



## Decolourization and Degradation of Azo Dyes Blood Red and Turquoise Blue by Selected Soil Bacteria

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### Authors' contributions

This work was carried out in collaboration between all authors. Author DS designed the experiment, and wrote the first draft of the manuscript. Author RL managed the literature searches, analyses of the study performed the spectroscopy analysis and author TL managed the experimental process, performed HPLC. All authors read and approved the final manuscript.

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### ABSTRACT

Two soil bacterial isolates (*Bacillus* sp. and *Staphylococcus* sp.) were isolated from agricultural soil and used in the decolourization and degradation of two synthetic textile azo dyes blood red and turquoise blue. Of the two bacterial species employed, the isolate b (*Staphylococcus* sp.) caused better decolourization of blood red (72.40%) and turquoise blue (66.63%) than isolate a (*Bacillus* sp.). The bacterial consortium gave better decolourization (67.77%) for turquoise blue than with individual culture. Difference in the number of HPLC peaks between the intact dyes and the bacteria treated dyes confirms the bacterial biodegradation of azo dyes blood red and turquoise blue.

**Keywords:** Azo dyes; decolourization; biodegradation; blood red and turquoise blue.

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## 1. INTRODUCTION

Among the most concern causing environmental pollutions that threaten our biodiversity, water pollution is a major one where effluents from dye-based industries serve as principal source. With ever increasing demand for textile products, the textile industry and its wastewaters have been increasing proportionally making it one of the main sources of pollution worldwide [1]. The wastewaters discharged from textile industries are the most polluting wastes both in terms of volume and composition. After the dyeing process, the excess dyes remaining in the effluent must be treated before release into the environment. Conventional treatment systems for colour removal are not effective due to the complex structure and synthetic origin of dyes and other chemicals in the textile effluent.

Microbes that are versatile and adaptive to the changing environment are the self sustaining and cost-effective components to overcome the problem of textile effluent decolouration and degradation. A wide variety of microorganisms are reported to be capable of decolouration and degradation of dyes and industrial effluents [2]. Microbial bioremediation is the process in which micro organisms are stimulated to rapidly degrade hazardous organic contaminants to environmentally safe level in soils, subsurface materials, water, sludge and residues [3]. Microbial diversity offers an immense choice of environment friendly options for mineralization of contaminants or their transformation into less harmful non-hazardous compounds.

The present study has been designed to evaluate the decolourization and degradation potential of selected bacteria isolated from soil habitat, individually and in combination on the commonly used textile azo dyes Blood red and Turquoise blue.

## 2. MATERIALS AND METHODS

### 2.1 Procurement of Dyes and Preparation of Stock Solution

The textile azo dyes Blood red and Turquoise blue were procured from a dye store at Kottar, Nagercoil, Tamilnadu. Stock solutions of the dyes were prepared (2 g/L) and steam sterilized.

### 2.2 Soil Sample

Vertical Soil Sample was collected from a paddy field. Using sterile spatula, 6 inch top soil was

removed, placed in sterile polythene bags and stored at 4°C until further analysis.

### 2.3 Isolation and Screening of Bacteria

The soil sample was subjected to serial dilution following the previously described method [4]. Using spread plate method,  $10^{-5}$  and  $10^{-6}$  dilutions were plated on nutrient agar plates  $\text{g L}^{-1}$  (beef extract 3; peptone 5; sodium chloride 5; agar 15, distilled water 1000 ml and 1 g  $\text{L}^{-1}$  of the 2 dyes at pH 7.4) and incubated at 30°C for 3 days.

