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Biodegradation and decolorization of two different azo dyes, Reactive Blue 221 and Direct Black 38, and assessment of the degraded dye metabolites

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ABSTRACT

Azo dyes are xenobiotic compounds. When released along with industrial effluents, dyes affect aquatic species creating an environmental imbalance. Although numerous researches have been done on microbial degradation of dyes, however, an effective biological treatment method to deal with this hazardous compound is still lacking. The study focuses on the biodegradation and decolorization of two commercially important azo dyes, Reactive Blue 221 and Direct Black 38, using indigenous bacteria isolated from textile effluent and sludge samples. Reactive Blue 221 was effectively decolorized to 74.58% by consortium HBC 3, while pure culture of bacterial isolate PB9 decolorized 61.55% of Direct Black 38. The bacterial isolates used to build the consortium HBC 3 were identified up to genus level as *Corynebacterium*, *Aeromonas*, and *Vibrio* by conventional microscopic and biochemical methods, while PB9 was identified as *Lysinibacillus sphaericus* strain VITHPB9 by performing 16S r-RNA gene sequencing analysis. Different physicochemical parameters were optimized in order to obtain maximum decolorization of both the dyes. Analyses of the results of Fourier-transform infrared spectroscopy and high-performance liquid chromatography indicated the degradation of both the dyes. Finally, phytotoxicity assay conducted with seeds of *Vigna radiata* and *Cicer arietinum* confirmed that the degraded dye metabolites were nontoxic.

Keywords: Azo dyes; Biodegradation; Decolorization; Consortium; Pure culture; Phytotoxicity assay

1. Introduction

Dyes are natural or synthetic colored substances. They give permanent color to different substances on application. The chromophore group present in a dye structure is responsible for the color it imparts. Dyes have a lot of applications in different industries including cosmetics, pharmaceutical, leather, paper, food, and textiles [1]. Among these, textile dyeing and finishing industry is the largest consumer of dyes [2]. Increasing customer demand for synthetic dyes led to the worldwide production of dyes to reach a level more than 7×10^5 tonnes [3,4]. Approximately 70% of the total dyestuffs used are azo dyes, thus representing the largest group of synthetic dyes [5–8]. The structure of these dyes consist of a single or multiple azo (N=N) bonds (monoazo, diazo, triazo, or polyazo) and sulfonic ($-SO^{3-}$) groups [9,10]. More than 3,000 azo dyes have been discovered so far [11,12]. Textile dyeing and finishing industries are the largest users of azo dyes. However, 10% of the dyes used fail to bind to the textile fibers thus are released directly into the environment as effluents or sediments [13]. These have toxic effects, and exposure to the biotic environment can cause lethal effects viz., genotoxicity, mutagenicity, and carcinogenicity to the fauna and flora [14]. Aquatic ecosystem gets affected as the intensity of light penetration and gas dissolution in water decreases [15–17]. While in humans, the electron withdrawing azo (N=N) and sulfonic ($-SO_3$) groups present in azo dyes are distributed throughout the body immediately

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after absorption. These groups either exert an action themselves or are subjected to metabolism. Some of the harmful effects reported in humans are urinary bladder cancer (Direct Black 38 (DB 38)), chromosomal aberrations (Acid Violet 7), mutagenic, cytotoxic, genotypic effects, formation of micronuclei, and fragmentation of DNA in human hepatoma cells (Disperse Blue 291), inhibition of function of human serum albumin (Reactive Brilliant Red), and mutagenic (Direct Blue 15) [18–23].

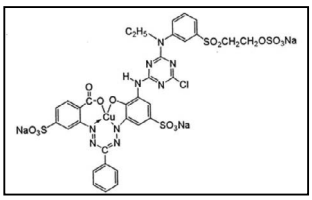
Many physicochemical methods have been employed so far for the decolorization of dyes in wastewater, which include membrane filtration, flocculation, froth flotation, adsorption on activated carbon, ozonation, electrocoagulation, and reverse osmosis. These methods are costly, produce waste material that is difficult to dispose, and have poor efficiency and applicability [24]. The biological and/or combination of biological and physiochemical treatment systems are considered to be much more effective compared with other conventional methods, to remove dye molecules from the water body [4]. This gained a lot of attention because of its cost-effectiveness, simple setup and handling, comparatively low sludge volume, environmental benignity, and has more applications [6,25]. Microbes which include several bacterial, fungal, algal as well as yeast species are able to metabolize azo dyes [26,27]. Bacterial cultures can easily metabolize the electrophilic azo bond of the dye under anaerobic conditions. This results in the production of colorless amines which are more toxic than the parent azo dye [28]. From the early 1970s studies have been carried out on the isolation of pure bacterial cultures such as *Bacillus subtilis*, *Aeromonas hydrophila*, and *Bacillus cereus* capable of degrading azo dyes [29]. However, isolation of a pure bacterial strain from dye containing textile effluent is a tedious process. In some conditions the isolate requires long periods of time before it is capable of utilizing the azo dye as sole substrate. Xenobiotic azo dye degradation is often studied or carried out using mixed culture, that is, bacterial consortium. It has been reported that the degradation rate as well as mineralization rate can be increased to a greater percentage when cometabolic activities within a microbial community complement each other. Jadhav et al. [30] reported that a bacterial consortium consisting of *Galactomyces geotrichum* MTCC 1360 and *Bacillus* sp. VUS efficiently decolorized Brilliant Blue G. Similarly, another bacterial consortium composed of three potential isolates belonging to *Chryseobacterium* and *Flavobacterium* genus was able to decolorize dye mixture better than the individual isolates [31].

