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EXTRACTION AND CHARACTERIZATION OF BIOFILM PRODUCED BY VS3D STRAIN AND ITS DYE ADSORPTION EFFICIENCY

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ABSTRACT

In the present investigation, four soil samples were collected from various locations of erode district. The soil samples were subjected to serial dilution technique which resulted in the isolation morphologically distinguishable five different bacterial strains designated as BS3B, BS1F, BS1F, VS3D, and VS3A. The five bacterial strains were screened for its biofilm production activity using tube ring assay and confirmed as best biofilm producers. The isolates were subjected to biofilm production using LB broth medium supplemented with 0.3 % chitin flakes. The formation of biofilm was confirmed by SEM imaging which showed the presence of biofilm cells on chitin flakes. Bio adsorption of dyes by the biofilm produced by the above isolated were performed by the absorption spectrum of visible spectrophotometer read at 528 nm. Based on the spectral data VS3D strain was found with potential dye adsorbing activity. The potential strain VS3D was tested for its dye adsorbing activity using different concentration of extracts (50, 100, 200, and 300 µg/ml).The results showed dye adsorbent potential of biofilm produced by the bacterial isolated VS3D. The biofilm produced by the strain adsorbed 50µg/ml concentration of dye when observed after 120 hrs of incubation time.

KEYWORDS: Bacterial strains, biofilm, tube assay, textile dye, SEM, decolorization.

1. INTRODUCTION

Dye effluents from the dye consuming and manufacturing industries such as paper, food, textile, etc. are one of the most problematic water pollution cases. Textile dye effluents contain reactive dyes in a concentration range of 5–1500 mg L^{-1}. Discharge of these effluents in the environment leads to some serious health problems as some of these dyes are carcinogenic in nature and affect the living organism's life. Dyes on surface water also acts as a barrier to the penetration of sun light and aeration of water body, and thus also leads to the reduction of photosynthesis activity. The toxic nature of the dye effluents also causes death of soil microorganisms, which are used for irrigation purposes and thus, also affects the productivity of agriculture.[1]

Reactive textile dyes are highly water-soluble anionic dyes. They differ from the other classes of dyes since they covalently bind to textile fibers and cannot be easily removed by conventional treatment processes due to their stability under light, heat, oxidizing agents and biological degradation.[2] Therefore, the treatment of dye-contaminated effluents is currently a primary environmental concern.[3]

Therefore, the removal of the dyes from wastewater is required. Adsorption techniques are widely used to remove certain classes of pollutants from waters, especially those that are not easily biodegradable.[4] Biosorption technology utilizing different types of biomasses is environmentally important since they can be used to remove toxic compounds from contaminated effluents. The dye biosorption potentials of many different biological materials have previously been reported upon, including *Thuja orientalis*[5], *Aspergillus wentii*, *Aspergillus niger*[6], *Trametes versicolor*[7], and *Phaseolus vulgaris* L.[8] However, there are very few reports available about effectiveness of bacterial biofilim on dye adsorption.

2. MATERIALS METHODS

2.1 Soil sample collection: Soil samples, 4 in number were collected in sterile containers from Erode district, Tamil Nadu, India. Basic physical parameters like pH, temperature were recorded and stored. Samples were collected by scraping of the soil surface with sterile spatula and about 10g of soil were obtained at a depth of 2-5cm.The samples were collected at a distance of about at least 1km each. All samples were kept aseptically in sterile plastic bags and stored at 4ºC. Bacteria present in the soil were isolated by serial dilution and spread plate technique.

2.2 Isolation of bacterial strains: One gram of soil sample was suspended in 10 ml of sterile distilled water (10^{-1}) in a test tube. The test tube was further subjected to serial dilution process and different dilutions were prepared from this. One hundred micro liter aliquots of dilutions 10^{-1} to 10^{-6} were taken in six different petriplates over which melted LB agar medium (PH 7.0) was poured. The plates were incubated at 30°C for 2-3 days. Finally the bacterial colonies were isolated based on their colony morphology.

A total of 5 bacterial colonies with visually distinguishable morphologies were randomly selected and isolated by directly streaking on LB plates and incubated for another 12-18 hours. The isolated colonies were then re-streaked on LB agar plates to obtain pure cultures. The viability of the isolated cultures was checked in LB broth and those found to be viable were screened for biofilm formation. Then the culture was stored at 4ºC for further work.