### 2.4 Bacterial Sub-culture

The single isolated colonies were subcultured on agar plates with minimal medium composed of  $\text{g l}^{-1}$  (D-glucose 1.4; ammonium sulphate 0.8; potassium phosphate dibasic 1.27; sodium chloride 4.0; magnesium sulphate 0.42; potassium chloride 0.29; EDTA 0.5; yeast extract 0.6; calcium chloride 0.02; ammonium nitrate 1; sodium carbonate 0.1; HCl 1 mol  $\text{L}^{-1}$  and distilled water 1000 ml at pH 7.5 and incubated at 30°C for 3 days. Biochemical characterization was done and the strains were identified based on Bergey's Manual of Determinate Bacteriology [5].

### 2.5 Decolourization Assay

Two bacterial isolates (isolate a and b were selected for the decolourization experiments. Individual strains of bacteria were inoculated into pre-sterilized 250 ml conical flasks containing 100 ml nutrient broth and 2 ml of the respective dye stock solution. Flasks were labeled as A,B,C and D. Flask A was the control with nutrient broth and dye solution but without bacterial inoculum. Flask B was inoculated with 100 ml of isolate a, Flask C with 100 ml of isolate b, flask D with 50 ml of each of the isolates (bacterial consortium). The inoculated flasks were incubated at 37°C for 10 days.

The degradation of the dyes was monitored at 24 hours interval. 2 ml bacterial culture was aseptically withdrawn from each flask and centrifuged at 6000 rpm for 30 minutes. The optical density of supernatants was determined spectrophotometrically to determine the rate of decolourization. Percentage dye decolourization (% DD) was calculated using the formula

$$\% \text{ DD} = \frac{\text{OD}_{\text{zeroday}} - \text{OD}_{\text{sample}}}{\text{OD}_{\text{zeroday}}} \times 100$$

## 2.6 HPLC Analysis

The bacteria treated samples were filtered through a 0.45 mm membrane filter and subjected to High performance Liquid Chromatography (Reverse phase HPLC Cyberlab, USA) analysis in a C<sub>18</sub> Column (250 mm x 4.6 mm) equipped with a C<sub>18</sub> guard column. Solvent used was acetonitrile and water in the ratio 65:35 which was degassed using sonicator for 50 times at 40°C. 20 µl of the filtered and centrifuged sample was injected using injection needle into the injection head. The compounds were eluted with isocratic elution of acetonitrile vs water at the flow rate of 1 ml/minute. The elution was monitored and absorbance recorded at 680 nm for blood red dye and 560 nm for turquoise blue.

## 3. RESULTS AND DISCUSSION

Of the two strains screened, one was *Bacillus* sp. (isolate a) and the other was *Staphylococcus* sp. (isolate b). Both the isolates were Gram positive and showed similar responses for the sugar

fermentation tests. Isolate a was catalase positive whereas isolate b was negative (Table 1). Blood red dye treated with isolate a gave the maximum decolourization (70.04%) on the 10<sup>th</sup> day whereas it was 56.66% for turquoise blue. Isolate b gave 72.40% and 66.63% decolourization for the two dyes respectively on the 10<sup>th</sup> day. The bacterial consortium gave 69.23% for blood red and 67.77% for turquoise blue. In all the treatments, maximum decolourization was reached on the 10<sup>th</sup> day. The rate of decolourization gradually increased from the initial days of incubation to the 10<sup>th</sup> day (Table 2).

It was observed that the *Staphylococcus* sp. performed better than *Bacillus* sp. in decolourizing both the dyes. The results agree with the previous findings [6] wherein *Staphylococcus aureus* gave 37.5% after 90 days of incubation in decolourizing indigo dye containing textile effluent. *B. cereus* performed well because they are nutritionally versatile and carries an efficient enzymatic system for the cleavage of azo bonds which cause rapid decolourization of different azo dyes [7].