In this paper, bacterial consortium and pure bacterial culture were used for the biodecolorization and biodegradation of two azo dyes Reactive Blue 221 (RB 221) and DB 38.

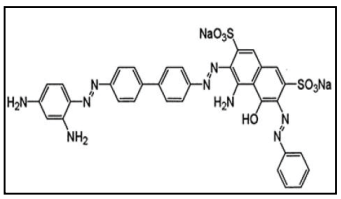
2. Material and methods

2.1. Chemicals

Azo dyes RB 221 and DB 38 in pure form were used for the studies and were collected from a textile industry in Tiruppur, Tamil Nadu (India). All the solvents, chemicals, and media used in this study were obtained from HiMedia Laboratories Pvt. Ltd., Mumbai, India and Sisco Research Laboratories Pvt. Ltd., Mumbai, India.



Reactive Blue 221



Direct Black 38

2.2. Dye stock solution

Stock solutions of the dyes were prepared by dissolving 5 g each of RB 221 and DB 38 in 1,000 mL of deionized water separately. Further working standards were prepared from these stock solutions and used to carry out further experiments.

2.3. Sample collection

Effluent as well as sludge samples were collected aseptically from a textile industry at Ranipet (Latitude 12°56'0"N, Longitude 79°20'0"E), Tamil Nadu, India in sterile containers. The temperature and pH of the area from which the sample has been collected were measured using a laboratory thermometer and a pH meter (Hanna digital pH meter, model-671-p). The collected sample was transported and stored in the laboratory at 4°C for further studies.

2.4. Isolation and screening of dye decolorizing bacteria

Primary isolation from the collected effluent samples was carried out by serial dilution in distilled sterile water up to 10^{-8}, followed by plating of dilutions in Luria Bertani (LB) agar plates and the plates were incubated at 37°C for 24 h [32]. After incubation, isolates with distinct colony characteristics were selected and further purified and maintained on LB agar plates.

Primary screening of these isolates was performed in decolorizing media consisting of (g L^{-1}): yeast extract 2, $Na_2HPO_4{\cdot}2H_2O$ 12.8, KH_2PO_4 3, NaCl 1, and NH_4Cl 1 to identify their decolorizing ability [33]. This is done by inoculating 2% (v/v) of overnight grown cultures in 50 mL of decolorizing media and incubated for 24 h at 37°C. Dye addition was made to the overnight grown culture flasks at a concentration of 5 g L^{-1}. The dye added culture flasks were kept under static as well as shaking conditions (130 rpm) at 37°C in a shaker incubator (Scigenics Biotech Pvt. Ltd., Chennai, India) for another 24 h. The isolates showing effective dye decolorization were selected for further decolorization experiments.

2.5. Decolorization assay

Decolorization assay was carried out in 100 mL of LB broth inoculated with bacterial isolates at 37°C for 24 h. After 24 h, respective dyes, that is, RB 221 and DB 38 were added separately to each flask and incubation was done at 37°C under static conditions. At regular time intervals 3 mL of sample was taken and centrifuged at 10,000 rpm for 10 min. Supernatant was then analyzed by UV–Vis spectrophotometer (Hitachi, U2800) at 601 nm (λ_{max} of RB 221) and 520 nm (λ_{max} of DB 38), respectively. Decolorization rate was

expressed as percentage decolorization and calculated using the following formula:

$$\%\text{Decolorization} = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100$$

2.6. Compatibility test

Compatibility test of organisms degrading RB 221 at comparatively higher percentage has been carried out for the development of consortium. Compatibility analysis was slightly modified from the ones as described earlier [34]. In Muller Hinton agar plates the corresponding organisms were swabbed and streaked against each other and incubated at 37°C for 24 h. After incubation, plates were observed for zone of inhibition.

2.7. Optimization of the rate of dye degradation

Decolorization assay was carried out at various physicochemical parameters to optimize the degradation rate. The different parameters studied were pH (4.5, 7.5, and 10.5), temperatures (28°C, 37°C, and 42°C), salt concentrations (2%, 5%, and 8%), carbon sources (1% each of mannitol, starch, maltose, glucose, and lactose), and nitrogen sources (1% each of yeast extract, malt extract, urea, ammonium nitrate, and ammonium chloride).

2.8. Identification of potential isolates

The potential isolates that were used to construct the consortium were identified and characterized up to the genus level by performing various morphological and biochemical tests according to the *Bergey's Manual of Determinative Bacteriology*. The tests include indole test, Methyl Red-Voges–Proskauer (MR-VP) test, catalase test, oxidase test, urease test, and nitrate test and staining techniques including gram staining, endospore staining, and negative staining were performed. Further, the individual isolate responsible for decolorization of DB 38 was selected for 16S rRNA gene sequencing. Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit on ABI 3730xl Genetic Analyzer. Basic local alignment search tool with the database of NCBI was then used to find highly similar sequences. First 10 sequences with maximum identity score were aligned using multiple alignment software program Clustal W. Distance matrix was generated and the phylogenetic tree was constructed using MEGA 7.

