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Identification and Characterization of Indigenous Bacteria with Textile Dye Degrading Potential

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ABSTRACT

Although there are natural dyes available, the rapid expansion of the textile industry has led to the development and widespread use of synthetic dyes. Synthetic dyes provide several functional advantages, but their excessive persistence, effluents and toxicity can cause a serious hazard to the water streams and environment due to their synthetic origin and intricate structure. Consequently, the increasing demand for these dyes necessitates effective strategies for mitigating dye-associated water pollution. The present work aimed to explore indigenous bacterial isolates directly from dye contaminated sites and reveal their dye degrading capabilities. In this study, four samples were collected from dye-contaminated sites to isolate and identify bacterial strains capable of decolorizing media supplemented with reactive red and reactive blue dyes. The isolated strains were purified, and their morphological characteristics were studied. Strains exhibiting substantial decolorization activity were selected for further analysis based on their performance in dye-reduction assay. Among the isolates, strains RB4-1, RB6-1, RD4-1, BB5-2, and BB5-1 demonstrated 91%, 88%, 89%, 83%, and 89% reductions in dye absorbance, respectively. Following 16S rRNA gene sequencing, these strains were identified as *Salmonella enterica, Enterobacter cloacae, Citrobacter farmeri, Proteus mirabilis,* and *Klebsiella pneumoniae*. In conclusion, the identified strains were capable of decolorizing dye with potential to be used as sustainable and cost-effective agents for bioremediation.

Keywords: Synthetic dye, Textile effluents, Decolorization, Biological method.

INTRODUCTION

The demand for synthetic dyes and dyeing processes has increased dramatically over the past decades. Because of this escalating demand, the production of textile goods and the volume of associated wastewater has risen proportionally, making textile effluents one of the major contributors to global water pollution. Water pollution control has therefore become a central focus of contemporary

scientific research. Colored organic compounds not only elevate the organic load of wastewater but also negatively affect its aesthetic quality. Currently, an estimated 100,000 dye formulations and 800,000 tons of dyes are produced worldwide, and approximately 10–15% of these dyes are discharged directly into surface water systems (Periyasamy, 2024). To satisfy the growing need for textile coloration, synthetic dyes are generated through diverse and complex chemical formulations (Fobiri, 2022). These compounds exhibit high stability and resistance to degradation, and their elevated concentrations have been shown to exert toxic effects on aquatic organisms, posing an ongoing threat to biodiversity (Chequer et al., 2013). These dyes have the ability to absorb the light leading to a reduced approach of light in contaminated water and resulting in reduced light reaction for life in water (Yusuf, 2019). Some of these dyes have also been reported as carcinogenic or mutagenic for microbial life and leading to anaerobic microbial activity (Ismail et al., 2019). The stability of synthetic dyes is due to the hydrocarbon origin of their aromatic rings. The amine portion of aromatic ring is highly toxic, and further degradation is required to reduce its toxicity. Azo dyes are said to be aromatic compounds with one or more -N=N- groups and are of great concern because of their visible and bright color, bio recalcitrance and adverse effect on humans and animals (Hashemi and Kaykhaii, 2022). The colored wastewater entering the water bodies is also the major cause of water-borne diseases like nausea, ulceration of skin, renal damage, hypertension and severe irritation of respiratory tract (Akpor et al., 2014).

Enzymes are capable of degrading the aromatic rings of the dyes and most of the time, these enzymes are oxidative in nature. To treat textile effluents, many physiochemical techniques have been proposed including reduction and chemical oxidation. Other methods include adsorption of dyes from textile effluents (Adane et al., 2021). However, all these techniques require large amounts of raw material, produce secondary products that needs to be degraded later and are less cost effective.