**Table 1. Biochemical characterization of agricultural soil microbial isolates**

S. no.	Name of the test	Response of the colony	
		Isolate a	Isolate b
1.	Gram staining	Gram +ve Bacillus	Gram +ve Cocci
2.	Indole test	-ve	-ve
3.	Methyl Red test	-ve	-ve
4.	Voges Proskauer test	-ve	-ve
5.	Citrate utilization test	-ve	+ve
6.	TSI test	No reaction	No reaction
7.	Urease test	-ve	+ve
8.	Nitrate reduction test	+ve	-ve
9.	Catalase	+ve	-ve
10.	Coagulase	+ve	+ve
11.	Sugar fermentation test		
	(a) Arabinose	-ve	-ve
	(b) Adonitol	-ve	-ve
	(c) Dextrose	-ve	-ve
	(d) Fructose	+ve	+ve
	(e) Galactose	+ve	+ve
	(f) Mannitol	+ve	+ve
	(g) Rhaffinose	-ve	-ve
	(h) Sucrose	+ve	+ve
	(i) Trehalose	+ve	+ve
	(j) Rhamnose	-ve	-ve
	(k) Cellobiose	+ve	+ve
	Identified Organism	<i>Bacillus</i> Sp.	<i>Staphylococcus</i> sp.

+ve – Positive; -ve - Negative

**Table 2. Percentage decolourization (%D) of the azo dyes blood red and turquoise blue by the selected bacterial isolates**

Days	Isolate a		Isolate b		Isolate a & b	
	Blood red	Turquoise blue	Blood red	Turquoise blue	Blood red	Turquoise blue
0	-	-	-	-	-	-
1	8.16	2.15	4.19	3.15	1.09	3.33
2	18.36	3.22	16.66	9.47	12.08	6.66
3	22.44	7.52	30.20	16.84	21.97	13.33
4	26.53	12.90	35.41	27.36	34.06	20.01
5	38.77	18.27	42.70	37.89	43.95	26.66
6	44.89	25.80	47.91	43.15	44.05	32.22
7	47.95	37.63	58.83	49.47	53.84	40.04
8	59.18	41.93	68.75	37.89	62.63	53.33
9	69.38	50.53	68.79	61.05	67.03	63.33
10	70.04	56.66	72.40	66.63	69.23	67.77

The control showed no decolourization which confirmed that decolourization was a result of metabolic activities of the inoculated microbe and due to abiotic factors. The Synergistic effect of the microbes was more pronounced in decolourizing turquoise blue achieving 67.77% which was greater than those in individual treatments. Synergistic effect was also noticed in a previous study [8] in decolourization and degradation of textile dyes using a sulphate reducing bacteria (SRB).

In all the three sets of experiments, there was almost no change in the colour pattern after 10<sup>th</sup> day of inoculation. But in previous reports there is greater colour removal even after 90 days of incubation. Decolourization of certain sulfonated azo dyes occurs under aerobic conditions even after 2 months [9]. The decolourization potential of microbes was greater in blood red than turquoise blue. It suggests that the ability of microorganisms to decolourize different dyes vary depending on the structure and complexity of the dye [10,11].

It was assumed that the low decolourization percentage during initial hours of incubation may be due to adsorption. The primary mechanism of dye removal appears to be adsorption of dye on the microbial biomass followed by biodegradation. The acclimation of bacteria to grow in the presence of inhibitory dyes reduced the toxic effect of the dyes and partially restored the physiological activity of the cells. A microbial population could possibly become adapted to withstand and decolourize increasingly concentrated waste water [12,13].

HPLC analysis of the control samples showed 3 peaks of retention time 1.663, 2.118 and 2.210

minutes for blood red dye and 4 peaks with retention time 1.619, 1.903, 2.135, 2.200 minutes for turquoise blue. The number of peaks was reduced to 2 and 1 for the dyes blood red and turquoise blue respectively when treated with *Bacillus* sp. *Staphylococcus* sp. treated blood red dye recorded 4 peaks and turquoise blue recorded 2 peaks. HPLC analysis of the azo dyes treated with the bacterial consortium recorded 2 peaks for blood red and 5 peaks for turquoise blue (Table 3 and Figs. 1-10).

