



Biodegradation of Textile Dye by Indigenously Isolated Bacteria *Bacillus* and *Pseudomonas* Sp. and Their Discard After Bio-Treatment

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Indigenous bacterial isolates of *Bacillus* and *Pseudomonas* sp. were used to biodegrade the textile dye (light green) in the laboratory. The 50, 100 and 150 ppm of textile dye light green were completely decolorized after 24 h, while 200 ppm after 30 h of incubation. Both the isolates decolorized 76 to 79 % of dye after 24 h at pH 7 to 7.5 and temperature 37 °C. The crude enzyme extract of these isolates was also employed for decolorization of textile dye. Crude enzyme extract 0.5, 1 and 1.5 mL of *Bacillus* sp. showed 10, 45 and 74 % of decolorization, while 2 and 2.5 mL showed 100 % decolorization. Bacterial elimination was also done by inducing acetic acid stress. The results indicated that both the indigenous bacteria showed colony forming ability (CFA) after 36 h only when the concentration of acetic acid was 20 mM and further higher concentration did not favored colony forming ability. Even the incubation of 60 mM of acetic acid for 24 h was not favorable for colony forming ability which indicates the elimination of bacteria due to acetic acid stress. The relative sensitivity of *Sorghum vulgare* Pers. CV SSG5000 towards the textile dye and its biodegraded products were studied. Thus, phytotoxicity studies revealed that the biodegradation of the textile dye by the *Bacillus* and *Pseudomonas* sp. led to detoxification of the textile dye light green. In conclusion, the use of indigenous bacterial isolates had great potential for the biodegradation of textile dyes.

Keywords: Decolorization, Crude enzyme, Acetic acid, Germination, Phytotoxicity.

INTRODUCTION

Textile wastewater is the major problem in the world as production of colorant reached 8×10^6 kg per year, of which 10-15 % goes into effluents during different textile processes^{1,2}. These colorants are primarily designed to resist fading of colour in the presence of intense light and moisture conditions to make good quality fabric. Discharge of such dyes in wastewater is a great threat to the natural ecosystems because these are toxic as well as carcinogenic. Thus, there is a dire need to develop a cost effective strategy to deal with such dyes which are harmful to life^{3,4}. In this scenario, biological processes to treat such dyes could be environment friendly and cost effective procedures⁵. For example, degradative enzymes can be utilized to degrade textile effluents. These enzymes have low energy requirement, minimum operation cost and eco-friendly nature which make them suitable for such treatments. Laccases are the enzymes which are used for biotreatment of dyestuffs. Information regarding effluent treatment by laccases is available in the literature but potential of other enzymes such as oxidases and reductases is yet to be explored¹.

The textile effluents treated with enzymes can be reused in dyeing processes^{2,6}. A number of bacterial species belonged to genera *Bacillus*, *Exiguobacterium*, *Lysinibacillus*, *Micrococcus*, *Proteus*, *Pseudomonas*, *Sphingomonas* and *Staphylococcus* were reported to be used efficiently in bio-treatment of textile dyes^{2,7-10}. But it is essential to eliminate these indigenous bacteria after the bio-treatment of textile effluents prior to discharge. For this purpose suicidal genes were used which were also risky to the environment. Moreover, environmental stresses like weak acid, oxidative stress, antibiotics and heat can be used to eliminate such indigenous bacteria. Among them the use of weak acid is a good choice because of simplicity of its usage and being economical. Acetic acid is widely used as preservative to prevent microbial growth on food stuff and can be used for programmed cell death¹¹.

Considering the reported information in the present study we isolated indigenous bacteria to bio-decolorize the textile dye (light green), while acetic acid was used in low concentrations for the removal of indigenous bacteria.

EXPERIMENTAL

Wastewater, sludge and affected soil samples were collected in screw capped sterilized bottles for the isolation of bacterial strains from Hudaira drain near Nishat Mills Limited, Ferozpur Road, Lahore, Pakistan. One sample of soil, four samples of sludge and four samples of wastewater were collected from 0, 5, 500 and 1000 meter away from the main outlet¹².