2.3 Tube assay

Primary qualitative biofilm screening was done using tube staining assay. The isolated 5 bacterial *viz*, BS3B, BS1F, OS1B, VS3A and VS3D bacterial isolates were subjected for the biofilm producing ability by test tube assay. The overnight cultures (100µl) were inoculated in 10 ml LB broth and incubated for 72 hours at 37°C. The tubes were decanted and washed with Phosphate Buffer Saline (PBS) (pH 7.3) and dried. The tubes were stained with 0.1% crystal violet. Excess stain was removed by washing the tubes with deionized water and biofilm formation in tubes were then observed and documented.

2.4 Biofilm support material: Shrimp shell chitin was purchased from Hi-media (India). The purchased chitin was boiled for 1-2 hr then washed with distilled water for removing the attached dust particles on the surface of chitin. The washed chitins were heat dried in an oven at 100° C for about 48 h and then they were made into small flakes of about 4-6 mm size and these chips were used as biofilm support materials. Then these chips were thoroughly washed with distilled water and sterilized at 15 psi for 30 min for further process (Figure 1).

2.5 Biofilm formation: The isolates BS3B, BS1F, OS1B, VS3A and VS3D were subjected for its biofilm producing ability in chitin flakes medium. The prepared chitin chips were used as a biofilm supportive material. About 0.3 % of chitin chips were taken in LB medium and the pre-grown exponential cells were fed into each conical flask containing chitin chips.

2.6 SEM analysis: Visual identification of the biofilm formation on supportive material (chitin flakes) were confirmed by Scanning Electron microscope. The selected bacterial strains were inoculated in 30 ml of LB medium with 0.3 % of sterile chitin flakes. The inoculated conical flasks were incubated at room temperature for 72hr. Then the biofilm chitin flakes were washed twice with 50 mM phosphate buffer (pH 7.0) for 20 min. The washed biofilms were subsequently dehydrated in a gradient of ethanol solutions (80% ethanol) for 10 min each, and stored in 10% ethanol. These bone chips were dried, coated with gold particles and used for SEM analysis. The control chitin chips lacking cells were also prepared as described above. The gold coated samples were examined for SEM (FEI-SIRION scanning electron microscope) images at 20 kV.[9]

2.7 Dye stock preparation

The chemicals and reagents used in this study were of analytical grade (Himedia, India). The stock solution of dye (Reactive Red) was prepared by dissolving 1000mg of dye in 1 litre of sterile distilled water, which was further diluted for individual working concentrations.

2.8 Screening of potential dye adsorbing strains

The dye adsorbing potential of studies, isolated bacterial strains BS3B, BS1F, OS1B, VS3A and VS3D were performed by inoculating the respective bacterial strains LB broth supplemented with 30µg/ml of dye and 0.3 % of chitin. All the inoculated culture flasks were incubated at room temperature for 120hrs. The culture was collected from the culture flaks at regular time intervals (12 hrs) intervals and the culture free supernatant was collected by centrifuge at 8000 rpm for 10 min. The adsorption was estimated by measuring the dye concentrations in the culture filtrate at regular time intervals for every 12hrs. The dye adsorbent ability of biofilm was read at 528 nm using a UV-VIS spectrophotometer (Systronics, Model-2203). The dye uptaking ability of the biofilm was calculated by following formula.

$$\text{Decolourization (\%)} = \frac{(\text{Initial Absorbance} - \text{final Absorbance})}{\text{Initial Absorbance}} \times 100$$

2.9 Effect of different dye concentrations on dye adsorption: The effects of different dye concentrations on dye adsorption were studied with different concentrations of dyes such as 50, 100, 200 and 300 µg/ml of dye. Selected bacterial strain VS3D was inculcated in 30ml LB medium supplemented with 0.3 % of chitin with different concentrations of dye. All the inoculated flasks were incubated at room temperature. The culture was collected from the culture flaks at regular time intervals each 20 hrs the culture free supernatant was collected by centrifuge at 8000 rpm for 10 min. The adsorption was estimated by measuring the dye concentrations in the culture filtrate at regular time intervals for every 20hrs. The dye adsorbent ability of biofilm was read at 528 nm using a UV-VIS spectrophotometer.

2.10 FTIR analysis: To obtain the IR spectra of FC and EPS by IR spectroscopy (Shimadzu 8201PC, Japan) the autoclaved FC and EPS were dried overnight at 608°C.