HPLC chromatogram revealed that there were changes in the height and number of peaks achieved in the treated samples than in the control. The observed disappearance of the peaks in the HPLC chromatogram for both the dyes showed that the dyes were actually degraded. The results fall in alignment with previous observations [14], wherein the control dye Rubine GFL showed a single peak at retention time of 2.971 min while there was disappearance of the major peak and formation of two new major peaks at retention time of 3.047 and 3.317 min and three minor peaks at 2.265, 4.123 and 4.663 min retention time. The difference in retention time of the control dyes and metabolites formed after bacterial degradation confirms the biodegradation of the dyes. The study of relation between chromatogram pattern and decolourization pattern showed that there is reverse relationship between the two. HPLC pattern of blue dye showed greater changes from the control than the red dye which corresponded to the microbial decolourization also in which colour removal was more pronounced in the blue dye as observed from the final percentage of decolourization.

Table 3. HPLC analysis of azo dyes treated with bacterial isolates

Name of the dye	Control			Isolate a			Isolate b			Isolate a & b		
	RT (min)	Height (mv)	Area (mv.s)	RT (min)	Height (mv)	Area (mv.s)	RT (min)	Height (mv)	Area (mv.s)	RT (min)	Height (mv)	Area (mv.s)
Blood red	1.663	1030	6782.6	1.642	462	2853.9	1.700	1233	6555.4	1.630	387	2125.5
	2.118	117	741.3	2.237	82	1157.6	2.217	112	1368.2	2.205	61	778.0
	2.210	202	2778.2				2.357	67	492.2			
							3.405	44	356.3	1		
Turquoise blue	1.619	372	3571.7	1.663	947	4358.1	1.664	825	5148.7	1.682	1615	8176.8
	1.903	97	631.4				2.202	63	905.4	1.897	194	851.3
	2.135	105	1080.0							2.063	263	1386.9
	2.200	118	1245.9							2.136	237	2946.3
										3.010	103	713.3

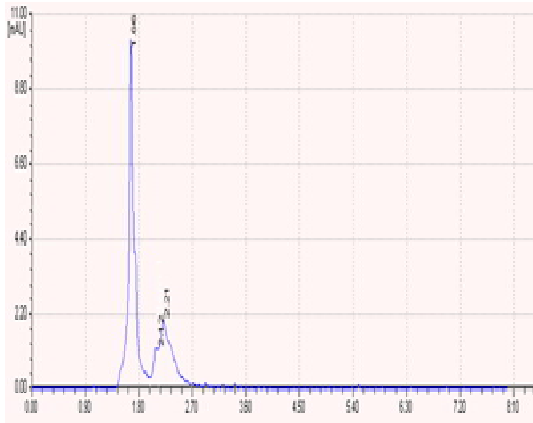


Fig. 1. HPLC chromatogram of azo dye blood red (control)

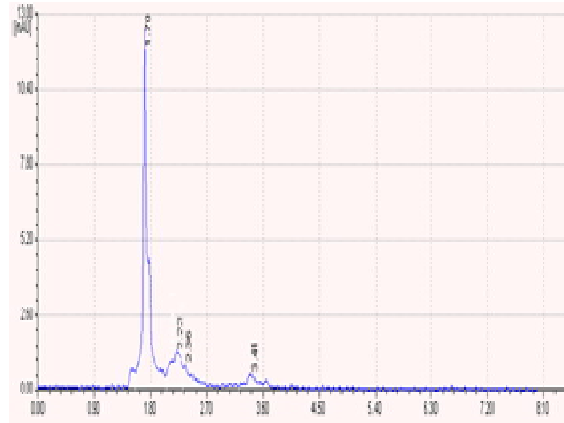


Fig. 2. HPLC chromatogram of azo dye blood red treated with *Staphylococcus* sp.