Isolation, screening and identification of the indigenous bacterial isolates: The isolation of indigenous bacteria was carried out through serial dilution method using nutrient agar medium^{13,14}. Isolated bacterial strains were screened out by incubating them on nutrient agar medium containing various concentrations of textile dye light green 50, 100, 150 and 200 ppm. The stock cultures of screened bacterial isolates were maintained on the nutrient agar medium and stored at 4 °C. The screened bacterial strains were identified on morphological, biochemical and physiological properties following the protocol given in Bergey's Manual of Determinative Bacteriology¹⁵.

Bio-decolorization and bio-degradation characterization: Decolorization ability of bacterial isolates was analyzed by using spectrophotometer (SpectroScan 80D UV-visible) at optimum wavelength 340 nm. The experiments for decolorization were performed at pH 7 to 7.5 and temperature 37 °C on nutrient broth (in triplicates). The decolorization activity was expressed in terms of percentage decolorization using the following formula¹⁶:

$$\text{Decolorization (\%)} = \frac{A_o - A_f}{A_o} \times 100$$

where A_o is initial absorbance and A_f is absorbance at incubation time.

HPLC (SHAMZDU-LC20AT) was used to characterize the biodegraded metabolites of light green dye. The analysis was carried out on column C_{18} with methanol:acetonitrile (1:1) as mobile phase at one milliliter per minute flow rate and used UV detector at 254 nm¹⁷.

Enzymatic decolorization of textile dye: The bacterial cells were harvested by centrifugation at 8000 rpm for 10 min and then washed with 10 mM phosphate buffer of pH 7. It was then suspended in 50 mM phosphate buffer of same pH. The cells were sonicated (cold) for 15 min at 70 % amplitude to disrupt the bacterial cells. The cell fragments were removed by centrifugation at 15,000 rpm for 20 min. The supernatant was used as crude enzyme extracts (CEE) and dissolved in 10 mL of 50 mM phosphate buffers. The enzyme assays was carried out in a cuvette (4 mL). One hundred ppm of light green dye was mixed in 100 mL phosphate buffer (50 mM).

The crude enzyme extract (CEE) was taken 0.5, 1.0, 1.5, 2 and 2.5 mL in cuvettes separately and then 1 mL of dye was added in all the cuvettes. Absorbance was measured at 340 nm after 0.5 h using spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme required to decolorize 1 ppm of dye in 0.5 h under the assay conditions (pH 7 and temperature 37 °C). All the assays were conducted in triplicate².

Bacterial elimination by induced environmental stress: Bacterial elimination was done by inducing acetic acid stress. The concentrations of acetic acid (0, 20, 40, 60, 80, 100, 120, 140 and 160 mM) were prepared and added separately in isolated cultures on nutrient broth medium. These cultures were spread on nutrient agar after 12, 24 and 36 h to ascertain the colony forming ability (CFA) by which bacterial elimination was assessed indirectly¹¹.

Phytotoxicity studies: In order to ascertain the toxicity of dye and biodegraded metabolites, phytotoxicity tests were performed on *Sorghum vulgare* Pers. CV SSG5000. The biodegraded metabolites of light green dye were extracted and dissolved in 10 mL distilled water for these tests. The phytotoxicity tests were carried out on seeds of sorghum (20 seeds), a common agricultural crop of Pakistan. The study was carried out at room temperature by watering 5 mL of dye. Control set was carried out using irrigation water at the same time. Germination (%), plumule and radical length (cm) were recorded after 7 days⁹.