2.9. Extraction and analysis of degraded dye metabolites

Extraction of metabolite was achieved by solvent extraction method, using different solvents such as petroleum ether, butanol, chloroform, ethyl acetate, and acetonitrile. Ethyl acetate showed best results and was further chosen for extraction of degraded dye metabolites. The culture broth after decolorization of RB 221 was collected. The degraded product was obtained by centrifuging the culture broth at 10,000 rpm for 20 min. The supernatant containing the degraded product was collected. It was then extracted using equal amount of ethyl acetate and kept in separating funnel for 24 h. The extracts were collected and then evaporated to dryness for further analysis.

Characterization of the pure dye as well as degraded dye metabolites was done by Fourier-Transform Infrared (FT-IR) analysis (Thermo Nicolet AVATAR 330) in the mid-region of 400–4,000 cm^{-1} with 16 scan speed. The samples for this purpose were prepared by mixing with spectroscopically pure KBr in the ratio of 7:93. Sample holder was used to fix the pellets and then analysis was carried out. For high-pressure liquid chromatography (HPLC) analysis (Waters, Model no. 501) both the pure dye and degraded metabolites were dissolved in HPLC grade carbinol. The samples were then run on a C_{18} column (symmetry 4.6 × 250 mm) with a flow rate of 1 mL min^{-1} and the UV-detector was set at 601 nm for RB 221 and its degraded metabolite, while at 520 nm for DB 38 and its degraded metabolite. The mobile phase used was carbinol/water (50:50, v/v).

2.10. Phytotoxicity assay

Phytotoxicity assay of the degraded metabolite as well as the pure dyes (RB 221 and DB 38) was carried out using the seeds of *Vigna radiata* and *Cicer arietinum*. Different concentration (500 and 1,000 mg L^{-1}) of pure dye and its degraded metabolite were used to treat the seeds and toxicity was analyzed. Seeds treated with distilled water were used as control. The treatment was conducted for a period of 7 d. Sprouted seeds of *V. radiata* were planted in clay pots and their phenotypic characters such as height, color, and numbers of leaves were observed. On the other hand, *C. arietinum* seeds were sprouted and shoots were dissectioned and stained with safranine for analyzing the cell structure abnormalities under microscope (100× oil immersion) (Olympus Opto Systems India Pvt. Ltd., Noida, India).

3. Statistical analysis

All the tests were performed in triplicates and data were analyzed by one way as well as two way analysis of variance (ANOVA) comparison test. The results were established based on probabilities. Results were considered to be significant when probability (*P*-value) was less than or equal to 0.05.

4. Results

4.1. Isolation and screening of bacterial isolates

From the samples collected (industrial effluents and sludge), a sum of 23 morphologically distinct bacterial isolates were recovered. All the 23 isolates were primarily screened for dye decolorization. Among them seven isolates (HB1, HB2, HB 3, HB4, HB5, HB6, and HB12) showed the ability to degrade RB 221, while only one isolate (PB9) showed degrading ability toward DB 38 (Figs. 1(a) and (b)). These isolates were further used in the quantitative decolorization assay.

4.2. Decolorization assay

The maximum absorbance for RB 221 was observed at 610 nm and that for DB 38 was observed at 520 nm. The isolates HB4, HB3, and HB12 showed the highest percentages

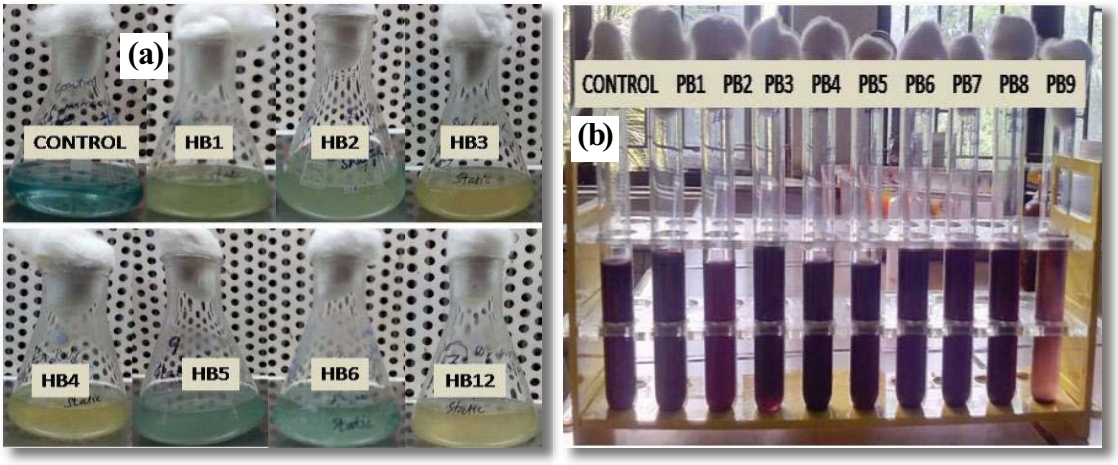


Fig. 1. Primary screening results (a) RB 221 degrading isolates (HB1, HB2, HB3, HB4, HB5, HB6, and HB12); (b) DB 38 degrading isolate PB9.

of decolorization (69.92%, 58.69%, and 55.4%, respectively), followed by HB1, HB2, and HB6 (53.68%, 53.3%, and 41.23%, respectively). The isolate HB5 (31.11%) showed the least decolorization against RB 221. A consortium HBC 3 was created by using the most potent isolates (HB4, HB3, and HB12) for decolorizing RB 221. The consortium HBC 3 decolorized RB 221 up to 74.58% within 24 h under static conditions. Since only PB9 showed the ability to decolorize DB 38, pure culture approach was used to decolorize DB 38. PB9 decolorized 61.55% of DB 38 within 24 h under static condition (Fig. 2).

