ACTA BOTANICA PLANTAE

www.actabotanica.org

Original Research Article

ISSN: 2583-7400

Isolation and Molecular Identification of Dye Degrading Bacterial Flora from Textile and Tanneries Effluent for Bio-augmentation

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Citation: Sidra Shaheen, Samina Iqbal, Fatima Qazi, Aliza, Mohammad Zafar, Atia Gohar, Laraib Tahir, Kashaf Fatima, Saeeda Bano, Muhammad Shakeel (2025). Isolation and Molecular Identification of Dye Degrading Bacterial Flora from Textile and Tanneries Effluent for Bio-augmentation. *Acta Botanica Plantae.* **https://doi.org/10.51470/ABP.2025.04.02.32**

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Received 28 April 2025 | Revised 19 May 2025 | Accepted 18 June 2025 | Available Online 08 July 2025

ABSTRACT

Untreated textile effluent contains potentially hazardous compounds and poses serious health risks to both humans and aquatic life. Chemical approaches are effective in reducing the dye effluent, but these also produce more toxic byproducts. Biological processes provide more environmentally friendly alternatives and produce easily degradable chemical compounds. Dye-degrading bacteria may decolorize the colors in the effluent using anaerobic and aerobic conditions, producing easily degradable chemicals posing minimal to no environmental risk. This study aimed to isolate, identify, and characterize dye-degrading bacteria in industrial textile effluent.

Industrial textile effluents from two sources were incubated in nutrient agar media along with red reactive dye. Pure colonies were isolated and, subjected to biochemical, morphological, and molecular characterization through the Sanger's sequencing.

Seven (07) bacterial isolates from Sample-1 and 08 from Sample-2 showed growth on dye-degrading media along with the zone of clearance around them, indicating the degradation of dye. Among these, two bacterial isolates showed maximum dye degradation activities and were named as FUUAST-SF1 and FUUAST-SF2respectively.The isolates were identified on the basis of morphological, biochemical and 16s rRNA sequence. The results of the molecular analysis revealed that the isolate FUUAST-SF1 had 96% identity with Bacillus licheniformis and FUUAST-SF2 had 90.93% identity with Bacillus subtilis, respectively. The identified novel bacterial strains can be used as an environmentally friendly and cost-effective method for the degradation of dyes produced by textile effluents.

Keywords: *Dye degrading bacteria, environmentally friendly, textile effluent, molecular characterization.*

1. Introduction

Industrial pollution has increased in recent decades at an alarming rate. The textile industry is one of the major contributors to the economic growth of a country (22). However, the expansion of the textile industry has led to major environmental concerns. Textile effluents are wastewater produced by the textile industry through a variety of wet processes, including bleaching, dyeing, finishing, scouring, sizing, and desizing (5). Its hazardous to the environment and public health because they contain a complex mixture of various chemicals, including metals, dyes, surfactants, salts, organic and inorganic compounds, and biocides (5) (9). In aquatic ecosystems, textile effluents can have several negative effects, including lowering dissolved oxygen levels, changing pH levels, raising chemical and biological oxygen demands (BOD and COD), obstructing photosynthesis, and having an impact on aquatic organism growth and reproduction (13) (21). Compounds found in textile effluents, such as dyes, can be hazardous to human health due to their ability to accumulate and magnify in the food chain. Dyes are also carcinogenic, mutagenic, and poisonous(11).

The treatment of colorful textile effluents has been suggested using several physicochemical methods (1) (27). These comprise adsorption on various materials, Fenton's reagent oxidation and precipitation, bleaching with chloride or ozone photodegradation or membrane filtration, coagulation, flocculation, flotation, electrochemical destruction, and mineralization (7). Due to their high cost and tendency to produce huge amounts of sludge, all of these physical or chemical processes contribute to secondary land pollution. Bioremediation is defined as the introduction of microorganisms to break down environmental pollutants. The use of microorganisms in bioremediation is an economical and environmentally benign method of getting rid of textile waste(27).

Dyes can be effectively degraded by microbes such as yeast, fungi, and bacteria (2)(17). Dye-degrading bacteria can degrade synthetic dyes, like azo dyes, which are frequently used in paper, textile, and other industries. These dyes pose a major risk to human health and the environment since they are frequently poisonous, carcinogenic, and resistant to standard treatment techniques (29). As a result, bioremediation with bacteria that break down dyes is thought to be a potential and environmentally beneficial substitute for dye removal (8). The isolates of dye-degrading bacteria include *Pseudomonas sp*., *Enterobacter sp*., *Bacillus sp*., and *Xenophilus sp*. (8) (28).