RESULTS AND DISCUSSION

Bio-decolorization and bio-degradation of textile dye: Morphologically distinct colonies were observed and found that 21 showed growth (50 ppm light green dye) out of total 76 colonies. All the 21 morphologically distinct colonies were screened at 100, 150 and 200 ppm light green dye. The isolate number 1, 3, 5, 7, 9 and 20 were screened on the basis of their ability to degrade the dye efficiently (more than 60 %) within 24 h. Among these, isolate number 5 and 9 were used for this study and were identified as *Bacillus* and *Pseudomonas* sp., respectively by standard physiological, morphological and biochemical tests following the protocol given in Bergey's Manual of Determinative Bacteriology¹⁵. The bacterial isolates *Bacillus* and *Pseudomonas* sp. showed remarkable performance towards bio-decolorization of textile dye. The 50, 100 and 150 ppm of textile dye were completely decolorized after 24 h while 200 ppm after 30 h by *Bacillus* and *Pseudomonas* sp. Both the isolates decolorized 76 to 79 % of dye after 24 h at pH 7.0 to 7.5 and temperature 37 °C (Table-1).

TABLE-1
BIO-DECOLONIZATION (%) OF TEXTILE DYE (LIGHT GREEN) WITH RESPECT TO TIME AND CONCENTRATION OF THE DYE

Sr. No.	Bacterial isolates	Bio-decolonization (%)				
		Time (h)	50 ppm	100 ppm	150 ppm	200 ppm
1	<i>Bacillus</i> sp.	12	64	62	58	54
		24	cd	cd	cd	79
		30	ee	ee	ee	cd
2	<i>Pseudomonas</i> sp.	12	67	64	59	51
		24	cd	cd	cd	76
		30	ee	ee	ee	cd

cd = complete decolorization, ee = end of experiment

The HPLC analysis of textile dye collected at 0 h of incubation showed peak at 2.622 min. After 24 h of incubation, when maximum biodegradation had occurred, five major peaks were observed at 2.802, 3.113, 3.480, 8.849 and 8.931 min, which showed that a single compound after bio-degradation reduced to five different compounds (Fig. 1a, b). The textile dyes indisputably pass on high load of carcinogenic and mutagenic pollutants to the aquatic ecosystems. Bioremediation being eco-friendly is preferred over the conventional effluent treatment methods because they have high operational cost and produce much more sludge¹⁸. In this study indigenous isolated bacteria *Bacillus* and *Pseudomonas* sp. were used to bio-decolorize textile dye. The results revealed the efficacy of *Bacillus* and *Pseudomonas* sp. for the bio-decolorization and biodegradation of textile dye is quite significant. It also makes these isolates as potential candidates for commercial applications. Various researchers have already conducted bioremediation studies using other textile dyes. They obtained similar results for decolorization of orange T4LL, reactive orange16 and Congo red^{13,17,19}.

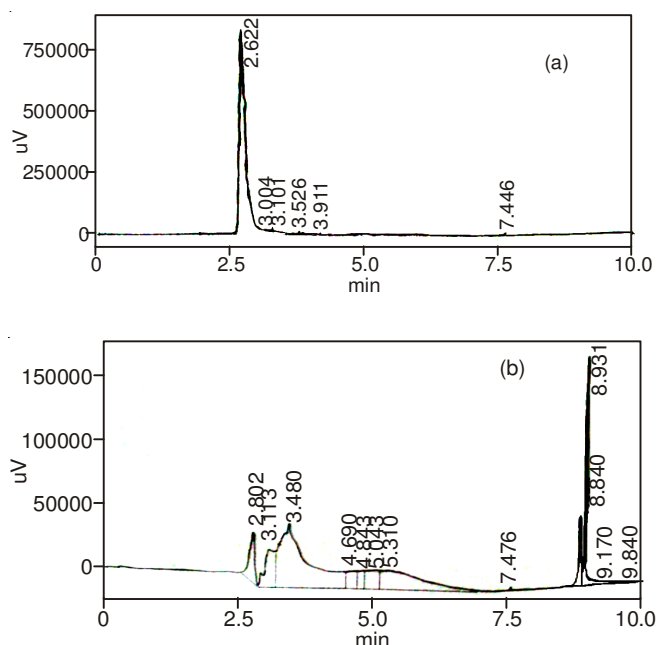


Fig. 1. HPLC chromatogram (a) control textile dye light green at 0 h (b) degraded metabolites of textile dye light green after 24 h