Approximately 0.01 g of the dried biomass/EPS was mixed with 0.1 g of KBr and pressed into a tablet form by pressing the ground Mixed material with the aid of a bench press. The resulting pellet was transparent and was used to test the surface functional groups by IR spectroscopy (Shimadzu 8201PC, Japan) where it was scanned between 4000 and 400 cm1 at a resolution of 4 cm1.

2.11 XRD analysis: The powder X-ray diffraction spectrum (XRD) was analyzed with XPERT-PRO system. To study the interaction between control biofilm and dye treated biofilms samples were used for X-ray diffraction studies. For XRD analysis both the biofilm samples were dried with hot air oven at 80°C for 24h and the dried biofilm samples were powdered with the help of mortar and pistil. The diffraction spectra were recorded over the range of 10° to 60° (2θ) with a step length of 0.02° (2θ).

3. RESULTS

3.1 Soil sample collection and isolation of bacterial strains: In our study, 4 soil samples were collected from different sites of Erode District such as (Sample I - 11°38'57.5"N 78°09'35.9"E, Sample II - 11°38'53.9"N 78°09'41.8"E, Sample III - 11°38'52.4"N 78°09'39.2"E and Sample IV - 11°38'52.8"N 78°09'41.0"E), Tamil Nadu, India (Table 1). Five bacterial isolates BS3B, BS1F, OS1B, VS3D, and VS3A were screened randomly by visually distinguishable morphologies (Figure 2a and 2b). Isolated bacterial strains were subjected to various studies. The Bacterial isolate VS3D showed best biofilm formation qualitatively during Test tube assay.

Table 1: Geo-location of soil sample collected sites and screening of potential biofilm producing bacterial isolates.

S. No	Sampling Location	Geo-Location	Soil characteristics pH & temp.	Primary screening (tube assay)	Bacterial strains
1	Erode Town	11°38'57.5"N 78°09'35.9"E	7.2 and 32	++	2(BS1F, BS3B)
2	Erode Town	11°38'53.9"N 78°09'41.8"E	7.2 and 32	++	1 (0S1B)
3	Erode Town	11°38'52.4"N 78°09'39.2"E	7.1 & 33	++	1 (VS3A)
4	Erode Town	11°38'52.8"N 78°09'41.0"E	7.4 and 33	+++	1(VS3D)

3.2 Morphological and biochemical characterization

The morphological and biochemical characteristics of the selected strains were studied (Table 2). The isolated bacterial strains produced circular, light yellow mucoid colonies on LB medium (Figure 2b). The biochemical results showed that all the isolates was motile, non-pigmented and gram negative rod. They showed negative result in Indole production, MR and oxidase but gave positive results for VP, catalase, H_2S production and citrate utilization tests. The results of morphological characteristics and biochemical tests revealed that the selected bacterial strains belong to Enterobacteriaceae family.

Table 2: Morphological and biochemical characterization of the selected isolates

S. No	Characteristic	BS3B	BS1F	OS1B	VS3A	VS3D
1	**Morphological characterization**					
	Gram reaction	-	-	-	-	-
	Cell shape	Rod	Rod	Rod	Rod	Rod
	Colony Morphology	Round and white	Round and white	Raised and grey	Flat and irregular	Flat and irregular
	Pigmentation	-	-	-	-	-
	Motility	+	+	+	+	+
2	**Biochemical characterization**					
	Catalase	+	+	+	+	+
	Indole	-	-	-	-	-
	MR	-	-	-	-	-
	VP	+	+	+	+	+
	Citrate	+	+	+	+	+
	H_2S	+	+	+	+	+
	Oxidase	-	-	-	-	-

Note: '+' positive result, '-' negative result

3.3 Screening of biofilm producing bacterial strains

The test tube assay was performed to identify the potential biofilim producing bacterial strains by qualitative manner. The biofilm production assay of the tested bacterial isolates showed positive results for biofilm production. The bacterial strains produced blue color ring on side wall of test tubes. The isolate strain VS3D showed visually highest production capacity as compared to other tested bacterial strains (Table 1 and Figure 3). Further, biofim producing ability of the bacterial strains was assessed by quantitatively. The strain VS3D showed higher amount of biofilim production (88 mg/50ml) followed by this, the strain BS3B (78 mg/50ml), BS1F (58 mg/50ml), OS1B (49 mg/50ml) and VS3A (37 mg/50ml) produced considerable amount of biofilm (Figure 4).