4.3. Optimization of the rate of dye degradation

The effective degradation of RB 221 by the bacterial consortium HBC 3 was found to occur at a temperature of 42°C, a pH of 7.5, salt concentration of 5%, and with carbon and nitrogen sources as starch and yeast extract, respectively, within 24 h under static conditions (Fig. 3).

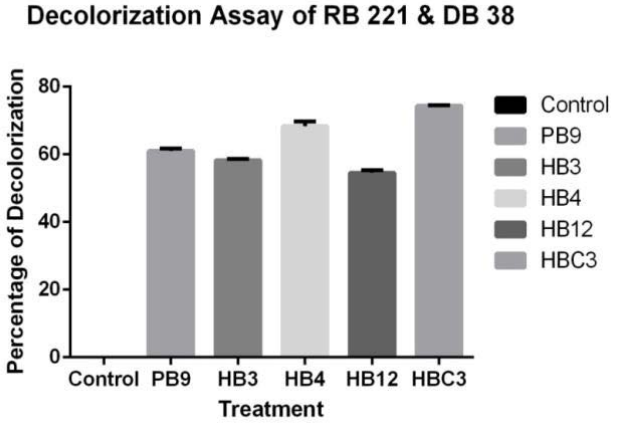


Fig. 2. Decolorization assay results: % decolorization of individual isolate PB9 against DB 38 and % decolorization of consortium HBC 3 as well as individual isolates HB3, HB4, and HB12 against RB 221.

Whereas the effective degradation of DB 38 by PB9 occurred at a temperature of 42°C, pH of 4.5, salt concentration of 5%. and with mannitol and malt extract as carbon and nitrogen sources, respectively, within 24 h under static condition (Fig. 3).

4.4. Identification of bacterial isolates

The identification and characterization of the isolates HB3, HB4, and HB12 as well as PB9 were done by performing gram staining, endospore staining, and biochemical tests (indole test, MR test, VP test, citrate utilization test, nitrate test, catalase test, and oxidase test) according to the *Bergey's Manual of Determinative Biology*. Based on the results obtained, isolates HB3, HB4, and HB12 were tentatively identified to belong to the genus of *Corynebacterium* sp., *Aeromonas* sp., and *Vibrio* sp., respectively (Table 1).

Isolate PB9 was identified using both conventional tests and 16S rRNA sequencing. It was identified to be *Lysinibacillus sphaericus* strain JD1103 (100%). The 16S rRNA gene sequence of this strain is available under the GenBank accession number KY411697 (Fig. 4).

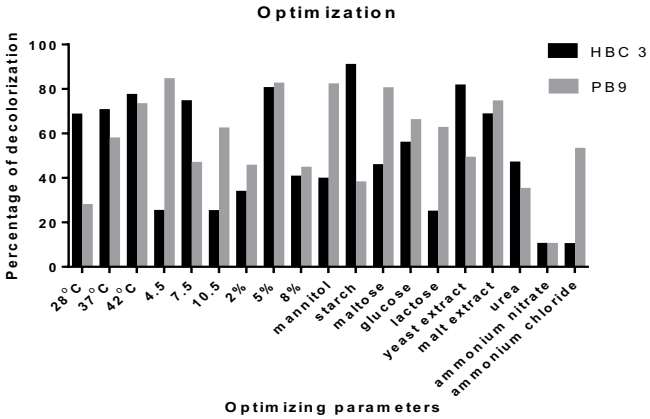


Fig. 3. Optimization of the rate of dye degradation by HBC 3 against RB 221, optimization of the rate of dye degradation by PB9 against DB 38.

Table 1
Biochemical tests and staining results

Test	HB1	HB4	HB12	PB9
Gram staining	+	−	−	+
Endospore staining	−	−	−	−
Indole test	−	−	−	−
Methyl Red test	−	+	−	−
Voges Proskauer test	−	−	−	−
Catalase test	+	+	−	−
Oxidase test	+	+	+	+
Nitrate reduction test	+	−	−	+
Citrate utilization test	−	−	−	−

4.5. Analysis of degraded dye metabolites

4.5.1. FT-IR analysis

Based on FT-IR analysis, pure dye RB 221 showed peaks at 3,423.11 cm^{-1} representing O–H group, 2,926.41, 1,600.60, and 994.41 cm^{-1} represented CH_2 group, C=C stretching, and =CH out of plane vibration, respectively. Peak at 1,142 cm^{-1} corresponded to C–OH stretching vibration. Peaks at 1,252.30 and 1,357.51 cm^{-1} indicated aromatic primary as well as secondary amine stretch (C–N), respectively. Azo group (N=N) showed peak at 1,398.42 cm^{-1}. Aliphatic chloro compound (C–Cl) stretch was observed at 735.21 cm^{-1}, while S=O stretching vibration was observed at 1,040.22 cm^{-1} (Fig. 5(a)). However, a marked difference of peak formation was seen in the FT-IR spectrum of degraded dye metabolite of RB 221. The O–H stretch was shifted to 3,525.88 cm^{-1} and occurred at a low intensity, while the C=C stretching occurred at 1,641.42 cm^{-1}. C–OH stretching occurred with a low intensity at 1,118.71 cm^{-1} and S=O stretching vibration occurred at 1,060.85 cm^{-1}. There was absence of many peaks which was present in the FT-IR spectrum of RB 221. Absence of peaks at 1,252.30, 1,357.51, and 735.21 cm^{-1} indicated the absence of aromatic primary and secondary amine groups and aliphatic chloro compound, respectively. Most importantly, disappearance of azo bond (N=N) peak at around 1,398.42 cm^{-1} primarily confirms the degradation of RB 221 (Fig. 5(b)).

