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# Optimization of parameters for decolorization of a textile azo dye, Remazol Black B (RBB) by a newly isolated bacterium, *Bacillus thuringiensis* BYJ1

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*Bacillus thuringiensis* BYJ1, a newly isolated bacterium from textile effluent contaminated soil was exploited to decolorize a textile azo dye, Remazol Black B (RBB). Various process parameters like initial RBB concentration, temperature, pH and cultural conditions on the process of dye decolorization were studied in order to determine optimum condition. Decolorization process was largely affected under aerobic process. Despite the toxic and inhibitory effects of dye, *B. thuringiensis* BYJ1 was able to tolerate as high as 1200 mg L<sup>-1</sup> of RBB. Optimum decolorization of RBB was achieved at 37°C and pH 7 under static culture condition. Decolorized dye products were analyzed by thin layer chromatography (TLC), UV-visible scanning and fourier transform infrared spectroscopy (FTIR) analysis. Seed germination assay was performed to analyze the effect of decolorized dye products. The plasmid isolation and its curing from *B. thuringiensis* BYJ1 was performed to study effect of plasmid on the process of biodegradation of RBB.

Key words: Azo dye, decolorization, immobilization, plasmid curing, Bacillus thuringiensis.

#### INTRODUCTION

Application of metabolic potentials of microbes in remediation of polluted sites is an efficient, effective, economic and eco-friendly way to solve the pollution related environmental problems. In bioremediation, we utilized microorganisms and/or their enzymes as tools to degrade both natural and anthropogenic materials in wastewater digesters, composters, landfills, natural terrestrial environments and natural or artificial aquatic ecosystem as an advanced bioremediation technology.

Synthetic dyes are vital for the textile dyeing and printing industries. Amongst all synthetic dyes, azo dyes are the most common, being used up to 90%. There are more than 8000 chemical products associated with the dyeing process listed in the Colour Index (Society for Dyes and Colourist, 1976) while more than 1,000,000 commercially available dyes exist with more than 7 x  $10^5$ 

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metric tons of dyestuff produced annually (Meyer, 1981; Zollinger, 1987). During dying process, almost 10-15% of the dyes are lost as component of textile effluent (Vaidya and Datye, 1982) and due to this, the pollution by dyewaste water has reached an alarming level. There are several dyes that exhibit toxic and mutagenic effect on aquatic as well as other living systems (Brown and Stephen, 1993). Sewage treatment plants that can deal with different kinds of sewage have been developed; however, technology is still unable to treat many chemicals. More worrying is the fact that current legislation only governs the amount of acidity, alkalinity, COD, amount of biochemical oxides of industrial effluents, but not the dye concentrations and extent of degradation of the compounds.

Azo dyes are the most important commercial colorants because of their wide color range, good fastness properties and pictorial strength as compared to anthraquinones dyes, the second most important group of the dyes. These include several structural varieties of dyes, such as acidic, reactive, basic, disperse, azo, di-azo, anthraxquinone-based and metal-complex, etc. The only thing in common is their ability to absorb light in the visible region of electromagnetic radiations.

The dye can be primarily removed from the textile effluent by adsorption (biosorption), precipitation and by microbial biotransformation and/or biodegradation in different conditions (Brown and Laboureur, 1983; El-Gundi, 1991). There are reports on bioremediation of textile azo dyes (reactive and disperse dyes) Direct Blue, Procion Navy Blue, Procion Green and Supranol Red by a bacterial consortium consisting of known strains of Pseudomonas and Bacillus (Asgher et al., 2007; Abraham et al., 2004). The mechanism and kinetics of process of azo dve biodegradation by a microbial consortium consisting of bacteria and white rot fungus was studied by Fang et al. (2004). This may help to manipulate the pathway of biodegradation or increase the rate of reaction by manipulating the kinetics parameters. There are various reports of microbial degradation of textile and laboratory dyes by fungi, actinomycetes, yeast, algae and bacteria (Patricia et al., 2005; Xu et al., 2007). Still the urge of researchers to find the new strains of bacteria with dye decolorizing capacities has not come to an end. The dye decolorizing capacity of indigenous bacteria, like Bacillus sp., Klebsiella, Planococcus SD.. Pseudomonas aeruainosa and Pseudomonas fluorescens, were optimized to generate effective process to degrade different dyes (Joe et al., 2011; Mohan et al., 2013; Bhatnagar et al., 2013; Shah et al., 2013). Recently, the area of focus in the research is to find or design the group of bacteria involved collectively in the textile dyes degradation as a co-metabolism approach of a natural process and consortium studies at the laboratory levels (Mahmood et al., 2012; Neelambari et al., 2013).