3.4 SEM analysis

Biofilm production was further confirmed by Scanning electron microscopic imaging (SEM). Scanning electron microscopy images revealed the formation of bacterial biofilm on the surface of the chitin flakes. It is observed that the bacterial biofilm cells are well distributed on the surface of chitin flakes. They are attached to the chitin flakes surface and developed into a biofilm within 72 h. (Figure 5).

3.5 Screening of biofilm producing strains with dye adsorption ability

Biofilm from the selected five bacterial strains were subjected for dye adsorption analysis. The biofilm extracted from VS3D showed more dye adsorption ability when compared with other biofilm producing strains. It showed 100% adsorption of dye after 3 day incubations at 30µg/ml concentrations of dye. The biofilm from BS3B, OS1B, VS3A and BS1F strains showed significant dye adsorption respectively (Figure 6). Back on the preliminary screening, strain VS3D was selected for further adsorption studies.

3.6 Effect of different concentrations of dye on adsorption: We have investigated the effect of different dye concentrations on dye adsorption ability of the extracted biofilm. The bioadsorption was monitored at different concentrations of dye ranging from 50, 100, 200 and 300 µg/ml in aerobic conditions at pH 7.0±0.2 with room temperature (Figure 7). Figure 7 showed the dye adsorbing ability of the biofilm extracted from the strain VS3D. The complete dye adsorption was observed after 120 h incubation at 50 µg/ml concentration of dye. While increasing the concentration of dye in adsorption medium, the dye reducing ability of biofilm was decreased significantly.

3.7 FT-IR analysis

The functional groups present on the surface of the biofilm are responsible for the adsorption of textile dye and other heavy metals due to the nonspecific interaction of synthetic dye compounds with them. To study the possible interactions between biofilm and textile dye, the FT-IR spectra from was recorded at a wavelength of 400 to 4000 cm^{-1}. The surface functional group changes of the biofilm were illustrated in Figure 9. While comparing control biofilm and dye treated biofilm, dye treated biofilm showed several distinct, medium and weak bands at different wavelength. Whereas, control and dye treated biofilm displayed some similar kind of banding patterns such as 3415 cm^{-1} (alcohols, phenols group), 2925 cm^{-1} (alkanes groups), 1319 cm^{-1} (nitro compounds) and 1403 cm^{-1} (aromatics groups).

On the other hand, few strange variations were also observed in dye treated biofilm while compared with control biofilm. The FTIR spectra of the dye treated biofilm showed a highly significant shift in frequency from 1116 cm^{-1} to 1095 cm^{-1}. Interestingly, few new peaks were observed in dye treated biofilm sample such as 654 cm^{-1} (alkyl halides) and 541 cm^{-1} (alkyl halides). However, few peaks were disappeared in dye treated biofilm samples as compared to control (1890 cm^{-1}, 1815 cm^{-1} and 926 cm^{-1}).

To confirm the dye and biofilm interaction, control and dye treated biofilm samples were subjected to XRD analysis. From the diffractograms patterns, control and dye treated bacterial biofilm show clear and perfect crystal peaks and we did not observed any clear peak for dye adsorption (Figure 10).

Figure 1: Prepared chitin flakes for biofilm formation

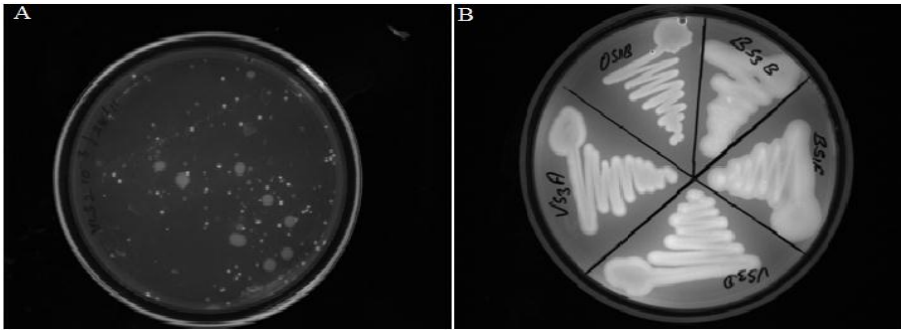

Figure 2: Isolation of bacterial strains from soil samples. 1) spread plat techniques and 2) pure culture on LB medium.