Similarly, FT-IR spectrum of pure dye DB 38 showed O–H stretching at 3,425.76, 1,498.68, and 1,177.25 cm^{-1} corresponded to C=C–C aromatic ring stretching and C–OH stretching, respectively. Aromatic primary amine (C–N) stretch was observed at 1,344.78 and 1,326.74 cm^{-1}, while secondary amine NH bend occurred at 1,612.27 and 1,571.79 cm^{-1}. Azo (N=N) group was represented at 1,571.59 cm^{-1}, and S=O stretching was observed at 1,043.96 cm^{-1} (Fig. 6(a)). Several changes in peak formation occurred in case of degraded dye metabolite

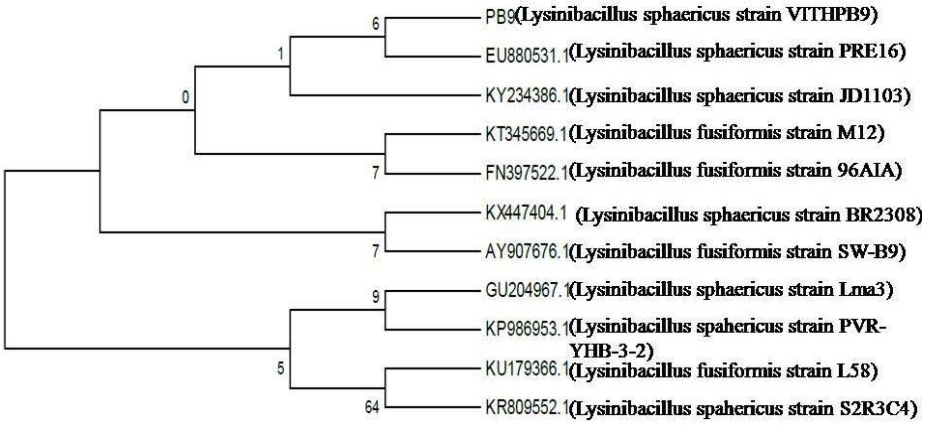


Fig. 4. Phylogenetic tree of *Lysinibacillus sphaericus* strain VITHPB9.

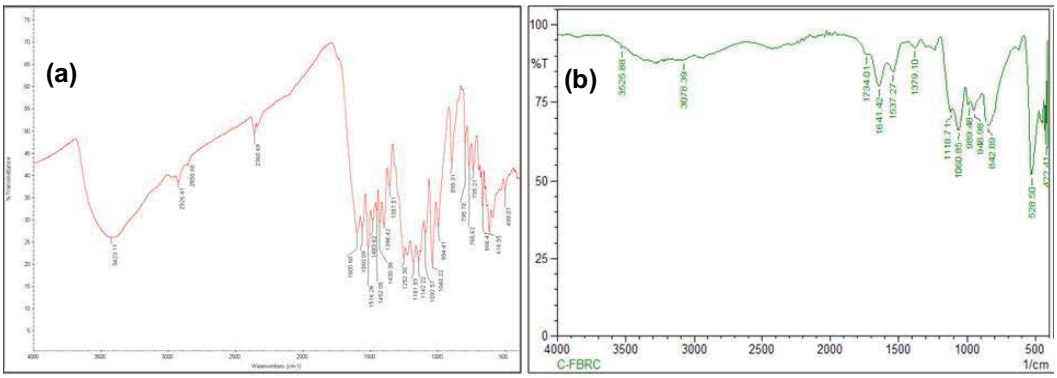


Fig. 5. FT-IR spectra of (a) pure dye RB 221; (b) degraded dye metabolite of RB 221 by HB3.

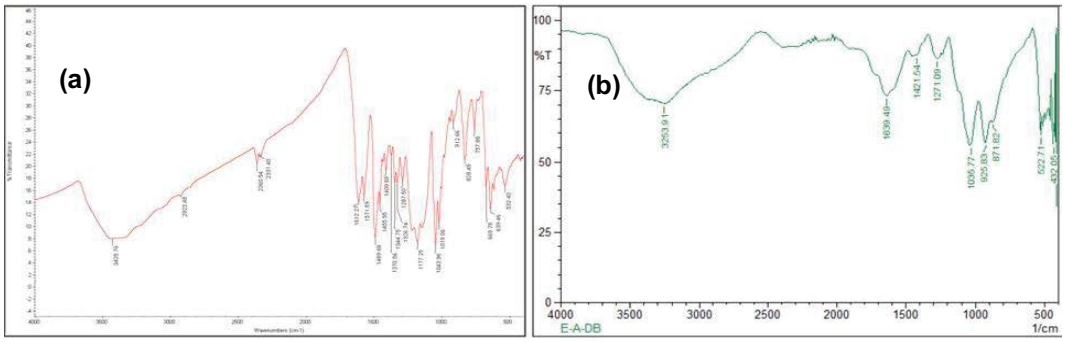


Fig. 6. FT-IR spectra (a) pure dye DB 38; (b) degraded dye metabolite of DB 38 by PB9.

of DB 38. O–H stretch was observed at 3,253.91 cm^{-1}, aromatic C–C stretch was shifted to 1,421.54 cm^{-1}. Secondary amine NH bend occurred at 1,639.49 cm^{-1}, while the aromatic primary amine peak was observed at 1,271.09 cm^{-1}. S=O stretch was represented at 1,035.77. However, there was absence of peaks at 1,498.68 and 1,177.25 cm^{-1} representing C=C–C aromatic ring stretch and C–OH stretching, respectively. Also, azo (N=N) bond was absent at around 1,571.59 cm^{-1}. Therefore, considerable changes occurred in compound structure of degraded dye metabolite of DB 38 confirming the degradation of pure dye DB 38 (Fig. 6(b)).