The development of a large scale establishment ofbiore-

mediation technology which should be efficient, economic and fast is the current demand of the developing countries like India.

The present study was focused on isolation, screening and identification of textile dye decolorizing bacteria from contaminated sites. Attempts were made to check the decolorization efficiency under free cells and immobilized cell condition. Process parameters were tested in order to optimize the condition of dye decolorization by isolated bacteria.

#### MATERIALS AND METHODS

#### Sample collection

Soil and effluent samples were collected from the dying and printing industry effluent release sites of Vatava, Ahmedabad, Gujarat, India. The Vatava GIDC is one of the largest industrial area of Gujarat state hosting more than 1200 dye manufacturing units. Autoclaved sampling bags were used to collect soil samples stored at 4°C till used.

#### Dyes, culture media and chemicals

The Remazol Black B (RBB -  $\lambda$ max 595nm) was procured from local dye manufacturing industries situated at Vatava, Ahmedabad, Gujarat (India). Other chemicals and medium components used in this study were of analytical and molecular grades.

Nutrient Broth (N-broth) purchased from Hi-Media, Mumbai consisted of (g L<sup>-1</sup>): Peptone (5), yeast extract (1.5), beef extract (1.5), NaCl (5) with pH 7.4  $\pm$  0.2. Nutrient Agar (N-agar) purchased from Hi-Media, Mumbai consisted of (g L<sup>-1</sup>): Peptone (5), yeast extract (1.5), beef extract (1.5), NaCl (5), Agar (15) with pH 7.4  $\pm$  0.2. Minimal salt medium (MSM) consisted of (g L<sup>-1</sup>): KH<sub>2</sub>PO<sub>4</sub> (2), K<sub>2</sub>HPO<sub>4</sub> (0.8), MgSO<sub>4</sub>.7H<sub>2</sub>O (0.1), NaCl (0.1), FeSO<sub>4</sub>.7H<sub>2</sub>O (0.02), NH<sub>4</sub>NO<sub>3</sub> (1.0), CaCl.<sub>2</sub>H<sub>2</sub>O (0.02) and glucose (0.5). The pH of the medium was adjusted to 7.0  $\pm$  0.2.

A stock solution of RBB (1% w/v) was prepared and autoclaved at 121°C and 15 lbs for 10 min and stored at 4 - 8°C temperature. Required dilutions were prepared from this stock for further use.

#### Enrichment and isolation of dye decolorizing bacteria

Isolation of dye decolorizing bacterial was carried out by inoculating 1 g of contaminated soil in 500 ml of Erlen Meyer flask consisting 200 ml of MSM supplemented with RBB (500 mg L<sup>-1</sup>) as a sole carbon source. The flasks were incubated on orbital shaker at 120 rpm for 30 days. After every 5 days of incubation, a loop-full of enriched medium was streaked on N-agar plates and incubated at 37°C for 24 to 72 h. Simultaneously, 1 ml of enriched culture was transferred to fresh MSM medium supplemented with RBB as described earlier. Such serial transfer was performed for 30 days. Isolation of bacterial strain was performed by successive streaking on nutrient agar plate supplemented with RBB till single isolated pure colony was obtained.

