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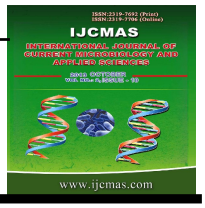

Original Research Article

Biodegradation of textile azo dyes and its bioremediation potential using seed germination efficiency

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A B S T R A C T

To decolourize indigo blue dye which was used mostly in all jean manufacturing textile industries using the soil and sludge isolate, *Bacillus* spp. Soil and sludge from textile effluent discharge sites were collected from different jeans manufacturing industries in Coimbatore district. Indigo blue dye degrading organisms were screened using a Zhou and Zimmermann (ZZ) screening media after serially diluted the collected samples. After screening and isolating the best dye decolourizer from the samples, optimization of dye degrading conditions were carried out. Different factors like temperature, pH, carbon source, nitrogen source, metallic salts, inoculum size and time course of decolourization was selected as optimization parameters. Phytotoxicity of dye and degraded dye was evaluated by measuring the relative changes in seed germination of three different plants Green gram (*Vigna radiate*), Kidney beans (*Phaseolus vulgaris*), Fenugreek (*Trigonella foerum*). Seven bacterial isolates were screened based on their ability to decolourize the indigo blue dyes from the soil and sludge samples. Dye decolourizer (DD4) decolourized upto 98% followed by DD2 with 81%. Both the isolates were identified as *Bacillus* sp1 and 2 after microscopic and biochemical characteristics. From the optimization analysis, the suitable optimized condition for the isolate, *Bacillus* sp2 was identified as sucrose (1%), ammonium chloride (0.25%), temperature (45^0C), pH (8.0), potassium dihydrogen-ortho-phosphate and inoculum dose (2%). Phytotoxicity of dye and degraded dye was evaluated after 6 days of study. No phytotoxicity was observed for the concentration of dye used in the study. Good germination and shoot, root length of the plants were observed for both dye and degraded dye exposed seeds after comparing with the control. The isolate from the effluent discharge site showed a potential of degrading dyes at faster rate with an application of good seed germinating efficiency. These properties thus found useful for the bioremediation of various textile industrial effluents, saving the ecosystem from harmful effects of various dyes.

Keywords

Indigo blue dye; *Bacillus* spp ; Different factors; Green gram (*Vigna radiate*); Kidney beans (*Phaseolus vulgaris*),; Fenugreek (*Trigonella foerum).*

Introduction

Textile processing industries largely employ azodyes (Correia *et al*., 1994). Azo dyes have been used increasingly in industries because of their ease and cost

effectiveness in synthesis compared to natural dyes. However, most azo dyes are toxic, carcinogenic and mutagenic (Pinherio *et al*., 2004). Azo bonds present in these compounds are resistant to breakdown, with the potential for the persistence and accumulation in the environment (Mahdavi Talarposhti *et al*., 2001). Waste water from textile industry is a complex mixture of many polluting substances ranging from organochloride based waste pesticides to heavy metals associated with dyes and dyeing process (Correia *et al*., 1994). Intensive irrigation of agricultural lands with water polluted with various industrial effluents severely affects soil fertility and plant growth. Dissolved substances in industrial effluents after the chemical and biological status of the soil and water may affect growth and productivity of plants. Plant growth parameters namely germination percentage, seedling survival and seedling height have been taken as criteria to assess plant response to specific pollutants. Dyes used are considered as carcinogenic and mutagenic and the effluents reduce the rate of germination and growth of crop plants (Nirmalarani and Janardhanan, 1988).

A higher concentration of untreated effluents inhibits elongation of shoot and root of seedlings. High concentration of solids in the effluent reduces the level of dissolved oxygen, which resulted in restriction of growth and development of the seedlings (Saxena *et al*., 1986). The inhibition of seed germination may be due to the high dissolved solids in the effluent that disturb the osmotic relation of the seed. Increase in the effluent concentrations affected the germination of paddy and the dry matter production. The dry matter accumulation reflects the actual physiological status of the plant and therefore an indicator parameter of plant growth (Sahai *et al*., 1983). Effect of the effluent from South India Viscose factory on different varieties of maize has been reported (Saxena *et al*., 1986). A considerable decrease in the biochemical parameters of onion grown in untreated dye solution has been reported. As the carbohydrate, protein and chlorophyll contents are related to plant growth, a decrease in their content is a clear indication of the toxic nature of the dye industry effluents. Increased proline concentration was also observed in plants exposed to textile dye effluent. Proline accumulation is a common feature of plants exposed to various kinds of environmental stresses like drought, salinity, low temperature and heavy metals (Ameta *et al*., 2003)

