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Isolation and characterization of textile dye degrading native bacterial strains from textile effluent contaminated sites

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Abstract
Synthetic dyes constitute the largest and most versatile class of synthetic dyes used in the textile, pharmaceutical, food and cosmetics industries and represent major components in wastewater from these industrial dying processes. Biological decolorization of azo dyes is an alternative and efficient tool for environmental management. Hence, in this study totally 40 bacterial isolates were screened from textile effluent contaminated soil samples. Out of 40 bacterial isolates, six potential bacterial strains were chosen based on their decolorization efficiency. Among the six bacterial strains, T11 strain was found to decolorize maximum number of dyes followed by T1, T4, T9, T7 and T10. The strain T11 decolorized reactive red 94%, reactive blue 95%, reactive grey 94% and reactive violet 100% after 120 h incubation at 300ppm concentration. Moreover, the selected bacterial strains were showed resistance to various heavy metals like Cr, Mn, Pb, Zn and Fe. The Phytotoxicity effect of degraded product was evaluated after 5 days of degradation. Phytotoxicity test revealed the nontoxic nature of the degraded metabolites. Based on the biochemical characterization and 16s rDNA gene sequencing analysis, the selected bacterial strain T11 was identified as *Bacillus firmus*.

Keywords: Textile dye, decolorization, heavy metals, bacterial strains

1. Introduction
The textile industry plays an important role in the world economy as well as in our daily life, but at the same time, it consumes large quantities of water and generates large amounts of waste water. The chemical reagents used in the textile sector are diverse in chemical composition ranging from inorganic to organic molecules (Subhatra *et al*. 2013). The release of a wide range of compounds from industries is creating disturbance to the ecosystem causing climatic changes, reduction of water levels in the ground as well as oceans, melting ice caps, global warming, ozone layer depletion due to photochemical oxidation, etc. (Varsha *et al*. 2011). Therefore, industrial effluents containing azo dyes must be treated before discharging into the environment to remove the dye toxicity from textile effluent (Rajaguru *et al*. 2002). Physical and chemical methods (adsorption, chemical transformation, incineration, photo-catalysis, ozonation etc) are not suitable for the removal of recalcitrant dyestuffs, because of high cost, low efficiency and in-applicability to a wide variety of dyes (Dawker *et al*. 2008).
A dye can be generally described as a colored substance that has an affinity to the substrate to which it is being applied. The dye is generally applied in an aqueous solution and may require a mordant to improve the fastness of the dye on the fiber. Both dyes and pigments appear to be colored because they absorb some wavelengths of light preferentially. In contrast with a dye, a pigment is generally insoluble and has no affinity for the substrate. Some dyes can be precipitated with an inert salt to produce a lake pigment. Archaeological evidence shows that particularly in Indian and the Middle East, dyeing has been carried out for over 5000 years. The dyes were obtained from animal, vegetable or mineral origin, with no or very little processing. By far the greatest source of dyes has been from the plant kingdom, notably roots, berries, bark, leaves and wood. But only a few have ever been used on a commercial scale. The first human-made (synthetic) organic dye (Mauveine) was discovered by William Henry Perkin, 1856. (Zollinger, 1987).
The microorganisms being highly versatile are expected to develop enzyme systems for the decolourization and mineralization of azo dyes under certain environmental conditions (Pandey *et al*. 2007). Along with the reductive enzymes, some investigators have

Demonstrated the oxidative enzymes such as lignin peroxidase, laccase and tyrosinase, in the decolourization and degradation of azo dyes (Bhatia, 2005). Biodegradation using microorganisms are gaining importance as it is cost effective, environmental friendly and produces less sludge (Bella Devassy Tony *et al*. 2009). In this study, we focused on the isolation and identification of dye-degrading microorganism from textile effluent contaminated site having a decolourizing ability and various intermediates formed have been analyzed during the degradation of Reactive Blue.

