table 2: Decolourization Efficiency of The Methyl Red Dye by The Isolates After 72 Hours of Incubation

BLANK	SAMPLE	Absorbance of 620nm		% Of Decolourization
		Initial Absorbance	**Final Absorbance**	
0.00	S1	0.72	0.67	92%
0.00	S2	0.72	0.63	86%
0.00	S3	0.72	0.596	82%

Table 3: Decolourization Efficiency Of The Crystal Violet By The Isolates After 72 Hours Of
Incubation

A similar investigation was carried out by Kumar and Saravanan,2015 (14) isolated 13 bacterial strains
from the textile effluent of Kanchipuram, Tamil Nadu, India revealed the potential biodegradation of
the malachite green used in their study and it was identified as Staphylococcus species. A confirmed
study by *Olukanni etal ,2006* proves that many strains of *Bacillus, Staphylococcus* and *Pseudomonas*
had a very good degrading potential of textile dyes and and can be used for bioremediation (15). A
study by Thangaraj sheela and senthil kumar,2020 proved that the bacterial isolate identified from the
textile effluent in Salem district proved to be the strain of *Bacillus cereus* and they have the capacity to
degrade the reactive Black white dye. (16). The spectrophotometric data in the present study showed a
great potential of MR, and CY dye decolorization by three of the isolates. The isolates S1 and S2 has a
higher degradative ability compared to S3 and they were further processed for sequencing analysis to
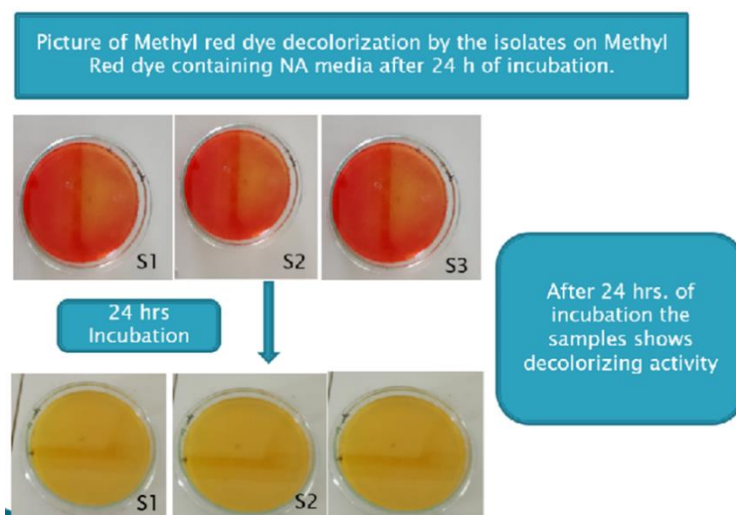find out the molecular phylogeny

Fig 1: Petri plates representing the decolorising efficiency of the three isolates on Methyl red dye
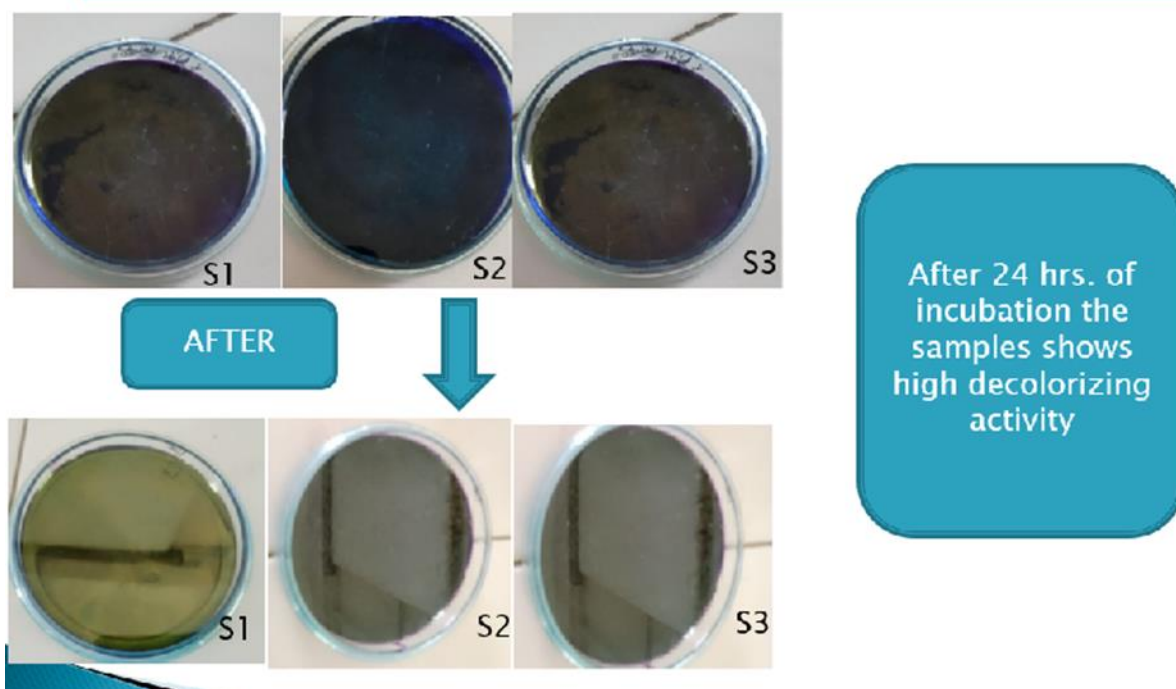containing NA media after 24hrs of incubation.