A lot of effort has been poured down to make eco-friendly and cost-effective methods for decolorization of textile wastewater. Bioremediation has proved to be quite effective when it comes to cleaning up the mess humans have made in the environment (Sharma, 2012). The usual strategy adopted for bioremediation is to activate the native microorganisms to enhance their degradation activity. Micro-organisms like bacteria, fungi, actinomycetes and algae have shown great potential in decolorizing the wastewater (Elnakeib and Zahran, 2024; Ramachandran et al., 2013). Enzymes are the key molecules that possess the ability to decolorize the textile effluents which takes place by breaking the electrophilic linkage in azo dyes. Azoreductase enzymes are responsible for this breakage and for the production of aromatic amines (Misal and Gawai, 2018). The reductive cleavage of azo bonds by an enzymatic biotransformation reaction is considered to be the initial step in the bacterial degradation of azo dyes. Aromatic amines which are the resulting toxic products are further broken down into simpler non-toxic forms by multiple steps bio-conversion (Mishra et al., 2020; Pandey et al., 2007).

Microorganisms naturally exposed to textile dye pollution are likely to develop adaptive mechanisms for dye tolerance and degradation; however, such indigenous bacterial population remain unexplored. The present study addresses this gap by isolating and characterizing novel bacterial isolates from native dye degrading bacteria from textile dye contaminated sites, providing insight into the degradation potential and applicability in bioremediation.

METHODOLOGY

Sample collection

Water and soil samples were obtained from the drainage and sediment habitat around different local dyeing centers in Lahore, Pakistan. The color, pH and physical appearance of the collected samples were recorded once they were transported safely to a laboratory in sterilized tubes. Samples were processed further as soon as they reached the laboratory.

Dyes and chemicals

Two dyes, Reactive red and Reactive blue were used in this study. Using UV-spectrophotometer, the maximum absorbance of each dye was recorded by preparing the dye concentrated solution and scanning it under a visible range of 350-750nm. All the ingredients required for media preparation were of analytical grade. The MSM medium was used with following composition: Glucose: 3g/l; $(NH_4)_2SO_4$: 2g/l; KH_2PO_4: 1g/l; K_2HPO_4: 10g/l; $MgSO_4.7H_2O$: 0.1g/l and NaCl: 5g/l.

Enrichment technique and isolation of bacteria

The isolation was performed using enrichment technique proposed by (Banat et al., 1996). Either 1g of soil sample or 1ml of collected water sample was used to inoculate 100ml MSM medium with 0.01g of each dye added to the medium. The Erlenmeyer flasks were placed on shaker at 30˚C for 3 days. The enriched cultures that showed considerable reduction in dye intensity were serially diluted up to 10^{-6}. Dilution of 10^{-6}, 10^{-5} and 10^{-4} were spread aseptically on nutrient agar plates and placed in incubator at 37˚C for 24 hours following the protocol of (Shah, 2014).

Effective isolates screening through dye reduction assay

Before testing the selected colonies for their ability to decolorize the azo dyes, each culture was revived in nutrient broth. Each purified strain was inoculated in MSM medium supplemented with 100ppm dye and placed in shaking incubator at 37˚C for 3 days. The dye medium which was uninoculated served as control. This assay was adopted after slight modification of the method performed by Shah (Shah, 2014). Decolorization activity was determined and expressed in terms of percentage. The λ_{max} for each dye was recorded and the decrease in absorbance was measured accordingly. Optical density (OD) was measured at 542nm and 601nm for reactive red dye and reactive blue dye respectively. One ml decolorized sample was withdrawn periodically and centrifuged at 10,000 rpm for 5 minutes. Then, absorbance was measured at λ_{max} of that particular dye. The equation which was used to express the decolorization by bacterial isolates is as follow (Pokharia and Ahluwalia, 2013).

Decolorization percentage = X-Y/X × 100

Where, X = initial absorbance; Y= Final absorbance of medium

Biochemical identification of selected isolates

The selected isolates were characterized by growth characteristics, gram staining and biochemical tests which included lactose fermentation, urease, citrate and catalase to know more about these strains and their origin.

Molecular identification of selected strains

Ribotyping through amplification and dideoxy sequencing of a portion of 16srRNA gene was done using primer described by (Qurban and Ameen, 2020). The resulting nucleotide sequences were aligned through NCBI BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

Bacterial strains in total samples

The four samples collected were designated as sample A, B, C and D. When they were inoculated in dye supplemented media, all four samples showed considerable visible color reduction (Figure 1). The smell of all the A, B, C and D samples was pungent and unpleasant and the appearance was grayish in appearance. The pH of all the collected samples turned out to be in between neutral to acidic.