Several physico-chemical techniques have been proposed for treatment of colored textile effluents. These include adsorption on different materials, oxidation and precipitation by Fenton's reagent, bleaching with chloride or ozone photo degradation or membrane filtration (Robinson *et al*., 2001). All these physical or chemical methods are very expensive and result in the production of large amounts of sludge, which creates the pollution. Therefore, economic and safe removal of the polluting dyes is still an important issue. Bioremediation through microorganisms has been identified as a cost effective and environment friendly alternative for disposal of textile effluent (Chen *et al*., 2003). In recent years a number of studies have focused on some microorganisms capable of degrading and absorbing dyes from wastewater. A wide variety of microorganisms are reported to be capable of decolonization of dyes (Chang and Kuo, 2000). According to Stolz, (2001) several microorganisms have been found to decolourize and mineralize azo dyes. The bacterial metabolism of azo

dyes is initiated by a reductive cleavage of azo bond in most cases, which results in the formation of amines. The initial process in microbial degradation of azo dyes is the cleavage of highly electrophilic azo bond leading to decolourization of azo dyes. The reductive cleavage of the azo bond resulted in formation of aromatic amines as end products (Nortemann *et al*., 1986)

Considering the impact of dyes on environment and significant crops, and the ability of microorganisms to metabolize azo dyes, in the present study an indigo blue dye used in jeans manufacturing textile industry was subjected to bacterial attack at optimized conditions. To find out the degrading ability of indigenous flora, the bacterial isolates were made from textile effluent discharge sites. Also, the bioremediation potential of treated dyes for germination of significant seeds was determined under standard *in vitro* conditions.

Materials and Methods

The entire research work was carried out from February 2013 to September 2013.

Soil and sludge samples were collected from different textile effluent discharge sites of Coimbatore district, Tamil Nadu, India for screening dye degrading bacterial isolates. A common dye used in textile industries, indigo blue was selected based on its usage in jean garments

Methodology

Isolation of dye degrading bacteria (Ponraj *et al*., 2011)

The dye decolorizing bacteria was isolated from the soil and sludge samples of textile dye effluent run-off site by serial dilution and plating appropriate dilutions on modified Zhou and Zimmermann (ZZ) agar medium.

Serial dilution technique

0.5g of soil sample and 0.5g of sludge sample was taken in a 100ml of sterile distilled water (10^{-2}). A series of tubes containing 4.5ml of sterile distilled water was taken. 0.5ml of the sample was transferred from 10^{-2} dilution to the distilled water in the tube and the sample was serially diluted using sterile pipettes from 10^{-2} to 10^{-5}.

Enumeration of Bacteria

Zhou and Zimmermann (ZZ) agar medium was prepared and sterilized. The serially diluted samples 10^{-3}, 10^{-4} and10^{-5} were plated on Zhou and Zimmermann (ZZ) agar media and the plates were incubated at 37^{o}C for 24 hours.

Isolation of organisms

The incubated plates were observed for the predominant types of organisms. Different colony morphology representing each type of bacteria was observed on the inoculated plates. The isolates were purified by streaking on nutrient agar plates.

Enrichment technique

All the isolated cultures were studied by inoculating them in an enrichment medium (effluent basal medium). The inoculated medium was incubated at 30° C for 3 to 6 days under shaking in an orbital shaker at 120 rpm.

Dye decolourization evaluation

Decolourization ability of each isolate was performed in 90 ml of ZZ medium containing 0.02g of indigo blue dye. About 10% (v/v) inoculum of each isolate was used separately. Uninoculated dye medium served as control. Inoculated medium and control was incubated at 30°C for 3 to 6 days under shake culture condition. About 2 ml samples were withdrawn aseptically and centrifuged at 8,000 rpm for 15 minutes. The clear supernatant was used for measuring absorption at 600 nm using UV-Vis spectrophotometer (Shimadzu, Japan). The percent decolourization of effluent was determined by using the formula,

$$D= [A0-A1)/A0] \times 100$$

Where,
D, decolourization in %;
A0, initial absorbance;
A1, final absorbance

Identification of dye degrading bacteria

Isolates showing maximum decolourization ability alone was identified based on microscopy, cultural characteristics and biochemical tests. During this analysis, all the isolates were identified to their genera level.