Enzymatic decolorization of textile dye by crude enzyme extract:

The one unit of enzyme activity was defined as the amount of enzyme required to decolorize 1 ppm of dye in 0.5 h under the assay conditions (at pH 7 and under temperature 37 °C). The crude enzyme extract (CEE) 0.5, 1.0, and 1.5 mL of *Bacillus* sp. showed 10, 45 and 74 while 2 mL and 2.5 mL showed 100 % decolorization. The crude enzyme extract (CEE) 0.5, 1.0, and 1.5 mL of *Pseudomonas* sp. showed 15, 47 and 78 % decolorization while 2 mL and 2.5 mL showed 100 % decolorization (Fig. 2). In the decolorization of textile dyes, a number of bacterial enzymes are involved. In fact, bacteria are the source of reductases and oxidases which are used to degrade the dyes. For example, *Escherichia coli* CD-2 was used to purify an oxygen insensitive azoreductase having characteristics of textile dye degradation. Once a textile dye is

reduced by a reductase, it cannot be re-oxidized to form colourant^{20,21}. The results indicated that crude enzyme extract decolorized textile dye light green is quite significant making the crude enzyme extract potentially more effective for commercial applications.

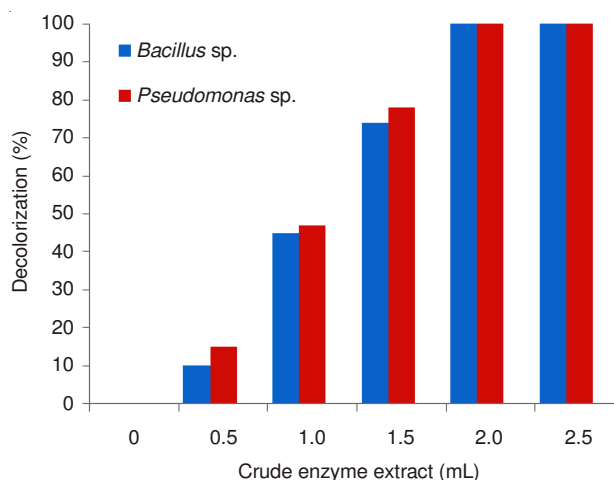


Fig. 2. Decolorization of textile dye light green by crude enzyme extract of *Bacillus* sp. and *Pseudomonas* sp

Bacterial elimination by induced environmental stress:

Bacterial elimination was done indirectly by inducing acetic acid stress. After 36 h only 20 mM concentration showed colony forming ability and further higher concentration of acetic acid did not favor colony forming ability. After 24 h *Bacillus* sp. showed colony forming ability both at 20 and 40 mM concentration of acetic acid. Contrary to 36 and 24 h, at 12 h all the concentration of acetic acid (0, 20, 40, 60, 80, 100, 120, 140 and 160 mM) exhibit the colony forming ability. Regarding *Pseudomonas* sp. after 36 h only 20 mM concentration showed colony forming ability and further higher concentration did not favor colony forming ability. In comparison, after 24 h *Pseudomonas* sp. showed colony forming ability both at 20 and 40 mM. Similar to *Bacillus* sp. *Pseudomonas* sp. also exhibited colony forming ability after 12 h in all concentrations of acetic acid (0, 20, 40, 60, 80, 100, 120 and 140 mM) (Table-2).

After the decolorization of textile dyes it is necessary to eliminate the used indigenous bacteria prior to discharge into the drain. Usually, environmental stresses like weak acid, oxidative stress, antibiotics and heat can be used to eliminate the indigenous bacteria. Among them the use of weak acid is a better choice because of simplicity of its usage, as well as it is quite economical. Acetic acid is widely used as preservative to prevent microbial growth on food stuff and is mostly used for programmed cell death. Acetic acid enters the bacterial cell by simple diffusion and inside the cell it leads to inhibition of the metabolic activities¹¹. It is clear from the results that both the indigenous bacteria (*Bacillus* sp. and *Pseudomonas* sp.) showed colony forming ability after 36 h only at 20 mM concentration of acetic acid and further higher concentration did not favor colony forming ability (Table-2). Even the incubation of 60 mM of acetic acid for 24 h is not favorable for colony forming ability. Thus, the use of acetic acids stress is quite effective in bacterial elimination.