4.5.2. HPLC analysis

HPLC analysis shows that the peaks found in the pure dye chromatogram of both RB 221 and DB 38 are not present in the degraded metabolite HPLC graph of both the dyes, respectively. Hence, it can be concluded that the toxic compounds present in the dye is no longer present in the degraded metabolite and is further converted to abundant number of other compounds which are represented by the numerous small peaks in degraded metabolite chromatograms of both RB 221 and DB 38, respectively (Figs. 7 and 8).

4.6. Phytotoxicity assay

Phytotoxicity assay was conducted to analyze the toxic effects of azo dyes compared with their respective degraded dye metabolites. Alterations in the phenotypic characters of *V. radiata* plants and *C. arietinum* seeds were studied. Control plants of *V. radiata* showed the highest growth and most number of leaves. Plants treated with both the pure dyes (RB 221 and DB 38) showed stunted growth and less number of leaves. Higher concentration (1,000 mg L^{-1}) showed less growth compared with the lower concentration (500 mg L^{-1}). Color of the leaves was also found to be changed from green to yellowish under pure dye treatment. However, plants treated with degraded dye metabolites (RB 221 degraded by HBC 3 and DB 38 degraded by PB9) showed rapid growth and more number of leaves in comparison with the plants treated with pure dye, with 500 mg L^{-1} of degraded metabolite showing more growth than those treated with 1,000 mg L^{-1} degraded metabolite (Table 2). Values taken were mean of three experiments ± SEM, and were found to be significantly different from the control experiment at $P < 0.05$, by one-way ANOVA.

Sprouted seeds of *C. arietinum* showed similar results. Seeds treated with degraded dye metabolites (500 and 1,000 mg L^{-1}) of RB 221 and DB 38 as well as control seeds showed 100% germination and uniform, healthy shoot growth. Microscopic observation revealed healthy shoot cells with proper shape. On the other hand, seeds treated with pure dyes RB 221 and DB 38 (500 and 1,000 mg L^{-1}) showed lower germination rate and the shoot growth was not uniform. Microscopic observation revealed abnormal cells with distorted shape (Fig. 9).

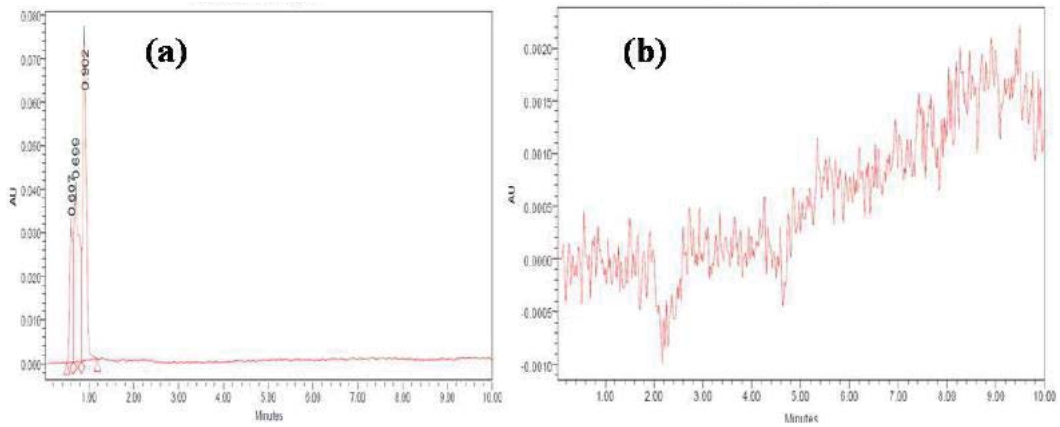


Fig. 7. HPLC chromatogram (a) Pure dye RB 221; (b) Degraded metabolite of RB 221.