Dye Decolourization optimization (Shah *et al*., 2013)

Decolourization of indigo blue dye by the selected isolate was optimized with respect to the effect of 1%, carbon sources (glucose, sucrose, lactose, mannitol), 0.25%, nitrogen sources (peptone, yeast extract, ammonium sulphate, ammonium chloride), temperature (25, 37C, 40 45), pH (5-9), metallic salts (Potassium dihydrogen orthophosphate, Magnesium sulphate, Calcium chloride, Ferric chloride) and inoculum size (1-5%). Decolourization experiments were carried out separately for each of these factors.

Time course of dye decolourization (Ponraj *et al*., 2011)

The time course of decolourization was carried out under optimum conditions obtained from the above studies. Indigo blue dye added media with optimized factors was inoculated with the best decolorizing isolate. To study the time course of decolourization, inoculated flasks were incubated up to 144h. For every 24 h the samples were removed and analyzed for decolourization activity.

Bioassay for dye toxicity/phytotoxicity: Seed germination test (Durve *et al*., 2012)

In this experiment, the effect of indigo blue dye at the concentration of 20mg was evaluated on germination of seeds of 3 different plants, Green gram (*Vigna radiate*), Kidney beans (*Phaseolus vulgaris*), Fenugreek (*Trigonella foerum*). The seeds were germinated in pots containing 10kg of paddy field soil. Three sets of 20 seed each of Green gram (*Vigna radiate*), Kidney beans (*Phaseolus vulgaris*), Fenugreek (*Trigonella foerum*) were treated every 24 hours with 10 ml of dye solutions and degraded indigo blue 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, the shoot length and root length of seedlings was

measured separately for dye, degraded dye and control samples. All analysis was conducted in triplicate and the results were presented as the mean of triplicate ± Standard deviations (SD)

Results and Discussion

Isolation, screening and identification of dye degrading bacteria

About seven isolates were obtained after screening dye degrading organisms from soil and sludge samples. All the isolates were evaluated for their dye decolorizing ability. Among the seven isolates, DD2 and DD4 showed maximum decolourization percentage of 81 and 98% respectively (Table-1). Based on microscopic and biochemical parameters, the two dye decolorizing isolates (DD2 and DD4) were identified as *Bacillus* sp1 and *Bacillus* sp2 (Table-2 and 3).

Dye decolourization optimization

Optimized conditions for effective decolourization of indigo blue dye by the isolate, *Bacillus* sp2 was analyzed and presented in Table-4. The range of activity on decolourization of indigo blue by *Bacillus* sp2 (DD4) in different carbon sources glucose sucrose, lactose and mannitol was 82.33%, 86.25%, 76.65% and 80.92% respectively. Maximum decolourization observed in sucrose (86.25%) containing media which indicates the optimized carbon source for the isolate, *Bacillus* sp 2. Decolourization of indigo blue by *Bacillus* sp2 (DD4) in different nitrogen sources like yeast extract, peptone, ammonium sulphate, ammonium chloride was found to be as 86.26%, 86.80%, 83.02%, 88.63% respectively. From this analysis, ammonium chloride was identified as the optimized nitrogen source for the isolate. Temperature and pH being the most significant factors for the optimization study; during the analysis, maximum decolourization was observed for the media incubated at 45^{o}C (92.66%) and media with pH 8.0 (87.23%). Interestingly, due to thermophilic property of this isolate, maximum decolourization was observed at 45^{o}C. Among the four different metallic salts, the isolate decolorized 84.96% of indigo blue dye in the presence of potassium dihydrogen orthophosphate. About 2% of inoculum was found to be suitable for decolorizing blue dye. Maximum of 91.3% was observed in the blue dye media inoculated with 2% of *Bacillus* sp2.

Time course of dye decolourization

From the optimization analysis, the suitable optimized condition for the isolate, *Bacillus* sp2 was identified as sucrose (1%), ammonium chloride (0.25%), temperature (45C), pH (8.0), potassium dihydrogen-ortho-phosphate and inoculum dose (2%) (Table-4).