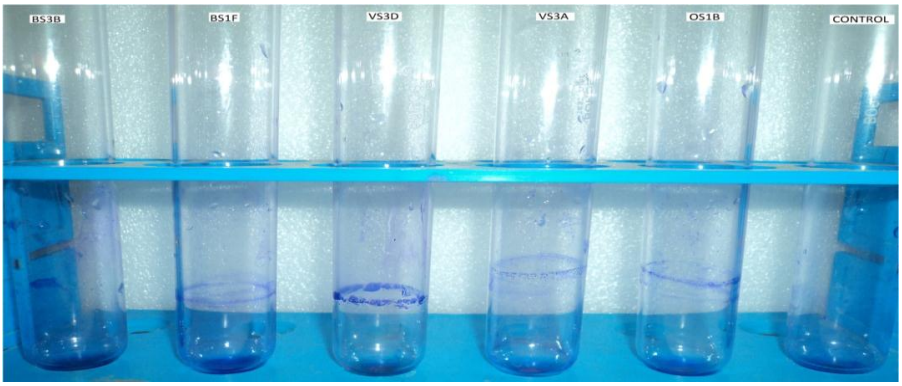

Figure 3: Qualitative and visual identification of biofilm producing bacterial strains.

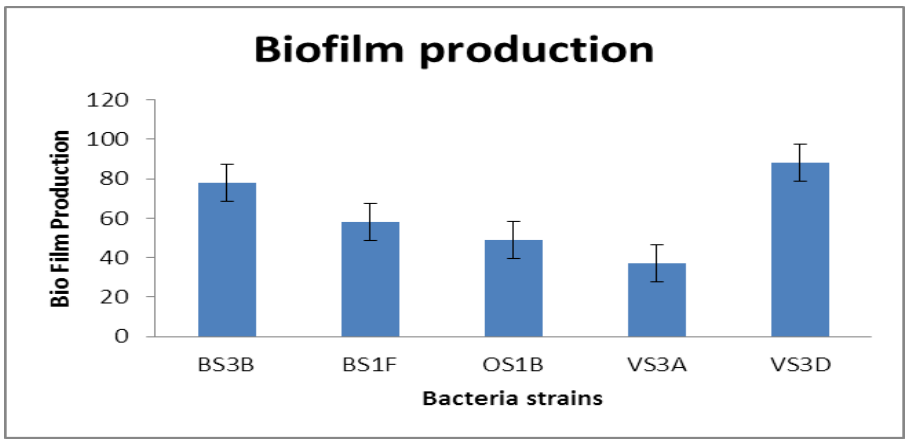

Figure 4: Quantitative estimation of biofilm from the selected five bacterial strains.

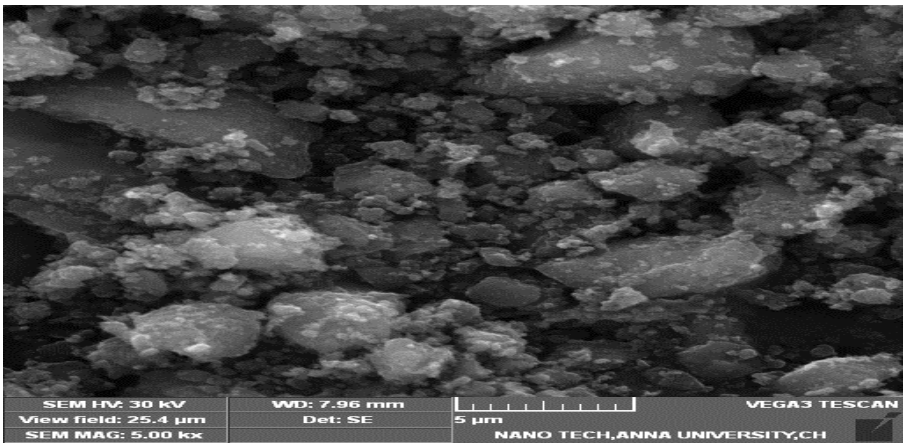

Figure 5: Scanning electron microscopy observation of biofilm formation on chitin flakes (arrowhead) after 72 h incubation