Figure 1. a) Reactive Red dye supplemented media before inoculation **b)** Reactive Red dye supplemented media after 72hrs inoculation **c)** Reactive Blue dye supplemented media after 72hr inoculation.

Twelve different microbes were isolated from each serial dilution of reactive red supplemented enriched

media. Three different bacterial strains were obtained from each sample A and B, four from sample C and two from sample D. With respect to reactive blue supplemented medium, three bacterial strains were isolated from each sample A and D. While four and two different strains were isolated from sample B and C respectively.

Dye reduction assay

Decolorizing ability of twenty-four initially screened microbes were analyzed in test tubes for the decolorization of azo dyes i.e. reactive red and Reactive blue. In sample A, the isolate RA5-1 showed the highest reduction in Reactive red dye i.e. 85% (Figure 2). In sample B, RB4-1 strain showed the highest value of reduction i.e. 91%, that is more than RA5-1 strain of sample A. The RAC4-4 strain from sample C showed the maximum reduction in dye that is 90% which is clearly more than RA5-1 strain but 1% less than RB4-1. RD4-1 from sample D showed 89% reduction which is more than strains of sample A but less than the strains derived from sample B and C. The value obtained from reactive red dye media in the tabular form are as follows:

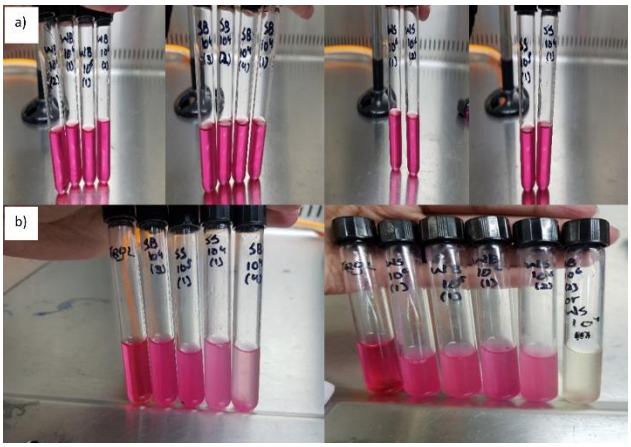

Figure 2. a) The reactive red dye containing media inoculated with purified strains **(b):** Decolorization of dye containing media after three days incubation. The left tube from the left is control (without inoculation).

From reactive blue dye supplemented medium, BA5-1 strain of sample a showed highest reduction i.e. 81%. Out of the four strains of sample B, BB5-1 represented the highest reduction among all other strains i.e. 89%, which is more than BA5-1 strains of sample A. BC6-1 of sample C showed the reduction of 79% which is less than both the strains of sample A and B that showed highest level of reduction.

Table 1. The optical density of the purified strains of reactive red dye and reactive blue dye. The OD values were presented as mean ± SD.

SAMPLE	STRAINS	REACTIVE RED		STRAINS	REACTIVE BLUE	
		OD_{542}	%		OD_{601}	%
Sample A	RA5-1	0.251±0.05	85%	BA5-1	0.246±0.03	81%
	RA5-2	0.270±0.01	84%	BA5-2	0.303±0.02	77%
	RA6-1	0.267±0.02	84%	BA5-3	0.259±0.02	80%
Sample B	RB4-1	0.149±0.02	91%	BB5-1	0.137±0.01	89%
	RB6-1	0.197±0.01	88%	BB5-2	0.221±0.05	83%
	RB6-2	0.288±0.04	83%	BB6-2	0.302±0.03	77%
Sample C	RC4-1	0.307±0.04	81%	-------		
	RC4-2	0.314±0.02	81%	BC5-1	0.298±0.04	77%
	RC4-3	0.300±0.01	82%	BC6-1	0.280±0.04	79%
	RC4-4	0.165±0.02	90%	--------		
Sample D	RD4-1	0.185±0.03	89%	BD6-1	0.356±0.05	73%
	RD5-1	0.255±0.02	84%	BD6-2	0.295±0.03	77%

In sample D, BD6-2 strain showed 77% reduction (Figure 3).