2. Materials and Methods

2.1 Soil sample Collection and bacterial isolation

The textile effluent contaminated soil samples were collected from the surroundings of dye industry, Tirupur region, Tamil Nadu, India. The sample was collected in a plastic container. Then the sample was brought to the laboratory as early as possible and was subjected for various microbiological studies. Bacterial isolations were carried out by serially diluting textile effluent and soil samples in sterile distilled water were subsequently plated onto LB plates (Cappuccino *et al*. 1996). The plates were incubated at 37±2˚C for 24 h and colonies with distinct morphology were picked up and purified by regular sub culturing. The strains were maintained on slants of LB medium.

2.2 Visual identification of decolourization

All the 40 morphologically distinct bacterial isolate were tested for their ability to decolorize textile azo dyes. Isolates were aseptically inoculated into test tubes containing 10ml of Nutrient broth containing 300 ppm of dye and incubated at 37ºC for 4 days. After 4 day incubation the tubes were observed for decolourization and was ranked on the basis of visual identification as intense (+++), moderate (++), Slight (+) and no decolourization (-).

2.3 Decolourization Assay

Fifty milliliter of nutrient agar sterile medium was amended separately with each of the textile dye (300 ppm) and subsequently inoculated with 2% bacterial suspension. The suspension contained $2.5x10^6$ cfu/ml spores. The flasks were kept in mechanical shaker and incubated at 30±1℃ for 60 hrs. Samples were drown at 12 hrs interval for observation. The samples were centrifuge at 10000rpm for 10 minutes and decolourzation was assessed by measuring absorbance of the supernatant with the help of spectrophotometer at wavelength maxima (λm) of respective dye. Two control flasks (Dye +medium without inoculums and medium with inoculaums without dye) were maintained. (N.Sriram *et al.* 2013)

$$\text{Decolourization (\%)} = \frac{\text{(Initial Absorbance–final Absorbance)}}{\text{Initial Absorbance x 100}}$$

2.4 Heavy Metal tolerance

The isolated strains were subjected to different metal resistance levels by the agar plate dilution method. The freshly prepared Nutrient agar plates were amended with heavy metal salts namely Fe, Pb, Mn, Cr, and Zn at different concentrations ranging from 100 to 1000 µg/ml and they were spot inoculated with a loop full of overnight grown cultures (T1, T4, T7, T9, T10 and T11). The plates were incubated at 28 ± 2° C for three to five days. The highest concentration of heavy metal salt supporting strains growth on Nutrient agar plates were defined as the maximum resistance level.

2.5 Phylogenetic analysis of the isolates

Total bacterial genomic DNA was isolated by phenol chloroform method (Sambrook *et al*, 1989). The 1.45 kb 16S rDNA fragment was amplified using 16S rDNA bacterial forward (5'AGAGTTTGATCTTGGCTCAG3') the reverse (5'GGYTACCTTGTTACGACTT3') primer set. PCR amplified product was purified by Gene Jet PCR purification kit. The sequence data were aligned and analyzed to identify bacterium and its closest neighbors using the NCBI web– based BLAST program (http:// www.ncbi.nlm.nih.gov/BLAST/). Closest known species were compared with percentages of identity. Sequences were aligned using the Clustal W program. Phylogenetic trees were constructed using MEGA5.1 software.

2.6 Phytotoxicity Analysis

In this experiment, the effect of Reactive blue160 dye at the concentration of 20mg was evaluated on germination of seeds of 4 different plants, Black gram,Green gram,Groundnut and Corn . The seeds were germinated in pots containing 10kg of paddy field soil. Four sets of 20 seed each of Black gram, Green gram, Groundnut and corn were treated every 24 hours with 10 ml of dye solutions and degraded reactive blue 160 dye solutions separately. Seeds germinated in pots treated distilled water were used as a control. All pots were kept under shade near sunlight for the period of 6 days. Germination of seeds treated with dye and degraded dye solutions was calculated after comparing with control. At the end of the germination experiment, degraded dye and control samples. All analysis was conducted in triplicate and the results were presented as the mean of triplicate ± Standard deviations (SD)

3. Results and discussion

3.1 Isolation of dye degrading bacteria from textile dye contaminated Soil

A total of 40 bacterial strains were screened from textile dye effluent contaminated soil samples. From 40 bacterial strains 6 (T1, T4, T7, T9, T10 and T11) strains were selected based on the primary visual identification and used for further studies (Table 1). Final screening of the microorganisms in liquid media with incorporated dyes resulted in isolation of six bacterial isolates capable of degrading various dyes with some isolates exhibiting capability of degrading a wide spectrum of dyes. Similar kind of screening process was reported by Neelambar *et al.* (2013).