Under this optimized conditions the time course of dye decolourization experiment was carried out using the isolate, *Bacillus* sp2.After 48 hours incubation maximum decolourization percentage (94.0%) was observed for the blue dyes (Figure-1). No stability in decolourization of the dyes by the isolate was observed (Figure-2). This may because of various other factors that include metabolic properties and growth phase of the isolate, or the inhibitory action of the dye concentration and dye structures.

Bioassay for dye toxicity
Seed germination test

The bioassay for dye toxicity or phytotoxicity was based on measuring the

Table.1 Decolourization of dyes using the soil and sludge isolates

S.No.	Dye decolourizers (DD)	Percentage of decolourization for blue colour dye (%)
1.	DD1	65.0
2.	**DD2**	**81.0**
3.	DD3	73.0
4.	**DD4**	**98.0**
5.	DD5	77.0
6.	DD6	68.0
7.	DD7	79.0

Table.4 Dye decolourization optimization

Indigo blue dye		OD of control	OD of sample	percentage
Carbon source	glucose	1.557	0.275	82.33%
	sucrose	1.268	0.214	**86.25%**
	lactose	1.046	0.319	76.65%
	mannitol	1.595	0.297	80.92%
Nitrogen source	Yeast extract	1.041	0.143	86.26%
	Peptone	1.046	0.138	86.80%
	Ammonium sulphate	1.056	0.120	83.02%
	Ammonium chloride	1.096	0.186	**88.63%**
Temperature	25ºC	1.110	0.365	67%
	37ºC	1.149	0.503	56.22%
	40ºC	1.116	0.166	85.12%
	45ºC	1.213	0.089	**92.66%**
pH	5	1.144	0.244	78.67%
	6	1.323	0.351	73.46%
	7	1.295	0.259	80%
	8	1.089	0.139	**87.23%**
	9	1.038	0.137	86.80%
Metallic salts	**Potassium dihydrogen orthophosphate**	1.024	0.154	**84.96%**
	Magnesium sulphate	1.019	0.172	83.12%
	Calcium chloride	1.015	0.185	81.77%
	Ferric chloride	1.009	0.192	80.97%
Inoculum size	1%	1.138	0.123	89.19%
	2%		0.099	**91.30%**
	3%		0.114	89.98%
	4%		0.119	89.54%
	5%		0.165	85.50%

Table.5 Bioassay of dye and degraded dye toxicity

Seed Type	Samples	Percentage of germination						Average shoot length (cm)*	Average root Length (cm)*
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6		
Green gram	Control (water)	-	28	64	76	100	100	17.9±0.351	3.5±0.351
	Degraded dye-1	-	24	52	56	76	80	16.1±0.833	2.6±0.436
	Dye -1 (Blue)	-	20	40	48	40	72	14.2±0.100	2.8±0.361
Kidney beans	Control (water)	-	20	80	88	92	96	24.3±0.577	2.2±0.265
	Degraded dye-1	-	0	64	68	88	88	19.8±0.764	3.5±0.500
	Dye -1 (Blue)	-	0	36	40	68	80	10.4±1.464	4.5±0.500
Fenugreek	Control (water)	-	24	52	52	84	90	6.6±0.721	3.7±0..416
	Degraded dye-1	-	16	48	52	72	85	5.9±0.907	3.9±0.100
	Dye -1 (Blue)	-	0	12	16	63	70	4.6±0.850	2.0±0.100