Fig 2: Petri plates representing the decolorising efficiency of the three isolates on Crystal Violet dye containing NA media after 24hrs of incubation.

Molecular identification of the Isolates:

Molecular taxonomic classification was performed for the strain S1 and S2. The partial 16s rRNA gene sequences of the strains were amplified and sequenced (Figure 3). The best sequenced portion of 16 srRNA exhibited highest identity with *Bacillus* and *Pseudomonas* Blast searching against the GenBank database was performed and the phylogenetic tree was constructed (Figure 4). During phylogenetic tree construction, strain S1 had formed a new branch and the homology indicates that the strain S1 is identified as *Bacillus tropicus* and the second isolate indicates homology with *Pseudomonas* and identified as *Pseudomonas moselli* (Fig 4)
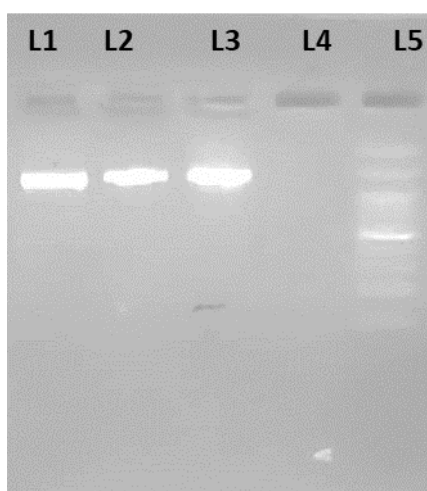

Fig 3: PCR generated amplicon confirmed in 1% Agarose gel electrophoresis

Fig 3: PCR Product representing Lane1: positive control; Lane 2: Sample 1; Lane 3: Sample 2; Lane 4: Negative control; Lane 5:100bp DNA ladder.
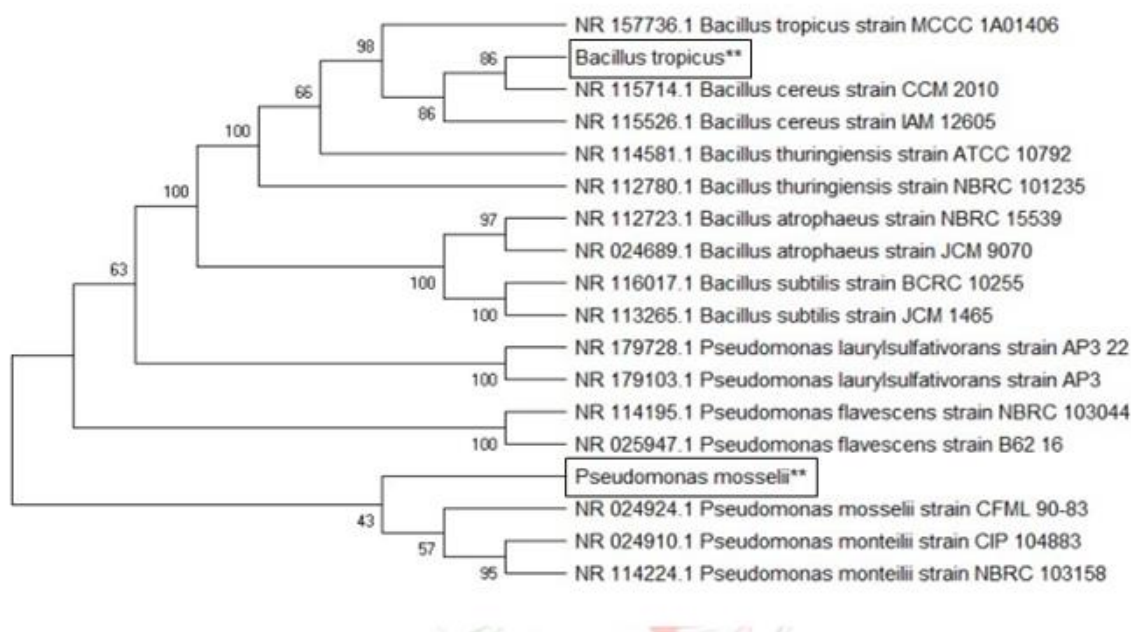
Fig 4: Phylogenetic tree construction using Neighbourhood joining method

4. Conclusion

In the current study the isolated bacteria from the textile effluents have a very good capability in degrading crystal violet and methyl red dyes. Molecular phylogenetic studies confirms that the isolated strains are *Bacillus tropicus* and *Pseudomaonas moselli* and these strains showed potential dye degradation activity. This potential capacity can be effectively used as an alternative strategy to the physical and chemical processes of treating textile effluents as they have a high potential for being able to decolorize methyl red and crystal violet dye. Detailed studies on process parameters for bioremediation and genetic manipulation are required to improve the potential of these organisms to solve the pollution problems caused by synthetic dyes.

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