#### Screening of dye decolorizing bacteria

The primary screening for dye decolorization ability of all bacterial isolates was carried out using solid MSM-agar containing RBB

(500 mg L<sup>-1</sup>). The actively growing cultures of individual isolates were spotted in the center of the agar plates and the plates were incubated at  $37^{\circ}$ C for 48 h. The organisms which showed bigger zone of decolorization were subjected to secondary screening process.

The secondary screening process was performed in liquid medium. Overnight grown culture of 0.5 OD (1%) was used to inoculate 100 ml N- broth supplemented with Remazol Black B dye (100 mg L<sup>-</sup>). The inoculated flasks were incubated under static conditions for 96 h at 37°C. Samples were withdrawn every 12 h from the flasks and decolorization assay was performed to determine the decolorization activity.

## Identification and phylogenetic analysis of *B. thuringiensis* BYJ1

The isolated dye decolourizing bacterium BYJ1 was identified by 16S rRNA gene sequence analysis. Genomic DNA was isolated from the pure culture pellet. The ~1.5 kb 16S rRNA gene fragment was amplified by high-fidelity PCR polymerase using consensus primers. The PCR products were cloned in plasmid and plasmid DNA was sequenced bi-directionally using the forward, reverse and an internal primer specific for bacterial domain. Sequence data was aligned and analyzed for finding the closest homologous for the query sequence. The nucleotide sequence analysis of the sequence was performed by BLASTN (Basic Local Alignment sequence Tool) site at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST). The alignment of the sequences was performed by using CLUSTALW program European **Bioinformatics** at site (www.ebi.ac.uk/Tools/clustalwomega) using the neighbor joining algorithm. A phylogenetic tree was constructed from the alignment by average distance of the percentage identity, using Jalview. A second clustering was done with the MEGA 5.2 software, with the same algorithm. After 500 iterations of bootstrapping, the BYJ1 strain was clustered in the Bacillus group with a bootstrap value of 98 (98% repeatability of the clustering). The sequence was refined manually after crosschecking with the raw data to remove ambiguities and was submitted to the NCBI.

#### Decolorization assay

The samples collected from all reaction mixtures were centrifuged at 8000 xg, 4°C, 20 min (REMI, RM-1214) and supernatants were used to determine the percentage decolorization. The decolorizing activity was determined by monitoring the decrease in absorbance at 595 nm ( $\lambda_{max}$  of RBB). The un-inoculated flasks containing dye was used as control. Decolorization activity (%) was calculated as: Decolorization activity (%) = [(A – B)/ A] x 100. Where, A = initial absorbance and B = observed absorbance.

## Effects of process parameters on decolorization of RBB by *B. thuringiensis* BYJ1

#### Effects of pH and temperature on decolorization of RBB

Nutrient broths containing 100 mg L<sup>-1</sup> RBB were prepared in 250 ml Erlenmeyer flask using different buffer solutions. The buffers selected for media preparation were 0.1 M citrate buffer (pH 4 and 5), 0.1 M sodium phosphate buffer (pH 6 and 7) and 0.1 M Tris buffer (pH 8, 9 and 10). Fresh nutrient broths were inoculated with actively growing culture of *B. thuringiensis* BYJ1 (1% v/v) and incubated under static culture conditions.

Nutrient broth with pH 7 was used for testing effect of temperature on the process of dye decolorization. Young culture of *B. thuringiensis* BYJ1 was inoculated in 100 ml of nutrient broth containing 100 mg L<sup>-1</sup> RBB and incubated at 10, 20, 30, 37, 40, 50 and 60°C temperature under static culture conditions. The decolorization activity for both sets was monitored at 0, 24, 48 and 72 h of incubation.

#### Effects of dye concentrations

Various concentrations of RBB (50, 100, 200, 400, 600, 800, 1000 and 1200 mg L<sup>-1</sup>) were prepared in different reaction mixture flasks and inoculated with actively growing culture of *B. thuringiensis* BYJ1 (1% v/v). The flasks were incubated at 37°C and incubated under static culture condition. Decolorization activity was measured at 0, 24, 48 and 72 h of incubation time as described above.