*Mean±SD

Figure.2 Time course of dye decolourization

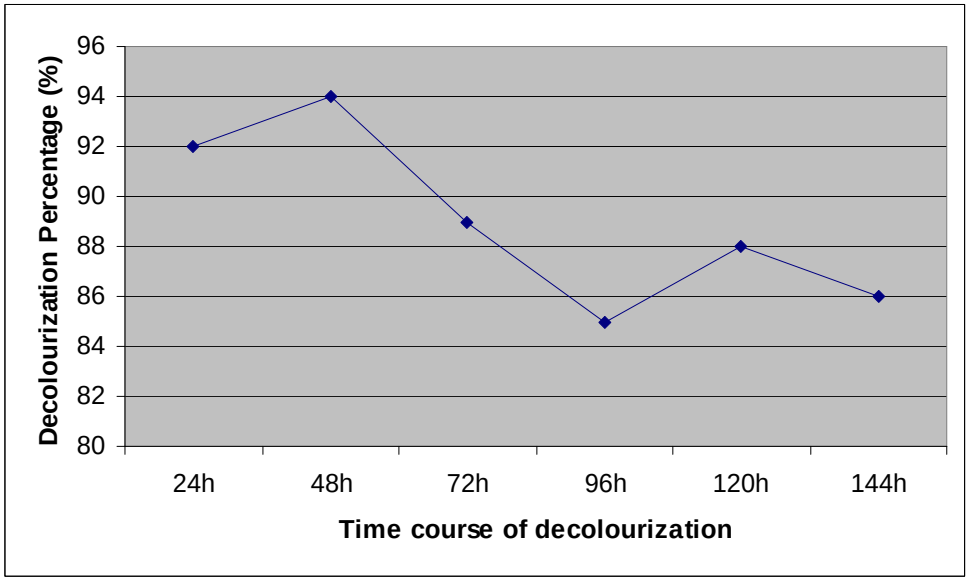

Figure.2(a) Bioassay of dye and degraded dye toxicity: Green gram

Figure.2(b) Bioassay of dye and degraded dye toxicity: Kidney beans

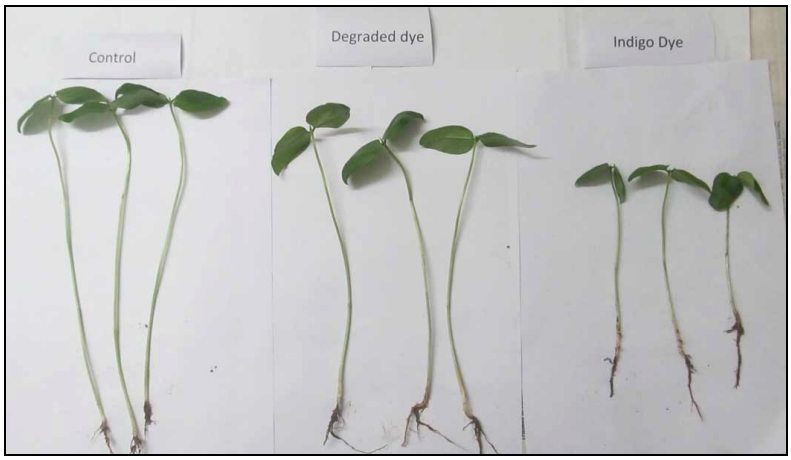

Figure.2(c) Bioassay of dye and degraded dye toxicity: Fenugreek

Figure.3 Indigo blue dye decolourization of *Bacillus* sp2

effect of indigo blue dye on seed germination, plant shooting and root elongation. The phytotoxicity of the dye was estimated by measuring the ability of dye and degraded dye to germinate the Green gram Kidney beans Fenugreek seeds as test plants (Table-5 and Figure-2a, b, c). The results show that the selected concentration of dye was not considered toxic to seed germination since good growth was observed when compared to that of control. 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, root and shoot growth, and chlorophyll content, 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.

Textile industries had faced the problems related to effluent discharge into the public accessing water bodies and aquatic sources for the past severalyears. The dye and its concentration in the effluent was reported to present different chemical compounds which may be carcinogenic to human and animal populations when the contaminated water was consumed. Several researchers have contributed to decolourize or degrade the dyes and its constituents for past two decades. In the present research one such

dye causing the similar problem in Coimbatore district was selected and its decolourization was measured using the strain isolated from the effluent discharge site itself. Interestingly, as expected under optimized conditions, good decolourization activity was measured for the isolate at a faster rate. Phytotoxicity of dye and degraded dye was evaluated after 6 days of study. No phytotoxicity was observed for the concentration of dye used in the study. Good germination and shoot, root length of the plants were observed for both dye and degraded dye exposed seeds after comparing with the control. The isolate from the effluent discharge site showed a potential of degrading dyes at faster rate with an application of good seed germinating efficiency. These properties thus found useful for the bioremediation of various textile industrial effluents, saving the ecosystem from harmful effects of various dyes.

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