Enzymatic degradation is the most promising remediation method for organic contaminants. Numerous enzymes involved in the breakdown of azo dyes are researched, including azoreductase, laccase, and peroxidases generated by fungi and bacteria (19). *Bacillus* species produce enzymes called lignin peroxidase and laccase, which are capable of breaking down azo dyes and other synthetic dyes. It is possible to separate these bacteria from soil or wastewater that has been tainted by the dyeing industry. *B. pumilus, B. subtilis*, and *B. cereus* are a few examples of *Bacillus sp*. that are capable of degrading dyes (12) (29) (33). These bacteria have potential uses in bioremediation and environmental protection since they can reduce the dyes' toxicity and hue. Wet industry expansion is producing high levels of wastewater and endangering the environment. Therefore, biological treatment is discouraged, since using commercially available wastewater-degrading bacteria drives up treatment costs. Research on the variety of native bacteria that break down wastewater has been scarce (4).

Therefore, the objective of the present study was to isolate and screen bacteria capable of degrading different types of dyes and to identify the isolated bacteria from textile effluents using morphological, biochemical, and molecular techniques, such as 16S rRNA sequencing. This study will not only contribute to our understanding of how these bacteria function and interact with dyes but also pave the way for the development of effective bioremediation strategies for dye-contaminated environments.

2. Materials and Methods

2.1. Samples collection

In order to isolate dye-degrading microorganisms effluent (Sample-1 and Sample-2) were collected in sterile bottles from a discharge panel of a local market and a textile industrial area, respectively. Both samples were aseptically collected and then kept in the refrigerator at 4°C till the isolation of bacteria.

2.2. Isolation of bacteria

The bacteria were isolated from the Sample-1 and Sample-2 effluent by pour plate method on nutrient agar media along with red reactive dye, and incubated at 37°C for 48 hours (32). The samples were serially diluted. One mL of each serially diluted sample was inoculated into the medium and colonies were repeatedly streaked on nutrient agar plates as well as slants. The isolated bacterial strains were stored at 4°C for further studies.

2.3 Selection of bacteria

Screening medium containing nutrient broth and agar media supplemented with red reactive dye was prepared for the selection of dye-degrading bacterial strains from Sample-1 and Sample-2. A loop full of dye effluent from the test tube of 7th dilution i.e 1:10000000 was added into the tube containing nutrient broth and incubated it at 37 °C for 24-48 hours. After the incubation time, a visible pellicle formed along with dye decolorization.

Dye degrading ability of bacteria was further confirmed by streaking a nutrient agar plate containing red reactive dye with both samples and then incubating it for 24 to 48 hours at 37°C.

2.4. Purification of isolated strains

Isolated organisms were purified through repeated plating using pour plate and streak plate methods on nutrient agar medium (29). The organism was considered pure when a plate yielded only one colony type, which was subsequently confirmed through microscopic observation.

2.5. Morphological characterization of isolated bacteria

Bacterial morphology of the selected strains was investigated in terms of color, form, margin, elevation, surface, organization, spore formation, and motility (18). The microscopic analysis of isolates was performed using the conventional Gram staining approach (3).

2.6. Biochemical identification of strains

The biochemical characterization of selected bacterial isolates was carried out by various biochemical tests that mainly includes fermentation of sugars and several hydrolytic activities (10). All biochemical reagents were prepared at the Pakistan Council of Scientific and Industrial Research (PCSIR), Karachi.

2.7. Identification of isolated bacteria by 16S rRNA gene sequencing

The 16S rRNA sequencing was performed for molecular characterization of the isolates FUUAST-SF1 and FUUAST-SF2. The CTAB method was followed to isolate the genomic DNA of the isolates, as described previously (31). Confirmation of DNA isolation was performed using agarose gel electrophoresis on 1% agarose gel. The set of primers F5´GAGTTTGATCC TGGCTCAG3´ and R5´TACGGTTACCTTGTTAC GACTT3´ was used to amplify the desired sequence of 16S rRNA gene. The PCR reaction was performed with the initial denaturation at 98°C for 3 min, 30 amplification cycles at 98°C for 12 sec, 55°C for 30 sec, 72°C for 30 sec, and final extension at 72°C for 5 min. The PCR product was then purified by using the Agencourt AMPure XP beads (BeckmanCoulter, USA). The PCR amplicon's forward and reverse DNA sequencing reactions were performed using the ABI PRISM BigDye Terminator Cycle sequencing kit (Applied Biosystems, USA) on an ABI 3500 Genetic Analyzer system (Applied Biosystems, USA). The sequence of the 16S rRNA gene of both the isolates was assessed and trimmed using the BioEdit software.