## Biodegradation of RBB and analysis of the transformed dye products

Nutrient broths containing RBB (100 mg L<sup>-1</sup>) inoculated with actively growing culture of *B. thuringiensis* BYJ1 (1% v/v) were incubated at 37°C under static and shaking culture conditions (120 rpm) for 96 h. The decolorized dye samples were harvested after every 12 h and percentage decolorization was calculated. Samples harvested after 96 h were subjected to different analysis.

#### UV-visible spectroscopic analysis

The supernatants were scanned in Picodrop® UV-Visible spectrophotometer at Department of Biochemistry, Anand Agriculture University, Anand, Gujarat, India.

#### FTIR and TLC analysis of the transformed dye products

The cell free supernatants obtained after decolorization under static and agitated culture conditions were extracted with equal volume of ether and dried. The concentrated samples were dissolved in methanol and used for FTIR analysis. The FTIR analysis of extract was performed using standard potassium bromide solution.

The samples were analyzed on TLC plates (MerK. GaA - 64271, Darmstadt, Germany). The solvent system used consisted of nbutanol : acetic acid : water (4:2:4 v/v). The resolved chromatogram was observed under natural light, short wave length UV (254 nm) and long UV (365 nm). The R<sub>f</sub> values of the dye spot and newly immersed spots were calculated using following formula:

R<sub>f</sub> = Distance traveled by solute/distance traveled by solvent

#### Seed germination study

The effect of dye and its degradation products on seed germination was analyzed by irrigating seeds with decolorized dye under static and agitated culture conditions. Seeds of mung (*Phaseolus mungo*) and groundnut (*Arachis hypogaea*) were surface sterilized by 0.1 % w/v HgCl<sub>2</sub> solution and rinsed in sterile distilled water to remove residual HgCl<sub>2</sub>. The seeds were incubated in sterile tubes and were irrigated with different treated dye supernatants of static and shaking samples along with the water and Remazol Black B dye (100 mg L<sup>-1</sup>) solution as controls for 13 days.



Figure 1. Decolorization of Remazol Black B by B. thuringiensis BYJ1.

#### COD removal and decolorization of RBB

Chemical oxygen demand (COD) analysis was carried out as per Standard Methods for the Examination of Water and Wastewater, APHA (Andrew et al., 1999). 250 ml Erlenmeyer flask containing 100 ml N-broth with RBB (100 mg L<sup>-1</sup>) was inoculated with 1% v/v inoculum of *B. thuringiensis* BYJ1 and incubated at 37°C under static culture condition for 96 h. Inoculated (media + BYJ1) and uninoculated (media + RBB) controls were also included. Samples were harvested at intervals and analyzed for decolorization.

#### Interaction of RBB and B. thuringiensis BYJ1

Erlenmeyer flask (250 ml) containing 100 ml Nutrient broth supplemented with RBB (100 mg L<sup>-1</sup>) was inoculated with 1% v/v actively growing culture of *B. thuringiensis* BYJ1 and incubated at 37°C. After 24 h incubation under agitated culture condition, the broth was analyzed by scanning electron microscopy to examine the interaction of RBB with *B. thuringiensis* BYJ1.

## Decolorization of RBB by immobilized cells of *B. thuringiensis* BYJ1

*B. thuringiensis* BYJ1 was encapsulated in alginate beads and its efficiency for RBB decolorization was measured. The alginate beads were prepared using sterile solutions of sodium alginate (3% w/v), calcium chloride solution (5% w/v) and young culture of *B. thuringiensis* BYJ1 (1.6 x  $10^7$  CFU/ml). Nutrient broth containing RBB (100 mg L<sup>-1</sup>) was inoculated with beads of immobilized *B. thuringiensis* BYJ1.