Evaluation of selected strains against different azo dyes

The strains that showed the best results were separated from the rest and were rested on shaker for further 24hrs. The strains were RB4-1, RB6-1, RD4-1, BB5-2 and BB5-1. These strains were further checked for their ability to decolorize Direct Brown, Direct Violet, Direct Turquoise and Reactive green. These strains after 24 hrs. of incubation showed considerable reduction in the intensity of dye supplemented media which is clearly visible in Figure 4.

Phenotypic identification of selected strains

The strains that showed the best results were further

characterized through biochemical tests.

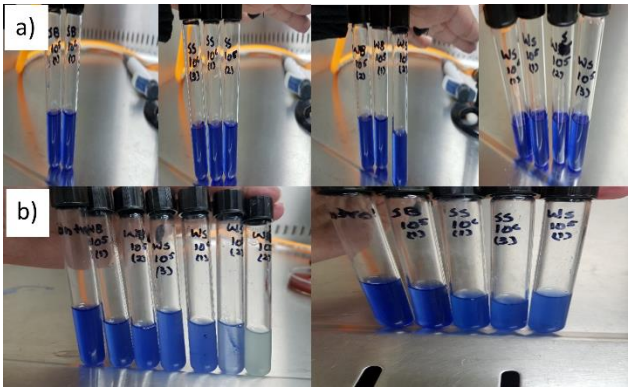

Figure 3. a): The reactive blue dye containing media inoculated with purified strains **b):** Decolorization of dye containing media after three days incubation. The first tube from the left is control (without inoculation).

Strain RB4-1 proved to be in negative in lactose fermentation, urease production and in gram staining. The strain RB6-1 was positive in lactose fermentation, citrate and catalase biochemical tests.

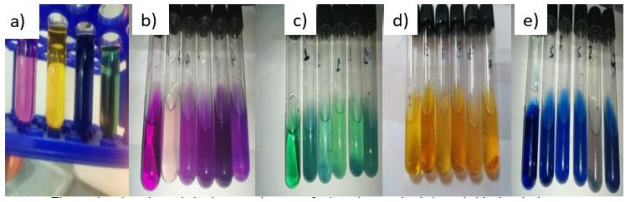

Figure 4. The evaluation of effective strains selected

The third strain named RD4-1 was negative in urease production and in gram staining. The fourth strain RB5-2 proves to be positive in urease, citrate and catalase production. The RB5-1 strain was positive in all biochemical tests except in gram staining where it showed up negative. The tests performed to identify the phenotype of the strains are summarized below in Table 2.

Identification of bacterial strains using ribotyping

After ribotyping, the strains RB4-1, RB6-1, RD4-1, BB5-2 and BB5-1 showed maximum similarity in alignment with the species which are mentioned below in Table 3. (Figure 5).

Table 2. Biochemical tests performed to identify the phenotype of efficient strains.

TESTS PERFORMED	RB4-1	RB6-1	RD4-1	BB5-2	BB5-1
Lactose Fermentation	Negative	Positive	Positive	Negative	Positive
Urease	Negative	Negative	Negative	Positive	Positive
Citrate	Positive	Positive	Positive	Positive	Positive
Catalase	Positive	Positive	Positive	Positive	Positive
Gram Staining	Negative	Negative	Negative	Negative	Negative
Morphology	Rods	Rods	Rods	Rods	Rods
Color	White and opaque	Off-white	Translucent white	off-white	Off-white

DISCUSSION

The textile industry utilizes large quantities of water throughout its manufacturing processes, particularly during dyeing and finishing processes. Wastewater generated from textile production is considered among the most polluting of all industrial effluents, owing both to the substantial volumes produced and to the complex and often highly contaminated composition of the discharged waste streams (Chequer et al., 2013). Synthetic dyes are the most concerning part of textile processing wastewater. During past few decades, synthetic dyes are dominating the textile market, with nearly 8×10^5 tons produced per year due to their wide range of color pigments and consistent coloration (Slama et al., 2021). The dyeing process contains many toxic chemicals, metals and non-soluble substances, such as wastewater, which is thrown into the environment.