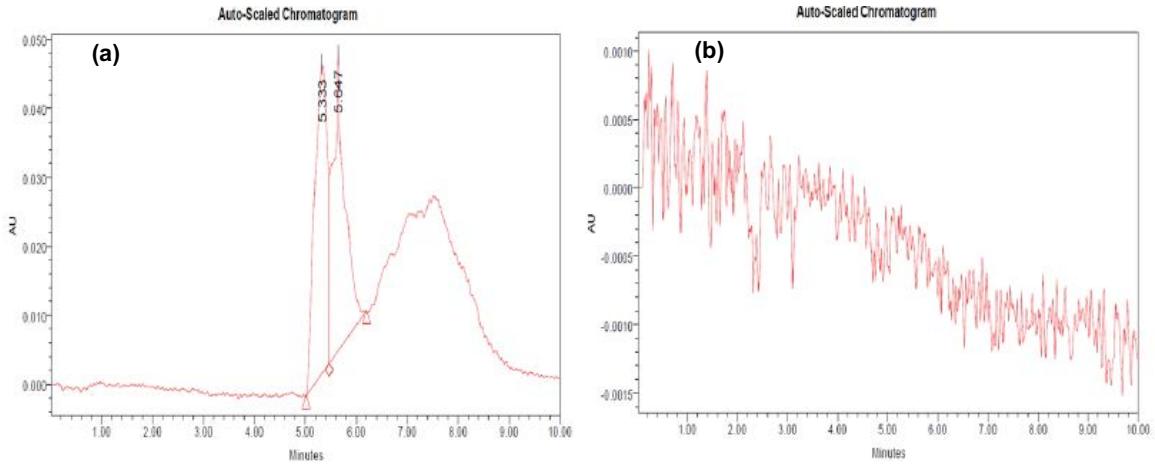


Fig. 8. HPLC chromatogram (a) Pure dye DB 38; (b) Degraded metabolite of DB 38.

5. Discussion

More than 200,000 tonnes of textile dyes are being discharged each year into the water bodies. These contribute largely to the higher pollution rate after industrial revolution [35,27]. Biological degradation of these dyes has proven to be more reliable, eco-friendly, and cost-effective compared with other methods known so far. A large number of organisms has been studied and experimentally proven to be effective against azo dye degradation. Among which bacteria is more studied and has been found to be more effective compared with other organisms. The bacterial strains for degradation can be isolated from the industrial effluents itself, as it has high concentration of the particular dye, to which the organisms are tolerant and can survive in it. Textile wastewater has been used for the isolation of dye decolorizing bacteria as reported by Das and Mishra [36]. They isolated two dye decolorizing bacteria showing activity against reactive green-19. Similarly in another study five dyes decolorizing bacterial isolates were obtained from water and soil samples of a waste disposal site of handloom industry [37]. The study focuses on the same aspect, that is, isolation of effective dye degrading bacteria from the industrial effluent and sediment samples. A total of 23 bacterial isolates were recovered from the samples. Among them seven bacterial isolates decolorized RB 221, while only one bacterial isolate is active in the decolorization of DB 38. Two different approaches, that is,

Table 2
Phenotypic parameters of *Vigna radiata* observed for phytotoxicity assay

Plant treatment	Day 7	
	Plant height (cm)	Number of leaves
Distilled water	20.5 ± 0.764	6.667 ± 0.667
Reactive Blue 221 (1,000 ppm)	12.667 ± 0.441	1.333 ± 0.333
Reactive Blue 221 (500 ppm)	14.167 ± 0.219	1.667 ± 0.333
Degraded product of Reactive Blue 221 (1,000 ppm)	17.1 ± 0.208	2.667 ± 0.333
Degraded product of Reactive Blue 221 (500 ppm)	19.567 ± 0.233	4.667 ± 0.333
Direct Black 38 (1,000 ppm)	13.2 ± 0.153	1.333 ± 0.333
Direct Black 38 (500 ppm)	14.767 ± 0.145	2.333 ± 0.333
Degraded product of Direct Black 38 (1,000 ppm)	16.2 ± 0.252	3.667 ± 0.333
Degraded product of Direct Black 38 (500 ppm)	18.567 ± 0.233	4.333 ± 0.333

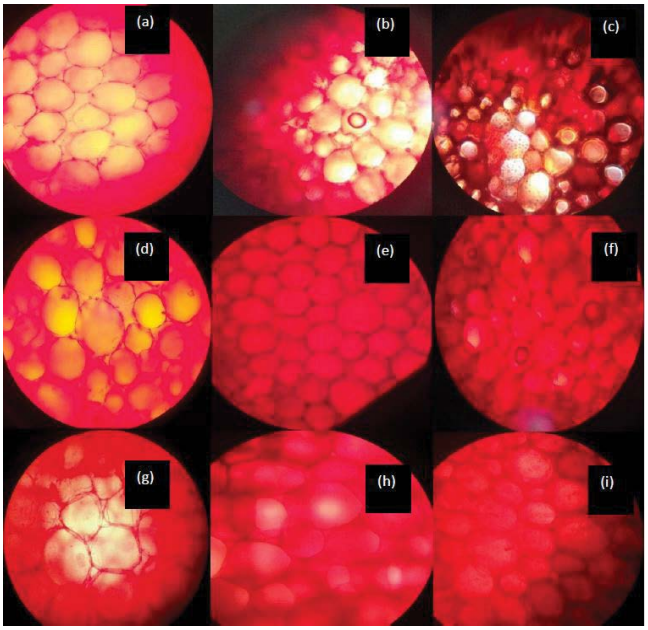


Fig. 9. Microscopic field observation of shoot cells (*Cicer arietinum*) under 100×. (a) Treatment with distilled water, (b) seeds treated with RB 221 (500 ppm) dye, (c) treatment with RB 221 (1,000 ppm) dye, (d) treatment with degraded sample of RB 221 dye (500 ppm), (e) treatment with degraded sample of RB 221 dye (1,000 ppm), (f) treatment with DB 38 (500 ppm) dye, (g) treatment with DB 38 (1,000 ppm) dye, (h) treatment with degraded sample of DB 38 dye (500 ppm), and (i) treatment with degraded sample of DB 38 (1,000 ppm).