#### Role of B. thuringiensis BYJ1 plasmid in decolorization of RBB

The bacterial isolates were grown in the presence and absence of dye Remazol Black B (100 mg  $L^{-1}$ ) in N-Broth for 24 h at 37°C. The cell pellets were obtained from the samples collected from both the reaction mixture.

The plasmids extraction was performed by modified alkali lysis methods from the culture grown in the presence and absence of dye. The isolated plasmid samples were subjected to agarose gel electrophoresis. The process was performed using 1% w/v agarose, TAE buffer and ethidium bromide (EtBr) to stain the gel for the visualization of plasmid DNA (Sambrook and Russell, 2001). *Plasmid curing of B. thuringiensis BYJ1 using acridine orange* 

The activated culture of *B. thuringiensis* BYJ1 was used for plasmid curing. The 100 ml sterile N-broth was prepared in which acridine orange was added at a final concentration of 100  $\mu$ g/ml. The media was inoculated with activated culture of *B. thuringiensis* BYJ1 (1 % v/v) and was incubated at 37°C for 48 h. After 48 h incubation, 100  $\mu$ l of sample was withdrawn and serial dilution was carried out. The diluted samples were spread N-agar plates and plates were incubated at 37°C for 24 h. After 24 h, a replica of all the plates with isolated colonies were prepared using N-agar with Ampicillin (10  $\mu$ g) plates and replica plates were incubated at 37°C for 24 h. After at 37°C for 24 h. After incubation, the antibiotic plates were compared with master plates and colonies which were absent on antibiotic plates were selected from master plate.

The plasmid cured colonies were subjected to plasmid isolation and agarose gel electrophoresis for conformation.

#### **RESULTS AND DISCUSSION**

#### Screening and isolation

seventy nine different types of bacterial strains were isolated through enrichment techniques. Among these 79 isolates, few showed less and some showed moderate decolorization activity during the screening procedures. A few of them decolorized dyes completely. The bacterial isolate C49 was one of them and the complete decolorization of Remazol Black B was achieved in 96 h (Figure 1).

#### Molecular identification of BYJ1

Based on 16S rRNA gene nucleotides homology and phylogenetic analysis, the bacterium, BYJ1 (Gene Bank



Figure 2. Phylogenetic tree of B. thuringiensis strain BYJ1.



Figure 3. Effect of pH on the decolorization process of RBB by *B. thuringiensis* (BYJ1).

Accession Number: KF145205; Bacillus thuringiensis BYJ1) was identified as *B. thuringiensis*.

Genomic DNA was isolated from the pure culture and using consensus primers, the ~1.5 kb 16S rDNA fragment was amplified using *Taq*DNA Polymerase. The PCR product was bi-directionally sequenced using the forward, reverse and an internal primer; sequence data was aligned and analyzed for finding the closest homologs for the microbe. The phylogenetic tree was drawn to scale, with the branch length in the same unit as those in evolutionary distance (Dhanve et al., 2009). The phylogenetic tree of *B. thuringiensis* BYJ1 (Figure 2) shows that the nearest homologous species was found to be *Bacillus anthracis* (Accession No. NR\_074453).

#### Effects of pH and temperature on decolorization of RBB

The pH is one of the important factors affecting the metabolic activities of the organisms. As shown in Figure 3, the dye decolorization activity of *B. thuringiensis* BYJ1 was optimum at pH 7.0. However, *B. thuringiensis* BYJ1 could resist and retain its ability to decolorize Remazol Black B at pH 8.0 also. This is promising feature because in India, most of the dye or textile effluents are neutralized by lime treatment before they enter to the biological treatment.