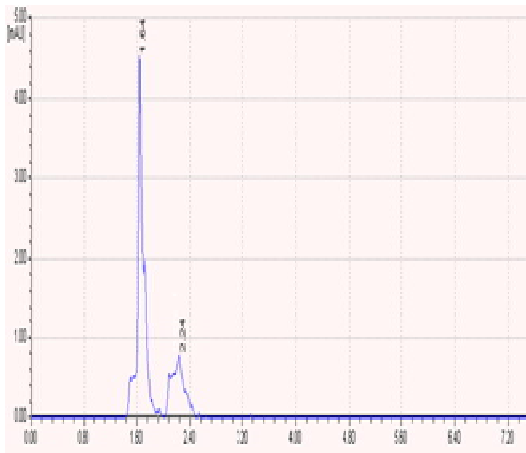


Fig. 3. HPLC chromatogram of azo dye blood red treated with *Bacillus* sp.

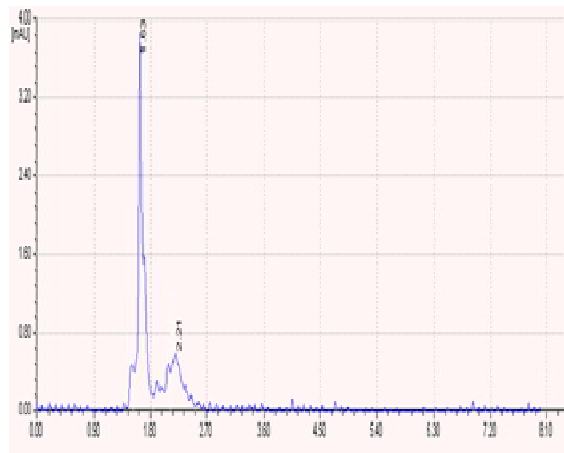


Fig. 4. HPLC chromatogram of azo dye blood red treated with *Staphylococcus* sp. and *Bacillus* sp.

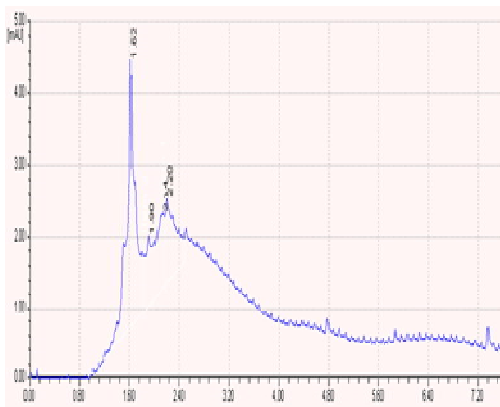


Fig. 5. HPLC chromatogram of azo dye turquoise blue (control)

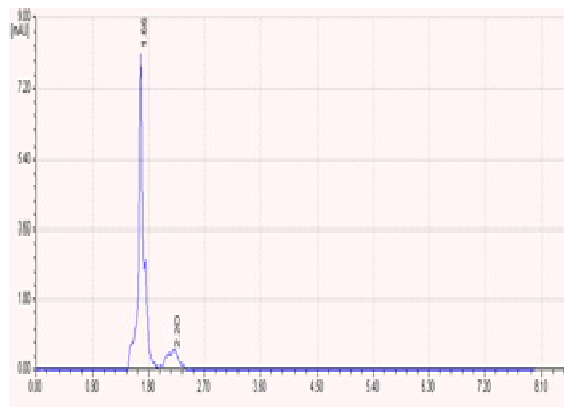


Fig. 6. HPLC chromatogram of azo dye turquoise blue treated with *Staphylococcus* sp.