3.2 Degradation of dye under the static condition

All the selected six bacterial strains were able to degrade more than one dye with ranging from 94-100% depending on the bacterial strains and dyes. Among the six bacterial strains, T11 showed maximum degradation of Reactive Red 94%, Reactive Blue 95%, Reactive Gray 94% and Reactive Violet 100% after 60 hrs incubation at 300ppm concentration (Figure 1). Except reactive violet dye, bacterial strains unable to reduce completely even after 60 h incubation. This may be due to the structure and complex nature textile dyes. Some azo dyes are more resistant to removal by bacterial cells and this may be attributed to their structural differences. These isolates probably have acquired natural adaptation to survive in the presence of the dyes (Khadijah *et al*. 2009). Chen *et al.* (2003) and Senan and Abraham (2003) reported isolation and screening of microorganisms capable of decolourising various azo dyes

from sludge samples collected from wastewater treatment sites contaminated with dyes. Similarly, Neelambar *et al.* (2013) also supported our result who has reported that the multi dye degrading ability of the native bacterial community.

3.3. Characterization of Heavy metal resistance isolates

Moreover, the selected bacterial strains showed tolerance to various heavy metals. Maximum resistance level of various heavy metals *viz* Fe, Pb, Mn, Cr and Zn have been determined and shown in (Figure 2). As the industrial effluent contains other heavy metals, resistance ability to various metallic salts has been determined. The results indicated that the strain T11 displayed resistance to different heavy metals at different concentrations. This might be due to the development of resistance mechanism of microbes to a variety of toxic heavy metals for their survival in the heavy metal contaminated environment [21]. On the other hand, dye degrading bacterial strains showed reduced colony forming efficiency and cell growth with increasing concentrations of heavy metals. The reduced microbial growth at high metal concentrations clearly indicated the alteration of metabolic and physiological behavior of microbes. Nonnoi *et al.* [22] also reported the reduction of microbial growth with exposed metal concentrations. Moreover, the macromolecular composition of biosorbent could also be manipulated by cultivation conditions (hard and soft acid and base principles) to produce stronger ligands of transition metals than those naturally present on the microbial surfaces (Huber *et al.* 1990).

3.4 Phylogenetic analysis of the strain

The molecular characterization of the selected strains was achieved by 16S rDNA gene sequencing. Selected dye degrading bacterial stain T11 showed a 99% relationship with *Bacillus circulans* KJ531945. The 16S rDNA sequence of the bacterial strain was submitted into GenBank (accession number KF011491). To assert the phylogenetic position of the selected rhizobacterial strain UPGMA tree was performed with NCBI obtained sequences in which *E. coli* (AB269763) used as an out group. Tree topologies were evaluated by performing bootstrap analysis of 100 data sets with the MEGA 6 software (Fig. 3).

3.5 Phytotoxicity studies

The untreated dyeing effluent may be unsafe to the ecosystem, when directly used for agriculture. Thus, it was of serious concern to assess the toxicity of the effluent before and after bacterial treatment. Hence, present phytotoxicity study was carried out with dye and dye degraded protects. The phytotoxicity studies showed that good germination rate as well as significant growth in degraded metabolites and plain water treated seeds as compared to the dye treated seeds. The raw dyes treated seeds showed less seed germination rate (53-69%). On the other hand water and degraded products showed increased seed germination rate 89-100% and 79-88% respectively. Similar study was conducted by Ren *et al* (1996) who demonstrated the toxicity of Polycyclic Aromatic Hydrocarbons (PAHs), Anthracene(ANT), Benzo[a]Pyrene (BAP) and Fluoranthene to the duckweed *Lemna gibba* L. and *Brassica napus* L. seeds. These authors used the germination efficiency as a measurement for toxicity. The results of this study suggested that the exposure of seeds to low concentration of dye was found less toxic to seed germination and growth of seedlings. However, more evidence from the literature survey reported that, germination and shooting percent could adversely get affected when the concentration of dye was increased significantly. This is in agreement with the previous work by Durve *et al*. (2012). They reported that both germination and shooting and rooting percent were drastically reduced when the concentration of dye was increased from 500 to 10,000ppm for *Vigna radiata* (whole moong), *Triticum* spp (Wheat) and *Brassica juncea* (Mustard) seeds.