Table 3. Identification of decolorizing bacterial strains through ribotyping

Strains	Bacteria with highest identity	Similarity
RB4-1	*Salmonella enterica*	98%
RB6-1	*Enterobacter Cloacae*	98%
RD4-1	*Citrobacter farmer*	99%
BB5-2	*Proteus Mirabilis*	95%
BB5-1	*Klebsiella pneumonia*	99%

The present work shows the ability of bacteria to

reduce synthetic dye contaminated environment. The high level of salt concentration in textile effluents makes it difficult for us to establish effective bio-treatments to eliminate the synthetic dyes. Many solutions have been put forward to deal with this constantly increasing issue including physical, chemicals and microbial methods.

Chemical methods produce by-products which are difficult to deal with. Physical methods are expensive and labor intensive. The use of microbes for the removal of dyes from the contaminated water proved to be a very effective and striking approach.

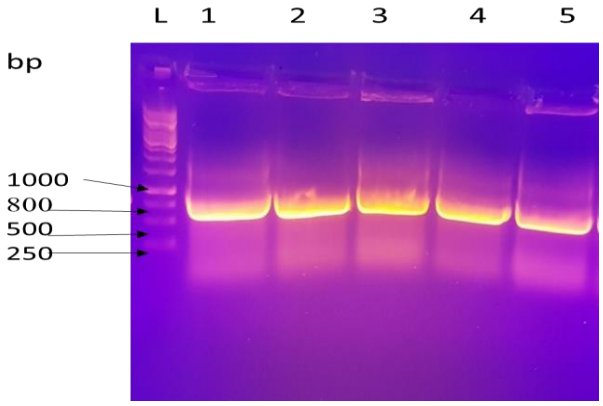

Figure 5. PCR amplification of bacterial strains for 16S rRNA ribotyping, L= DNA Ladder, Lane 1-5= PCR amplified products

This has led to the need to identify bacterial strains that are efficient in degrading the formulated dyes even under very high salt concentration. Halomonas strains were found from chemical contaminated costal sediments. These strains under high salt concentration were capable of degrading five azo dyes in the time period of 24hrs and up to 90% of the dyes were decolorized (Guo et al., 2008). In our project, effluent samples were collected for the isolation of bacteria that may be beneficial in the decolorization of two reactive dyes within 24 hrs.

In this study, the strains isolated and purified were also capable of degrading the Reactive red and Reactive blue dyes up to 90% within the span of 3 days on shaker. The decolorization rate was also analyzed by measuring the optical density values. The identification at molecular level was then carried out to know the exact strains that carried out the decolorization of both the dyes. The full sequenced genomes of decolorizing bacteria have also been obtained using genome sequencing. The exact mechanism of reduction of synthetic dyes has not been elucidated yet. The degradation depends entirely on the specific enzymes of microbes (Shrivastava et al., 2005), the location and conditions under which the degradation take place (Khanna and Srivastava, 2005), the performance of intracellular and extracellular enzyme (Tang et al., 2022). In our experiment, the inoculation of bacteria in dye containing medium showed the gradual reduction of dyes after 24hrs. In a report by (Balamurugan et al., 2011), Halomonas strains isolated from textile waste water had the potential of degrading seven azo dyes. They were able to degrade Remazol Black B, Sulfonyl Blue TLE, Remazol Black N, Maxilon Blue, Entrazol Blue IBC and Sulfonyl Scarlet BNLE (Asad et al., 2007). The strains identified in this study were able to decolorize Reactive red, Reactive blue, Direct Brown, Direct Violet, Direct Turquoise and Reactive green.

Conclusion

This research demonstrated an eco-friendly strategy to repulse water pollution, or the water contaminated with industrial waste containing the synthetic dyes. Different bacterial strains isolated from different dye polluted site were evaluated for Reactive Red and Reactive Blue dye decolorizing under shaking conditions. Five bacterial strains with more than 80% dye reduction were selected. The ribotyping identified these strains as *Klebsiella pneumonia*, *Enterobacter cloacae*, *Citrobacter farmeri*, *Salmonella enterica and Proteus mirabilis*. Hence, the strains discovered are effective in decolorizing the textile dye and with more characterization and implementation of different assays might be applied as a prime method for treating and purifying the water sources contaminated with waste commodities from textile industries.

Conflict of Interest

The authors declare that they have no conflict of interest.

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