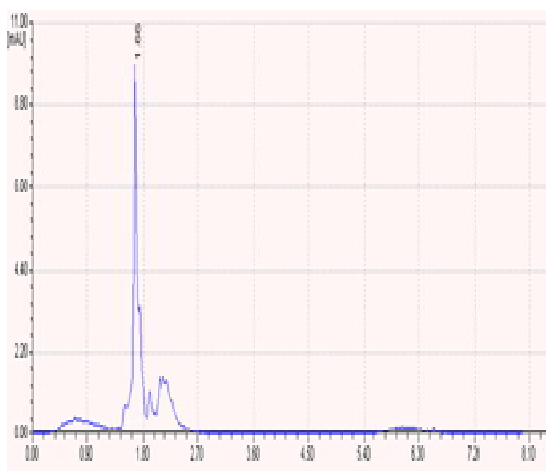


Fig. 7. HPLC chromatogram of azo dye turquoise blue treated with *Bacillus* sp.

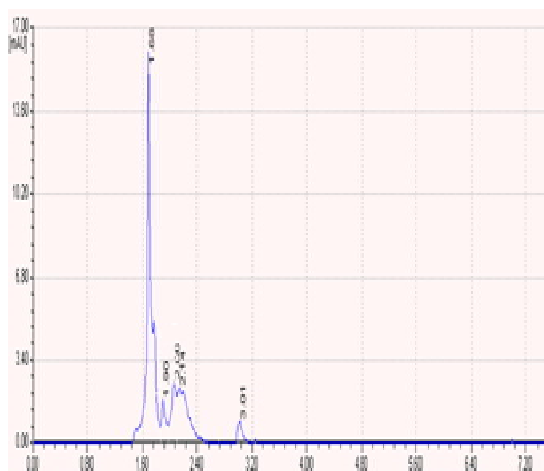


Fig. 8. HPLC chromatogram of azo dye turquoise blue treated with *Staphylococcus* sp. and *Bacillus* sp.

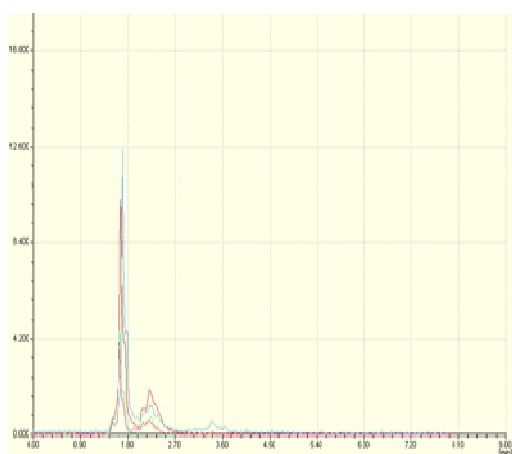


Fig. 9. Combined elution profile of blood red

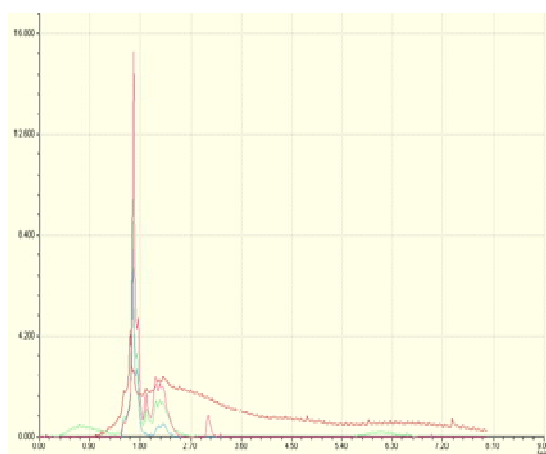


Fig. 10. Combined elution profile of turquoise blue

#### 4. CONCLUSION

The isolates used in the present study, *Bacillus* sp. and *Staphylococcus* sp. were able to persist and flourish in the dye environment utilizing the dye as the sole energy source though other nutrients are limited. Though both the isolates were found to be excellent bio-agents for the bioremediation of dyes, *Staphylococcus* sp. caused better decolouration than *Bacillus* sp. which indicates the possible use of these isolates in biodegradation of textile dyes and actual textile effluents. If the metabolites could be analyzed and the pathway of dye degradation explained the microbial degradation of textile effluent could

be successfully performed on a commercial scale.

#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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