Briefing of microbial degradation of textile (Azo) dye

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Abstract— **Release of textile azo dyes to the environment is an issue of health concern while the use of microorganisms has proved to be the best option for remediation. Thus, in the present study, a bacterial consortium consisting of *Providencia rettgeri* strain HSL1 and *Pseudomonas sp.* SUK1 has been investigated for degradation and detoxification of structurally different azo dyes. The consortium showed 98-99 % decolorization of all the selected azo dyes viz. Reactive Black 5 (RB 5), Reactive Orange 16 (RO 16), Disperse Red 78 (DR 78) and Direct Red 81 (DR 81) within 12 to 30 h at 100 mg L^{-1} concentration at 30 ± 0.2 °C under microaerophilic, sequential aerobic/microaerophilic and microaerophilic/aerobic processes. However, decolorization under microaerophilic conditions viz. RB 5 (0.26 mM), RO 16 (0.18 mM), DR 78 (0.20 mM) and DR 81 (0.23 mM) and sequential aerobic/microaerophilic processes viz. RB 5 (0.08 mM), RO 16 (0.06 mM), DR 78 (0.07 mM) and DR 81 (0.09 mM) resulted into the formation of aromatic amines. In distinction, sequential microaerophilic/ aerobic process doesn't show the formation of amines. Additionally, 62-72 % reduction in total organic car-bon content was observed in all the dyes decolorized broths under sequential microaerophilic/aerobic processes suggesting the efficacy of method in mineralization of dyes. Notable induction within the levels of azo reductase and NADH-DCIP reductase (97 and 229 % for RB 5, 55 and 160 % for RO 16, 63 and 196 % for DR 78, 108 and 258 % for DR 81) observed under sequential microaerophilic/aerobic processes suggested their critical involvements in the initial breakdown of azo bonds, whereas, a slight increase in the levels of laccase and veratryl alcohol oxidase confirmed subsequent oxidation of formed amines. Also, the acute toxicity assay with *Daphnia magna* revealed the nontoxic nature of the dye-degraded metabolites under sequential microaerophilic/aerobic processes. As biodegradation under sequential microaerophilic/aerobic process completely detoxified all the selected textile azo dyes, further efforts should be made to implement such methods for large scale dye wastewater treatment technologies**

Keywords- Remazol Black B; *Bacillus*; pH; Temperature; Static condition, Azo dyes, *P. rettgeri* strain HSL1, *Pseudomonas sp.* SUK1, bacterial consortium, decolorization, biodegradation, sequential microaerophilic/aerobic process, detoxification

I. Introduction

Textile dyes are chemicals of complex aromatic structures designed to resist the impact of detergents, sunshine and temperatures (Nigam et al., 1996). They are chemically and photochemically stable and are extremely persistent in natural atmospheres. The world-wide annual production of synthetic textile dyestuff has been estimated to be over 1 x 10^6 ton (Pandey et al., 2007). More than 10,000 different commercially available dyes are used in textile industry for dyeing and printing purposes (Meyer, 1981; Kadam et al., 2011). The fixation rates of several textile dyes are not 100 % and around 30-70 % of the amount of dyestuff used to get eliminated into effluent during the wet processing operations (Khaled et al., 2009; Bumpus, 1995). The estimated dyes concentration in the textile effluent has been reported to be in the range of 10-200 mg L^{-1} (Kadam et al., 2011). A wide variety of microorganisms are reported to be capable of decolonization of dyes [11-24]. The current study has evaluated the potential of isolated bacterial strain from textile effluent for their decolorization efficiency of the textile dye, Remazol Black B under *in vitro* conditions and optimization of the factors influencing the process. Textile dyes are chemicals of complex aromatic structures designed to resist the impact of detergents, sunshine and temperatures (Nigam et al., 1996). They are chemically and photochemically stable and are extremely persistent in natural atmospheres. The world-wide annual production of synthetic textile dyestuff has been estimated to be over 1 x 10^6 ton (Pandey et al., 2007). More than 10,000 different commercially available dyes are used in textile industry for dyeing and printing purposes (Meyer, 1981; Kadam et al., 2011). The fixation rates of several textile dyes are not 100 % and around 30-70 % of the amount of dyestuff used to get eliminated into effluent during the wet processing operations (Khaled et al., 2009; Bumpus, 1995). The estimated dyes concentration in the textile effluent has been reported to be in the range of 10-200 mg L^{-1}.

II. Material and Methods

Sampling and analysis of effluent

The effluent sample was collected from the middle point of the area. Standard procedures (Spot and Grab) were followed during sampling. The temperature and pH were determined at the sampling site. The pH was determined by using pH meter and temperature with laboratory thermometer. The sample was transported to laboratory at 4°C as in accordance with the standard methods [25]. The physicochemical parameters such as colour, Biological Oxidation Demand (BOD), Chemical Oxygen Demand (COD), Total Suspended Solids (TSS), and Total Dissolved Solids (TDS) were determined as soon as the sample was brought to the laboratory. Sample color was analyzed by

spectrophotometer (SHIMADZU UV-1800). BOD was determined by employing evaporation method by DO meter (WTW Germany) while COD was measured by COD instrument directly (Spectralab, India).

Microorganism and culture conditions: The method included two steps: Treatability test was carried out by enrichment culture technique and toxicity test in which, toxicity was assessed in a series of parallel flasks with geometrically increasing concentration of Remazol Black B. Microbial growth was measured daily by turbidity. The concentration at which the toxic components inhibited growth was noted and was used as a warning of an upper concentration limit. Isolation was done on nutrient agar plates incorporated with 100 mg/l Remazol Black B. Pure culture was maintained on the nutrient agar slants.