bacterial consortia and bacterial pure culture were used for treating two different dyes RB 221 and DB 38. The bacterial consortium HBC 3 composed of *Corynebacterium* sp., *Aeromonas* sp., and *Vibrio* sp. used here decolorized 74.58% of RB 221 within 24 h, which is much higher than the individual decolorization percentages of these organisms. Previous studies have reported that development of consortium has been more effective compared with individual organisms [36,38,39]. One study reported that a consortium developed with four bacterial cultures, namely *Bacillus flexus* strain NBN2, *B. cereus* strain AGP-03, *Bacillus cytotoxicus* NVH 391-98, and *Bacillus* sp. L10 decolorized 97.57% of Direct Black 151 and Direct Red 31 to 95.255% within 5 d [40]. Although, this study reported a higher percentage of decolorization but the time needed for decolorization was much greater than the present study. Another study reported that a bacterial consortium consisting of *Microbacterium* sp., *Leucobacter albus*, *Klebsiella* sp., and *Staphylococcus arlettae* decolorized Disperse Red 1 to 80% in 72 h [41]. The percentage decolorization in this case was almost comparable with the present study but the time taken for decolorization was much higher. The decolorization and degradation may be because of an unknown mechanism or a biochemical reaction which happens when these three organisms are grown together. In a consortium the different bacterial isolates attack the dye molecule at different positions resulting in higher rate of dye degradation. On the other hand, 61.55% of DB 38 was decolorized by only *L. sphaericus*. Thus, pure culture technique was used for effective decolorization of DB 38. Dye degradation using pure culture has the advantage of giving reproducible data and also it is easier to study the degradation mechanism in detail. There were several studies that reported the decolorization of reactive dyes by pure bacterial cultures including *Staphylococcus hominis* against Acid Orange [42], *Lysinibacillus* sp. against Remazole Red [25], *Rhizobium radiobacter* MTCC 8161 against Reactive Red 141 [43], *Pseudomonas vulgaris* against Scarlet RR, Navy blue HE2R, *Exiguobacterium* sp. RD3 against Navy blue HE2R [44] and isolated bacterium KMK48 against various sulfonated reactive azo dyes [45]. It is observed decades ago that by optimizing physical and chemical parameters, the efficiency of bacterial metabolism increases to a high level. Optimization of physical and chemical parameters increases the rate and percentage of degradation of azo dyes [46]. In 2016, Lalnunhlimi and Krishnaswamy conducted one study which showed the importance of different physical and chemical parameters including pH, temperature, carbon, and nitrogen sources on dye degradation. The study showed maximum decolorization by the consortium at a pH of 9.5, temperature 36°C, sucrose as carbon source, and yeast extract as nitrogen source. Similarly, in another study *Micrococcus luteus* strain SSN2 showed maximum decolorization of Direct Orange 16 at pH 8, 38°C, 3% NaCl under static conditions [47]. When different parameters were optimized, the decolorization rate increased to a greater percentage for both the dyes RB 221 and DB 38, and complete decolorization has been observed within 24 h under static conditions. Further, different analytical techniques such as FT-IR and HPLC confirmed the degradation of the dye molecule. In order to successfully apply these treatment methods for dye containing toxic industrial waste remedy, it is of utmost importance to analyze the toxicity of pure dye and the degraded metabolite. Previously, it has been observed that degraded dye metabolite can be more toxic than the primary supplement, that is, the dye in this case [4]. Different bioassays have been performed utilizing microorganisms, plants, animals in order to demonstrate the detoxification of the degraded dye metabolites. However, plant bioassays are more useful as plants and mammals have similar chromosomal morphologies, respond similarly to mutagens, less time consuming, and economical to use. Several studies have been carried out so far to determine the toxicity of degraded dye metabolites using plant bioassays. Kuberan et al. [48] conducted phytotoxicity assay using seeds of Paddy ADT43 to determine the nontoxic nature of degraded dye metabolites. Similarly, Saratale et al. [25] used plants such as *Sorghum vulgare* and *Phaseolus mungo* to assess the toxic effects of the parent dye as well as its degraded metabolites [25]. In this study toxicity of RB 221 was conducted with plants of *V. radiata* and *C. arietinum*. The phytotoxicity study in *V. radiata* plants showed that degraded dye metabolites were less harmful than pure dye, when phenotypic characters of the plant such as plant height and number as well as color of leaves were taken into account. The phytotoxicity study with *C. arietinum* showed similar results and dye was more toxic, whereas the degraded metabolite was less toxic with less cell damage.

6. Conclusion

This study mainly focused on the biodegradation and decolorization of azo dyes using indigenous bacterial isolates which were obtained from the industrial effluent and sludge samples. The organisms studied, proved to be efficient in degrading industrially used azo dyes, RB 221 and DB 38. A consortium HBC 3 was constructed from three bacterial isolates which showed effective degradation of RB 221. The isolates were compatible with each other and showed a higher percentage of decolorization of RB 221 greater than any of the individual isolates alone. On the other hand, only isolate PB9 was observed to decolorize DB 38 effectively. The bacterial isolates used to form the consortium HBC 3 belonged to the genera of *Corynebacterium* sp., *Aeromonas* sp., and *Vibrio* sp., while PB9 was identified to be *L. sphaericus* strain VITHPB9. Further the metabolites formed after dye degradation of both the dyes were found to exert nontoxic effects, when phytotoxicity assay was conducted with seeds of *V. radiata* and *C. arietinum,* respectively. Thus, this biological treatment process including the consortium HBC 3 and pure culture of *L. sphaericus* strain VITHPB9 has immense potential to be used in the industrial bioremediation purposes after testing in industrial scale bioreactors.

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