2.7.1. BLAST analysis and constructing a phylogenetic tree

The NCBI's online basic local alignment search tool (BLAST) was used to identify the sequence. The clean sequences of 16S rRNA gene of both isolates were utilized to perform BLAST searches against the NCBI GenBank database. The top fifteen sequences which were most closely related to the subject sequence were selected, and aligned with the isolate based on their maximum identity score using the ClustalW program. The distance matrix was constructed, and the phylogenetic tree was built using MEGA X software.

3. Results

3.1. Isolation of dye-degrading bacteria

Nutrient agar and nutrient broth were used to isolate and enumerate bacteria. After the propagation of bacteria, individual colonies were counted, and discrete colonies were immediately isolated according to colony morphology and transferred to other nutrient agar plate for further studies.

3.2. Selection of dye decolorization strains

Seven (07) bacterial strains from Sample-1 and eight (08) isolates from Sample-2 showed their growth on dye-degrading media along with the zone of clearance arcound them, indicating the degradation of dye. Among these two, different bacterial isolates showed maximum dye degradation activities, and were named as FUUAST-SF1 and FUUAST-SF2 from Sample-1 and Sample-2, respectively.

3.3. Morphological characterization of bacteria

The colony characteristics of two strains isolated from Sample-1 and Sample-2 of effluent water near local dye market and textile industries respectively, are shown in Figure1. The observed morphological characteristics of the isolated colonies are presented in Table 1. The morphological of both the strains was identical except that the FUUAST-SF1 colony was white colored, irregular in shape with lobate margin, whereas the FUUAST-SF2 colony was off-white in color, and circular in shape with curled margin.

3.4. Biochemical characterization of bacterial isolates

The presence and utilization of specific substances produced by selected isolates were performed by different biochemical tests (Table2). The results of biochemical characterizations of selected bacterial strains were identified on the basis of Bergey's Manual of Determinative Bacterology (6).

3.4.1. Utilization of carbohydrates

Various biochemical testes of FUUAST-SF1 and FUUAST-SF2 regarding the utilization of carbon sources mainly includes Glucose, Mannose, Lactose, Xylose and Sucrose were carried out according to Patel method (26). The results of the activity of carbohydrates by selected strains are described in Table2. Biochemically, both the FUUAST-SF1 and FUUAST-SF2 strains were similar which exhibited glucose, mannose, and sucrose utilization while lacking utilization of lactose and xylose.

3.4.2. Tests for nitrogenous compounds

The consumption of nitrogenous compounds by FUUAST-SF1 and FUUAST-SF2 were tested by performing various biochemical tests. These tests include Voges-Proskauer (VP), Catalase, Nitrate Reduction, Starch and Citrate utilization that performed as per the method explained by Patel (26). Both the strains were found positive for utilization of these nutrients.

3.5. Molecular characterization

DNA isolation, PCR amplification of 16SrRNA gene sequencing, and rRNA fragment sequencing were performed using the Sarkosyl method (30). For both the strains, a single band of high molecular weight DNA was observed which was used for 16srRNA sequencing. In PCR amplification, discrete bands of ~800 bp size were observed in the agarose gel electrophoresis 7 (Figure 2). In the DNA sequencing with the AB 3500 Genetic Analyzer, good quality (peaks well resolved) DNA sequences of 485 bp and 538 bp were obtained for the FUUAST-SF1 and FUUAST-SF2 strains, respectively.

The two isolates 16S rRNA gene sequences were utilized to perform BLAST searches against the NCBI GenBank database. The first fifteen sequences were chosen and aligned with the isolate based on their maximum identity score using ClustalW, a multiple alignment software application. The distance matrix was constructed, and the phylogenetic tree was built using MEGA X. The molecular analysis revealed that the isolate FUUAST-SF1 showed 96% identity with*Bacillus licheniformis*, andFUUAST-SF2 showed 90.93% identity with*Bacillus subtilis*. Maximum likelihood was used to infer the evolutionary history. MEGAX was used to perform evolutionary analysis. The bootstrap consensus tree produced from 1000 replicates is used to illustrate the evolutionary history of the taxa analyzed.

4. Discussion

Dye effluents are one of the major sources of water pollution in the world. The textile industry alone produces about 20% of the global industrial wastewater, which contains various synthetic dyes that are toxic and non-biodegradable. These dyes not only discolor water bodies and reduce their aesthetic value, but also harm aquatic life and ecosystems by affecting their oxygen levels, pH, and biodiversity. These dyes pose a major risk to human health and the environment since they are frequently poisonous, carcinogenic, and resistant to standard treatment techniques. Microorganisms known as dye-degrading bacteria can degrade synthetic dyes, like azo dyes, which are frequently used in paper, textile, and other industries (34). As a result, bioremediation with bacteria that break down dyes is thought to be a potential and environmentally beneficial substitute for dye degradation.