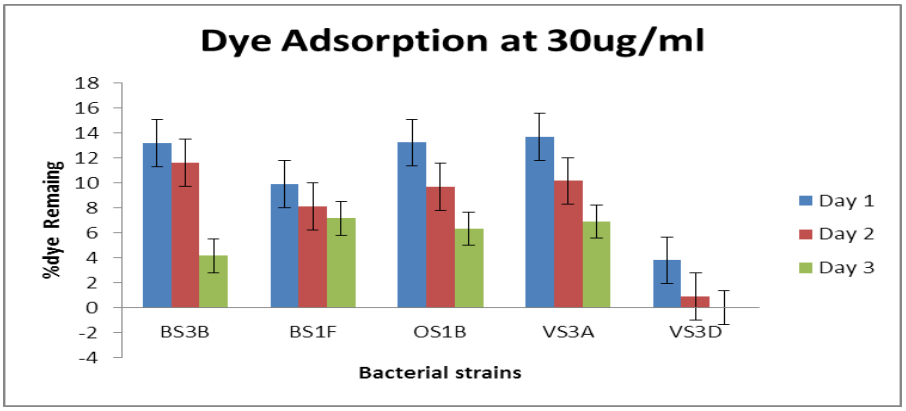

Figure 6: Screening of potential dye adsorbing biofilim producing bacterial strains

Figure 7: Visual observation of different concentrations dye adsorbing ability of biofilm extracted from VS3D strain

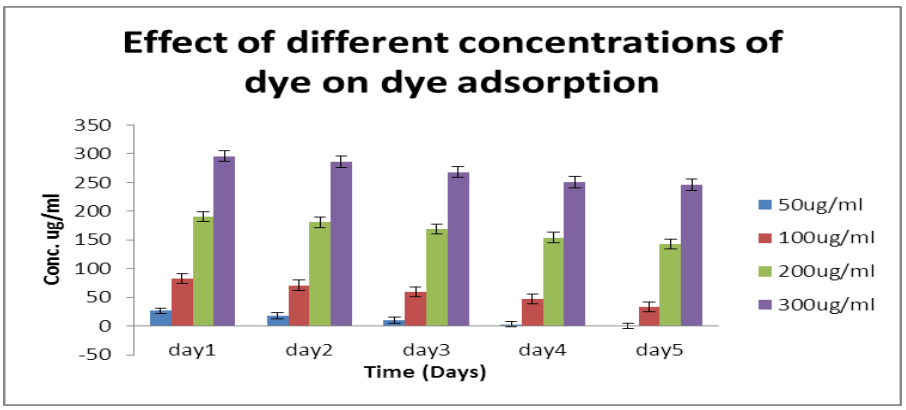

Figure 8: Effect of different concentrations of dye on dye adsorbing ability of the biofilm

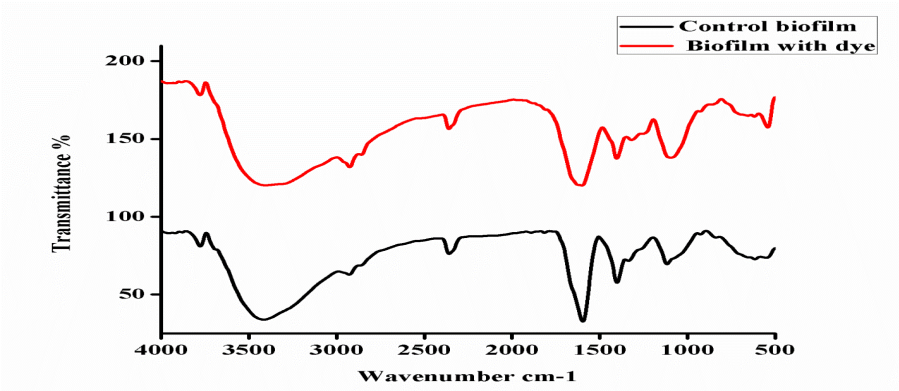

Figure 9: FT-IR spectra of the control biofilm and dye treated biofilm samples.