Conclusion

Screening of the microorganisms resulted in isolation of six potential bacterial isolates capable of degrading various azo dyes with some isolates exhibiting capability of degrading a wide spectrum of dyes. Moreover, selected strains showed multi metal resistance to different heavy metals. Phytotoxicity study revealed the non toxic nature of the dye degraded metabolites. Thus, they can be used as excellent bioagents for the bioremediation of toxic textile dyes which have been proved to be the smartest way to wrestle with dye effluent related pollution.

Table 1: Ranking of dye decolourizer based on visual identification

S.No	Name of the Isolates	Ranking of Decolorization
1	T_1	+++
2	T_2	++
3	T_3	++
4	T_4	+++
5	T_5	++
6	T_6	++
7	T_7	+++
8	T_8	++
9	T_9	+++
10	T_{10}	+++
11	T_{11}	+++
12	T_{12}	-
13	T_{13}	+
14	T_{14}	++
15	T_{15}	+
16	T_{16}	-
17	T_{17}	-
18	T_{18}	-
19	T_{19}	+
20	T_{20}	+
21	T_{21}	-
22	T_{22}	+
23	T_{23}	-
24	T_{24}	-
25	T_{25}	+
26	T_{26}	-
27	T_{27}	+
28	T_{28}	-
29	T_{29}	-
30	T_{30}	+
31	T_{31}	+
32	T_{32}	+
33	T_{33}	-
34	T_{34}	+
35	T_{35}	+
36	T_{36}	-
37	T_{37}	-
38	T_{38}	-
39	T_{39}	-
40	T_{40}	+

Note: Intense decolourization +++, Moderate decolourization ++, Slight decolourization + and - No decolourization

Table 2: Phytotoxicity analysis of dye and degraded product

Seeds	Treatment type	Percentage of germination					
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Black Gram	Control	-	30	55	78	100	100
	Degraded dye	-	28	46	66	79	88
	Reactive Blue	-	21	33	42	50	61
Green Gram	Control	-	24	62	78	89	94
	Degraded dye	-	18	46	53	69	88
	Reactive Blue	-	-	22	41	52	69
Ground nut	Control	-	24	54	58	77	89
	Degraded dye	-	16	48	55	68	79
	Reactive Blue	-	-	12	34	41	53
Corn	Control	-	28	64	76	100	100
	Degraded dye	-	24	52	58	73	86
	Reactive Blue	-	20	40	47	48	53