In the present study, two strains were isolated from different samples of textile effluent from local dye market (Sample-1) and textile industrial effluent (Sample-2), respectively. They were further identified on the basis of morphological, biochemical and molecular characterization. These isolates were screened on the medium containing red reactive dye due to which strains were screened out on the basis of clear zones that prove the dye degradation ability of bacterial strains. After screening, two different bacterial strains were selected from seven (07) bacterial strains of Sample-1 and eight (08) isolates of Sample-2 on the basis of zone of clearance, and were named as FUUAST-SF1 and FUUAST-SF2, respectively. It has been proved that dye-supplemented screening medium considered to be the most suitable medium for the direct selection of dye-decolorizing bacteria (14)(20).

Morphological characterization of the selected strains FUUAST-SF1 and FUUAST-SF2 resulted in Gram's positive bacteria of purple color for both the bacterial isolates. The biochemical tests of both isolated strains showed positive results with glucose, mannose and sucrose while negative results have been observed with lactose and xylose utilization of carbon sources. However, both strains showed hydrolytic activities with Vogues-Proskauer (VP), Catalase, Nitrate Reduction, Starch and Citrate utilization. On the basis of these morphological and biochemical studies,it was deduced that both FUUAST-SF1 and FUUAST-SF2 strains belonged to *Bacillus* sp.,which is in line with the agreement that several *Bacillus* species strains have been identified so far that have the ability to decolorize textile effluent and possess most potent characteristic for reducing dye from textile wastewater (24).

To further validate, the isolates FUUAST-SF1 and FUUAST-SF2 were subject to molecular characterization using 16S rRNA sequencing and phylogenetic analysis. Molecular characterization using 16S rRNA gene sequence analysis is a low-cost method for identifying basic to complex microbial strains at the species level. Phylogenetic analysis was done on both isolates using 16S rRNA gene sequences to identify microbial strains at the species level. The amplified gene sequences were matched with closely related sequences using the NCBI database via the BLAST search. The molecular characterization and phylogenetic analysis of the gene sequences using the greatest likelihood technique revealed that the two isolates FUUAST-SF1 and FUUAST-SF2, were more similar to *Bacillus licheniformis* (96%) and *Bacillus subtilis* (90.93%) respectively.

B. licheniformis has been identified to remove a range of dyes from effluent water. *B. licheniformis* produces azoreductases, which break down the azo dyes by cleaving azo bonds (25). The textile industry extensively uses azo dye orange II for coloring textiles. *B. subtilis* has also been identified to break down the dye at a temperature of 45.38 °C (15). *B. subtilis* breaks down azo bonds in the presence of glucose. Effluent water is a good source for isolating dye-degrading bacteria (23).

6. Conclusion

The present study was aimed to isolate and characterize the bacterial strains FUUAST-SF1 and FUUAST-SF2 isolated from textile effluents. After morphological and biochemical identification, these isolates were molecularly characterized using 16S rRNA molecular sequencing followed by phylogenetic analysis. The results of the molecular analysis revealed that isolate FUUAST-SF1 has a 96% identity with *Bacillus licheniformis* and FUUAST-SF2 has a 90.93% identity with *Bacillus subtilis.* These isolates were successful at decolorizing textile colors. The identified strains have potential to be used in environmental safety projects where industrial dye degradation is involved.Further research to explore the molecular mechanism of dye decolorization by the isolates FUUAST-SF1 and FUUAST-SF2 will add another value and support the experimental findings of this study.

Data submission

The 16s RNA gene sequence data of FUUAST-SF1 and FUUAST-SF2 strains have been submitted to NCBI GenBank vide accession numberPQ225974, and Pq225975.

Declaration

The authors have not any competing interests.

Funding

No specific external funding was received for this work.