TABLE-2
COLONY FORMATION ABILITY (CFA) OF INDIGENOUS BACTERIAL ISOLATES AT DIFFERENT CONCENTRATION OF ACETIC ACID

S.No.	Bacterial isolates	CFA of bacterial isolates after 24 h incubation Acetic Acid (mM)									
		Time (h)	0	20	40	60	80	100	120	140	160
1	<i>Bacillus</i> sp.	12	+	+	+	+	+	+	+	+	+
		24	+	+	+	-	-	-	-	-	-
		36	+	+	-	-	-	-	-	-	-
2	<i>Pseudomonas</i> sp.	12	+	+	+	+	+	+	+	+	-
		24	+	+	+	-	-	-	-	-	-
		36	+	+	-	-	-	-	-	-	-

Phytotoxicity studies: Biodegradation of textile dyes leads to production of various kinds of degraded products which could be harmful for plant growth. Therefore, it is virtually important to study the toxicity impact of these biodegraded products on plants. The relative sensitivity of *Sorghum vulgare* Pers. towards the textile dye light green and its biodegraded products was studied. The plumule and radicle length of *Sorghum vulgare* was 22 ± 1.14 and 6 ± 0.68 cm, respectively, whereas germination was 100 % in control. The germination was reduced to 36 % when seed were treated with 200 ppm of textile dye light green. Plumule and radicle length of *S. vulgare* decreased up to 4.3 ± 0.81 and 2.3 ± 0.11 cm respectively when seed were treated with 200 ppm concentration of green dyes. Similarly, the germination percentage of *S. vulgare* was 97 %, Plumule and radicle length was 7.5 ± 1.01 and 4.5 ± 1.04 cm, respectively when treated with biodegradation products of textile dye light green (Fig. 3). Results obtained with textile dye light green and its degraded metabolites are in agreement with results reported by Saratale *et al.*⁹ and Phugare *et al.*²². The results indicated that the extracted degraded metabolites by *Bacillus* and *Pseudomonas* sp. gives nontoxic products, resulting in good germination as well as plumule and radicle length of *S. vulgare* as compared to use of textile dye alone. Thus, phytotoxicity studies revealed that the biodegradation of the textile dye light green by the *Bacillus* and *Pseudomonas* sp. led to detoxification of the pollutant dyes.

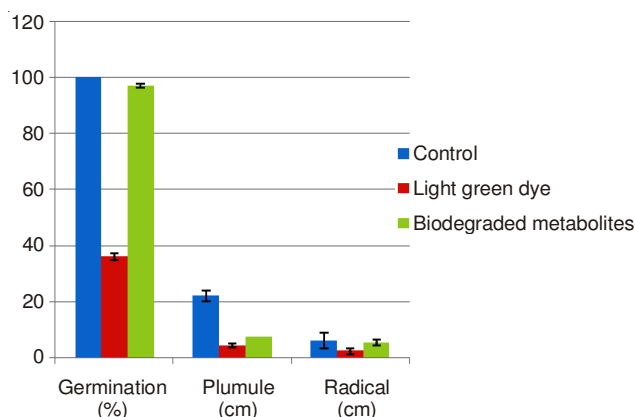


Fig. 3. Phytotoxicity test of treated and untreated textile dye light green

Conclusion

It is concluded from the present study that the use of *Bacillus* and *Pseudomonas* sp. for the removal of textile dye

light green could be an effective method, especially when they are eliminated after the treatment by acetic acid. Therefore, it is the most safe and cheaper method for the removal of textile dyes and discarding the bacteria after the bio-treatment.

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