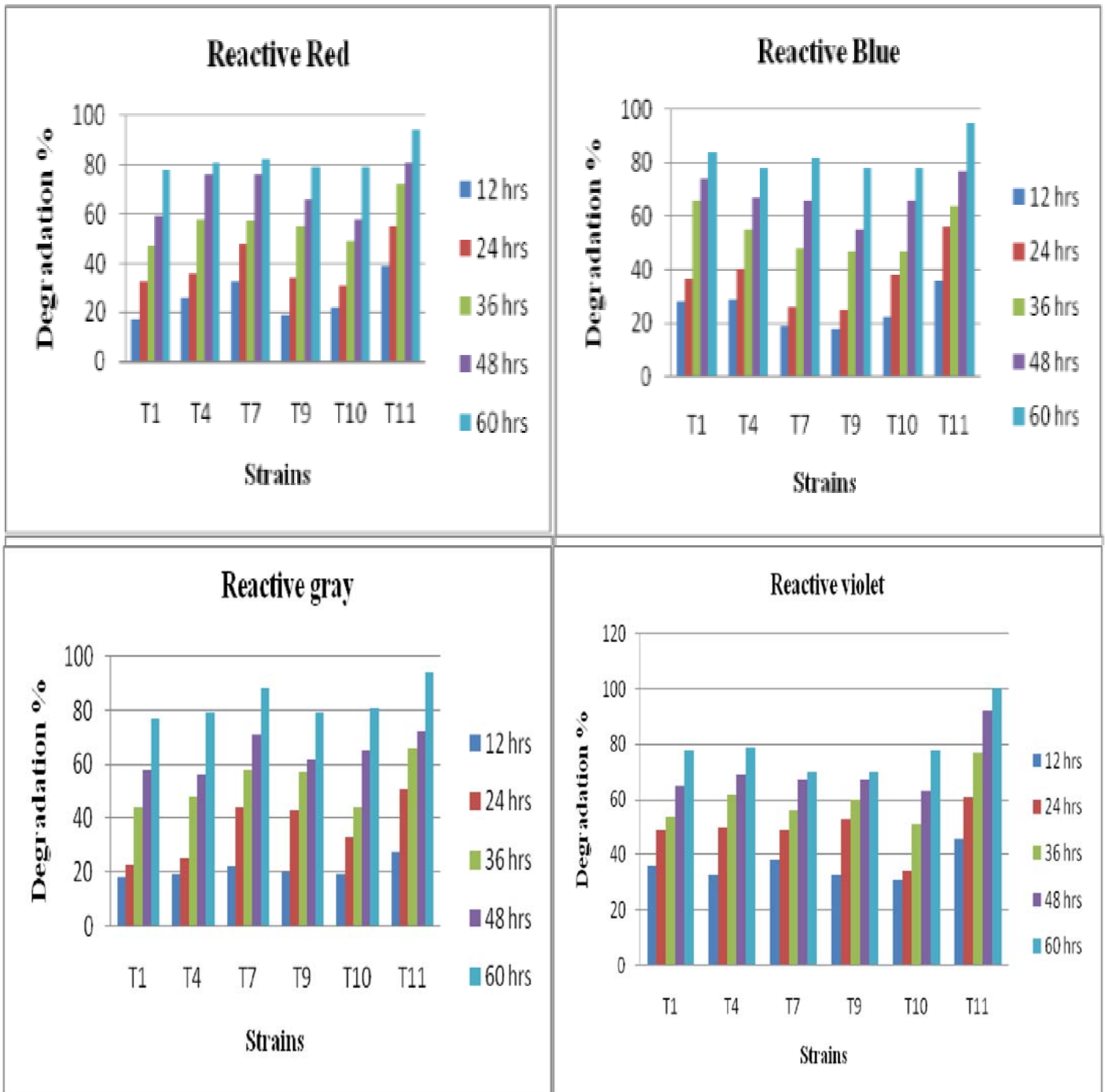
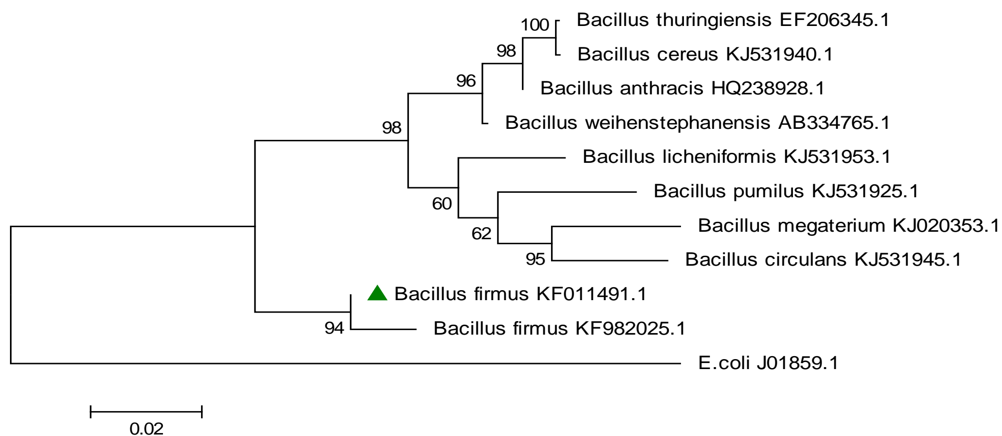


Fig 1: Effect of dye degrading bacterial strains on different azo dyes

Fig 2: Heavy Metal tolerance of the strains



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