The temperature is another critical and important factor that affects growth and metabolism of an organism. As shown in Figure 4, after 96 h incubation at different



**Figure 4.** Effect of temperature on the decolorization process of RBB by *B. thuringiensis* BYJ1.



Figure 5. Effect of initial dye concentration on the decolorization process of RBB by *B. thuringiensis* BYJ1.

temperature, the dye decolorization activity of *B. thuringiensis* BYJ1 was retained till 60°C. Notably the increase in temperature above 37°C decreased the dye decolorization efficiency of *B. thuringiensis* BYJ1. This can be attributed to loss of cell viability or inactivation of enzymes at relatively high temperature (Kapil et al., 2009).

#### Effects of RBB concentrations

Very few organisms, especially bacteria, can tolerate

textile dyes in high concentrations. The high concentration of dye may affect the growth and metabolism of an organism. Many times the concentration of textile dye above certain range proved to be toxic to the organisms (Bhimani et al., 2014). The mode of toxicity has not been characterized till now. There are different believes among different groups of scientists.

As shown in Figure 5, *B. thuringiensis* BYJ1 could tolerate Remazol Black B dye up to 1200 mg L<sup>-1</sup>, but the decolorization ability of *B. thuringiensis* BYJ1 was not affected much up to 200 mg L<sup>-1</sup> dye concentration and was giving 100% decolorization of dye. The increase in



Figure 6. Effect of aeration/agitation on the decolorization process of RBB by *B. thuringiensis* BYJ1.

dye concentration decreased the decolorization efficiency of *B. thuringiensis* BYJ1 up to 50%.

down products of RBB.

# Biodegradation of RBB and analysis of the transformed dye products

The reviews on azo dyes decolorization show that anaerobic biodegradation of azo dyes led to production of aromatic amines which are much more carcinogenic than the dye itself. That is why the aerobic biological treatment of dye degradation is preferred over anaerobic treatment but aerobic decolorization of dye is carried out by very few groups of organisms. The anaerobic treatment is powerful and faster as compared to aerobic treatment but its products are not acceptable to the receiving environment (Puvaneswari et al., 2006).

As shown in Figure 6, the complete decolorization of Remazol Black B was achieved in static condition after 96 h incubation while in shaking condition only 77% dye was decolorized.

#### UV-visible spectroscopic analysis

Remazol Black B dye control sample was analyzed for UV-visible scanning and showed peak at 595 nm. Samples were collected from static (ST) and shaking (SH) reaction mixture and centrifuged to remove biomass. Supernatant was subjected to UV-visible scanning and the peak was significantly reduced in both the samples as compared dye control. Static sample showed a new peak in UV range indicating existence of break-

#### FTIR analysis of the transformed dye products

The FTIR spectra obtained from the treated dye samples showed several peaks in the region where N–H and O–H stretching is normally observed, like 3318.68 and 3319.93 cm<sup>-1</sup> respectively, in Figures 7 and 8. The sample from aerobic (shaking) flask showed significant reduction in absorption and so reduction in peak size at 3300 cm<sup>-1</sup> region as compared to the sample from microaerophilic static condition corresponding to –NH- (stretching) in primary amine. In both spectrum, there is no peak found in 1590 – 1600 cm<sup>-1</sup> region which is fingerprint region for N=N stretching for azo bond (Figures 7 and 8). The absence of peak in the above region indicates breakdown of azo bonds of diazo dye Remazol Black B (Lamia et al., 2009).

Other band located at 1658.82 cm<sup>-1</sup> in the spectra of static sample (Figure 7) disappeared in aerobic treatment (Figure 8). The significant peak at 1658.82 cm<sup>-1</sup> is for  $NH_3^+$  deformation and suggest that possible alkenes conjugation with C=O and -C-N stretching vibration regions and it is well established that microaerophilic or anaerobic treatment of azo dyes produces aromatic amines. Moreover, no absorption peak in this region of the spectra of aerobic sample (shaking condition) indirectly indicates absence of such amines.

In both samples peaks at 595.35 and 625.22 cm<sup>-1</sup> shows production of sulfo-compounds as the region specifies bending vibration of S=O bond. The peaks at



Figure 7. FTIR spectrum of decolorized RBB dye by *B. thuringiensis* BYJ1 under static condition.