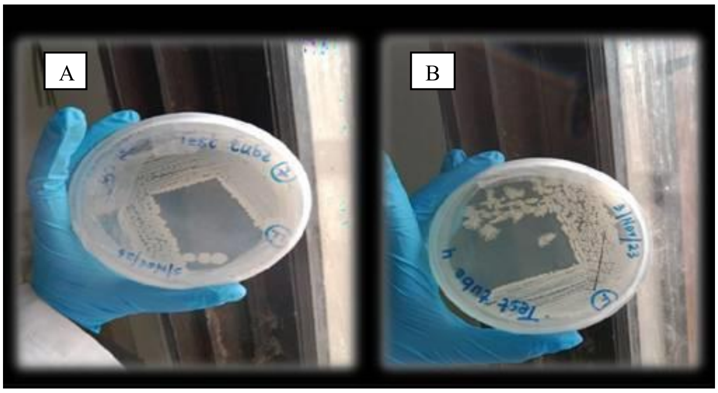

Figure 1: Bacterial colonies isolated on nutrient agar. (A) FUUAST-SF1 isolated from local dye market effluent (B) FUUAST-SF2 isolated from textile industry effluent

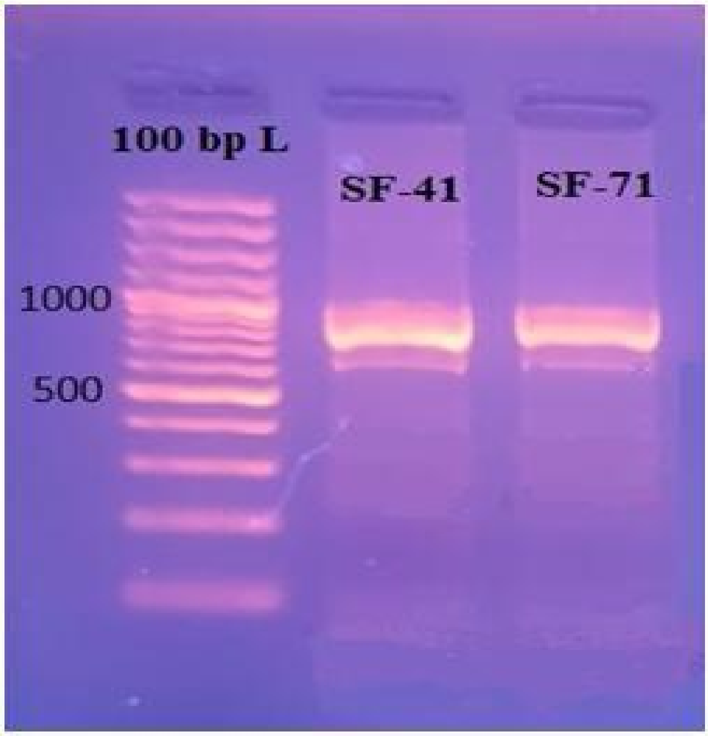

Figure 2: Agarose gel (2.0%) electrophoresis showing the bands of amplified 16S rRNA gene of the two strains FUUAST-SF1 (SF-41) and FUUAST-SF2 (SF-71)

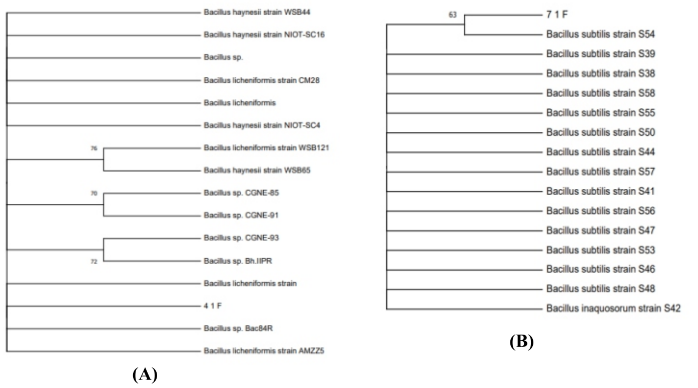

Figure 3. Phylogenetic trees of the isolated strains using 16S rRNA sequence by maximum likelihood method. (A)FUUAST-SF1 (here 4 1F) showed closeness to Bacillus licheniformis (96% similarity) (B)The FUUAST-SF2 strain (here 7 1F)showed closeness to Bacillus subtilis (90.93% similarity)

Table 1: Morphological Characteristics of Bacterial Isolates.

Morphology & Colony Characteristics	FUUAST-SF1	FUUAST-SF2
Gram type	Positive	Positive
Shape	Rod	Rod
Color	White	Off-white
Isolate form	Irregular	Circular
Isolate width	Medium	Medium
Margin	Lobate	Curled
Spore forming	Absent	Absent

Table 2: Biochemical characteristics of bacterial strains

Biochemical Characterization	FUUAST-SF1	FUUAST-SF2
Utilization of Carbohydrates		
Glucose	+	+
Mannose	+	+
Lactose	-	-
Xylose	-	-
Sucrose	+	+
Hydrolytic Activities		
Voges-Proskauer	+	+
Catalase	+	+
Nitrate Reduction	+	+
Citrate	+	+
Starch	+	+

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