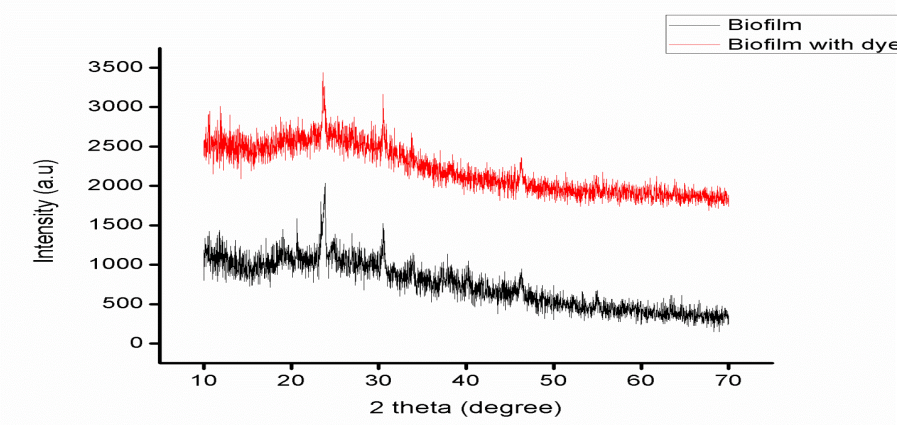

Figure 10: X-ray diffraction of dried powdered control biofilm and dye treated biofilm.

4. DISCUSSION

The textile wastewater is known to have strong color, large amount of suspended solids (SS), broadly fluctuating pH, high temperature, and high chemical oxygen demand (COD) concentration. Biological methods are generally cheap and simple to apply and have been recently applied to remove organics and color of textile wastewater. But the refractory pollutants caused by textile industries cannot easily be degraded by traditional biological process and remain in the effluent. Physico-chemical methods used for the removal of colour from effluents are effective, but they show several disadvantages in terms of operational problems, high cost, and sludge production. Moreover, these methods consume more energy and chemicals than biological processes. A number of studies have focused on microbial decolourization of azo dyes.[10, 11] Biological systems used for the treatment of wastewater containing dyes are more favorable due to their cost effectiveness, lower sludge production, and environmental friendliness.[12] The current focus is now shifting toward the development of environment friendly biological treatment systems.

In this study, five potential bacterial strains were isolated from different soil samples collected from Erode district of Tamil nadu. Previously, similar kind of biofilm producing bacterial strains was isolated from the municipal dump yard of Tenali, Andhra Pradesh.[13] On the other hand, few others researchers isolated biofilm producing bacterial strains from dye contaminated soils[14] and mounth swabbing.[15]

Biofilm producing ability of the bacterial strains was confirmed by test tube assay. Test tube assay is a simple and efficient protocol for primary identification of biofilm producing bacterial strains.[16] The use of crystal violet allowed us for direct visualization of biofilm formation in the test tube walls. This result clearly showed that the crystal violet bounded ring formation around the air-liquid interface of the tubes in all positive strains, somewhat with different thickness. The strain VS3D produced higher amount of biofilm production.

According to our results extracted biofilm from bacterial strain VS3D showed potential dye adsorbing ability as compared to other four tested bacterial strains. This result concludes that all the bacterial biofilm does not having the dye adsorbing ability. Biofilm-mediated bioremediation presents a proficient and safer alternative to bioremediation with planktonic microorganisms because cells in a biofilm have a better chance of adaptation and survival (especially during periods of stress) as they are protected within the matrix.[15] Owing to the close, mutually beneficial physical and physiological interactions among organisms in biofilms, the usage of xenobiotics is accelerated and, consequently, bio- films are used in industrial plants to help in immobilization and degradation of pollutants. The use of biofilms for water and wastewater treatments in the early 1980s. However, it is only during the past few decades that biofilm reactors have become a focus of interest for researchers in the field of bioremediation. However, dye adsorbing ability of the bacterial biofilm was reduced significantly with increasing concentrations of dye. Similar kind of adsorption was reported by.[15]

While analyzing the control and dye treated biofilm samples by FTIR spectrum, few new peaks were appeared in dye treated biofilm samples (alkyl halides functional group). However, few peaks were disappeared in dye treated biofilm samples. These results were clearly indicated the possible interaction between dye and biofilm. Similar to our findings,[17] observed few new peaks at Red MX 5B dye treated exopolysaccharide extracted from *Bacillus subtilis*. On the other hand, we did not observe any clear variations in control and dye treated biofilm samples in XRD analysis.

The results from this work indicate that the biosorption by bacterial biofilm in laboratory process could be a potential technology to remove dyes from textile effluent. However, further studies are needed to fully optimize this technology for environmental applications. Important questions currently under investigation include the establishment of the exact mechanisms of biosorption by biofilm, understanding the dye transformations, and

the development of ways to enhance efficiency of the bacterial biofilm.

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