Figure 8. FTIR spectrum of decolorized RBB dye by *B. thuringiensis* BYJ1 under shaking condition.

1019.39 and 1113.75 cm<sup>-1</sup> in static sample spectrum (Figure 7) and the peaks at 1020.96 and 1114.34 cm<sup>-1</sup> in shaking sample spectrum (Figure 8) were for C-H deformation. The peaks at 1449.95 cm<sup>-1</sup> in static spectrum and 1448.51 cm<sup>-1</sup> in shaking spectrum were for alkanes C-H deformation. The peaks at 2831.22, 2831.39, 2942.71 and 2942.94 cm<sup>-1</sup> were indicating alkanes C-H stretching.

There was considerable difference between the FTIR spectrum of treated samples in static and shaking

condition in terms of reduction in peaks' size and disappearance of peak. This indicated biodegradation of dye through two different ways due to difference in cultural conditions.

The fact was better conformed by toxicity testing of these samples on seed germination studies. If the organism uses same pathway for the degradation of Remazol Black B under both conditions, then the samples must have the same effect on seeds germinations. 
 Table 1. Seeds germination studies of dye samples.

Sample	Seed germination (%)	
	Phaseolus mungo	Arachis hypogaea
Water control	96	90
Dye control	22	09
Shaking sample (degraded dye)	98	84
Static sample (degraded dye)	69	37

#### Thin layer chromatography (TLC)

The decolorization was further conformed by analyzing the static and shaking sample on TLC plates. When TLC plates were observed under natural light, short wave length UV (254 nm) and long UV (365 nm), no spot was found corresponding to the dye spot (dye control having  $R_f$  0.66). This means that the textile dyes can be decolorized and degraded in both conditions.

#### Seed germination studies

Treated effluents of shaking and static conditions were subjected to seed germination studies using *Phaseolus mungo* and *Arachis hypogaea*. The sample treated in shaking condition showed almost equal seed germination capacity as with positive control water (Table 1). In contrast, the sample of the static condition was having less favorable effect on seeds germination. Dye solution which was used as negative control affecting negatively the germination of seeds (Patil et al., 2008). The biologically treated dye effluent under shaking condition can be used for irrigation purpose after suitable post treatment while statically treated effluents requires few more steps of treatment before disposal in the receiving body.

#### COD removal and decolorization of RBB

The treatments of environmental samples are done with the intention of reduction in COD and/or biochemical oxygen demand (BOD) values. The efficiency of any waste water treatment technology is finally assessed on the basis of its ability to reduce COD of the samples.

The initial COD load of the reaction flasks consisting of 100 mg L<sup>-1</sup> Remazol Black B was 18051 ppm. The COD of samples was decreased when treated with *B. thuringiensis* BYJ1, after 24 h of incubation there was 4076 ppm reduction in COD. After 48 h, 7844 ppm reduction was found with 70% dye decolorization and finally total 16087 ppm reduction on COD was achieved with 99% total dye decolorization.

# Interaction of dye Remazol Black B with *B. thuringiensis* BYJ1

The scanning electron micrograph of the organism in the presence and absence of dye revealed that in the presence of dye the thickness of the microbial cell increases significantly therefore dye Remazol Black B may get adherence on the surface of the *B. thuringiensis* BYJ1 and then must be degraded in small units.

It was found that when the isolates were grown in the presence of dye, their cell dimensions changed. From the electron micrographs, it was clear that during the decolorization process the dye gets deposited on the cell surface of the organisms. The decolorization may be done by membrane associated systems of decolorization and then simplified molecules of dyes may be internalized and further degraded. The cell length of *B. thuringiensis* BYJ1 was measured as 1.074  $\mu$ m (Figure 9) while the organism grown in the presence of dye was measured to be 1.131  $\mu$ m (Figure 10).

# Decolorization of RBB by immobilized cells of *B. thuringiensis* BYJ1

The immobilization is confinement technique which helps to separate active biological agents from other components of reaction mixture. In bioremediation, this technique is very useful for the treatment of toxic agents. The textile effluents contain variety of dyes in very high concentrations along with other toxic chemicals. More over the pH of the effluents is also one of the factors which greatly affect the growth and stability of the organism during the treatment. The encapsulation protects organism from all above challenges during biological treatment of dye effluents.

In immobilized state, the *B. thuringiensis* BYJ1 was more efficient in decolorization of dye over the free cells. In static conditions, 99% decolorization was achieved within 72 h in immobilized state while it took 96 h in free cells condition (Figure 11).

# Role of *B. thuringiensis* BYJ1 plasmid in decolorization of RBB

The ~5.5 kb plasmid was detected on agarose gel from



Figure 9. Scanning electron micrograph of *B. thuringiensis* BYJ1 cells grown without RBB.



Figure 10. Scanning electron micrograph of *B. thuringiensis* BYJ1 cells grown in the presence of RBB.

*B. thuringiensis* BYJ1 (Figure 12; L1). During the curing process, the culture of *B. thuringiensis* BYJ1 was treated with acridine orange. There were two colonies of *B. thuringiensis* BYJ1 found on the master plate of N-agar which were absent on replica of ampicillin plate. There was no plasmid detected on agarose gel from cells of cured colonies (Figure 12; L3 and L4).

When the treated samples of Remazol Black B by *B. thuringiensis* BYJ1 and cured *B. thuringiensis* BYJ1 cultures were analyzed, there was significant difference found in decolorization of dye. This was not in accordance with the observation made by Khaled et al. (2010), indicating least role of plasmid in decolorization

process. After 96 h, 92% dye was decolorized by *B. thuringiensis* BYJ1 with plasmid while 78% dye was decolorized by cured *B. thuringiensis* BYJ1 (Figure 13).

The dye decolorization efficiency of *B. thuringiensis* BYJ1 was affected due to loss of plasmid by curing experiment, but the organisms could decolorize RBB in lesser extent.

#### Conclusion

The reduction and degradation of azo dyes under aerobic and anaerobic conditions have been extensively studied



Figure 11. Decolorization of RBB by immobilized and free cells of *B. thuringiensis* BYJ1.



**Figure 12.** Agarose gel of plasmid from *B. thuringiensis* (BYJ1); L1 – BYJ1 with dye, L2 – BYJ1 without dye, L3 – BYJ1 cured colony 1, L4 – BYJ1 cured colony 2, L5 – DNA marker.

(Meiying et al., 2007). Remazol Black B is one of the difficult dyes to remove from the contaminated system. The organism *B. thuringiensis* BYJ1 was isolated from dye contaminated site and optimized for the dye degradation process. The organism could decolorizes dye in both static and shaking conditions but the static degradation products were not beneficial to plant germination while shaking dye transformed samples supported the

plant growth. This was very similar to the previous studies done by Patil et al. (2008).

The soil and water samples of dye contaminated sites can provide rich source of microbes with dye tolerance and degradative potentials and such microbes and their metabolic processes can be exploited to design the technology for effective treatment of dye/textile effluents. The system using indigenous soil bacteria like



Figure 13. Effect of *B. thuringiensis* BYJ1 plasmid on decolorization of RBB.

*B. thuringiensis* BYJ1 can be used to design such technology which can degrade dye in shaking condition and the treated sample can be used for irrigation of agriculture fields. Application of immobilization techniques will add to the efficiency of the technology.

The *B. thuringiensis* BYJ1 was detected with a plasmid. The presence of plasmid had raised the rate of decolorization of RBB while the loss of plasmid affected the decolorization potential of *B. thuringiensis* BYJ1. The further detailed molecular study in this direction may help to detect and understand the mechanism of genes involved in decolorization of RBB.

#### **Conflict of interest**

The author(s) have not declared any conflict of interests.

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