Chemicals: The textile dye, Remazol Black B (λ_{max} 595 nm) was obtained from local textile industries. A stock solution of the dye (1000 mg L–1) was prepared in deionized water and used for all studies.

Isolation, screening and identification of dye decolorizing bacteria from effluent: The textile effluent was collected in sterile collection tubes from the sludge and wastewater of the ditches at industrial site located in Textile Industries. The sample collected from the textile mill was screened for azo dye decolorizing bacterial strains by inoculating 10 ml of sludge solution into 250 ml Erlenmeyer flask containing 100 ml nutrient broth (g/L as mentioned above). The flasks were incubated at 37°C under shaking conditions (120 rpm). After 48 h of incubation, 1.0 ml of the culture broth was appropriately diluted and plated on Nutrient Agar containing 100 mg L–1 Remazol Black B. The Morphologically distinct bacterial isolates showing clear zones around their colonies due to decolorization of dye were selected for further studies. The pure culture stocks of these isolates were stored at 4°C on Nutrient Agar slopes containing 1000 mg L–1 of Remazol Black B. These isolates were screened for their ability to decolorize Remazol Black B in liquid culture. The Screening process in liquid media was carried out by inoculating a loop full of cultures exhibiting clear zones into Nutrient broth containing Remazol Black B under static conditions. After 24 h of incubation, 1ml. of cell suspension was transferred to fresh nutrient broth containing Remazol Black B to screen the strains with color removing ability. The Screening procedure in liquid medium was continued until complete decolorization of broth. A small amount of decolorized broth was transferred to nutrient agar plates containing Remazol Black B (100 mg L–1). The bacterial isolate which tolerated higher concentration of the azo dye was isolated by streak plate method. The azo dye decolorizing bacteria was identified from several aspects including morphology characters, biochemical tests as described in Bergey's manual of determinative bacteriology (Indole, Methyl Red, Voges-Proskauer test, Citrate, Catalase, Oxidase, Nitrate Reduction test, Hydrolysis of Casein, Starch, Urea and Gelatin). Assimilation of various sugars such as D-glucose, D-fructose, galactose, mannitol and D-maltose as sole carbon source was determined by inoculating the isolate into carbohydrate broth supplemented with respective carbon source. After inoculation the tubes were incubated at 37°C for 24-48 h.

Analyses of 16S rRNA sequences

Genomic DNA of the isolate was extracted with a GenElute DNA extraction kit from Sigma. The 16S rRNA gene of isolate was amplified using the universal primers 8F (5′-AGAGTTTGATCCTGGCTCAG) and 1541R (50-AAGGAGGTGATCCAGCCGCA-3′). The amplification was done by initial denaturation at 95°C for 5 min followed by 10 cycles of 93°C for 1 min, 63°C for 1 min, 71°C for 1.5 min; 20 cycles of 93°C for 1 min, 67°C for 1 min, 71°C for 2 min and final extension at 71°C for 5 min. The purified PCR product was sequenced in both directions using an automated sequencer by Perkin Elmer ABI Prism 377 DNA sequencer. The phylogenic relationship of the isolate was determined by comparing the sequencing data with sequences of some members of the genus *Bacillus* available through the GenBank database of the National Center for Biotechnology Information. The gene sequences of each isolate obtained in this study were compared with known 16s rRNA gene sequences in the GenBank database.

III. Decolorization Experiments

Bacillus spp. ETL-2012 was grown for 24 h at 37°C on nutrient agar. 10% inoculum (OD_{600} 1.0) the isolate was inoculated in nutrient broth to study the decolorizing ability of the culture. The dye was filter sterilized by using 0.2 µm filter (Sartorius Biolab, Germany) and added after sterilization of medium throughout the study. The dye (100 mg/l) was added immediately and incubated under static condition at 37°C. Aliquot (3 mL) of culture media was withdrawn at different time intervals and centrifuged at 8,000 g for 15 min. Decolorization was monitored by measuring the absorbance of the culture at λ_{max} of the dye i.e., 595 nm and change in pH was also recorded. Biomass in the dye containing medium was determined by wet weight method.

Decolorization at different dye concentration: In order to examine the effect of initial dye concentration on decolorization under static condition 50–1000 mg/l of Remazol Black was added to the sterile nutrient broth inoculated with 10% *Bacillus spp*. ETL-2012 of (OD_{600} 1.0) and incubated at 37°C under static condition. The % decolorization was measured. All decolorization

experiments were performed in three sets. Abiotic controls (without culture) were always included. The %decolorization and average decolorization rate was measured.

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

$$\text{Average decolorization rate} = \frac{\frac{C \times \%D}{1000} \times 100}{t}$$

Where C = initial concentration of dye mg/l, %D = dye decolorization % after time t [26].

Effect of pH on dye decolorization: Sterile nutrient broth of different pH 3, 4, 5, 6, 7, 7.5 and 8 was inoculated with 10% inoculum and incubated at 37°C under static condition. The dye concentration was 100 mg/l. All decolorization experiments were performed in three sets. Abiotic controls were also set. The %decolorization was measured as mentioned earlier.

Effect of temperature on dye decolorization: Sterile nutrient broth of pH 7.5 was inoculated with 10% inoculum and filter sterilized dye at 100 mg/l was added after sterilization. The broth was incubated at 28, 37, 40, 45 and 50°C. The experiment was carried out in triplicate. Abiotic controls (without microorganism) were always included. The %decolorization was measured.

Effect of carbon and nitrogen sources on decolorization: To study the effect of carbon and nitrogen sources on decolorization of Remazol Black B, semi synthetic medium was used [26]. It was further incorporated with different carbon and nitrogen sources (1% each) such as glucose, sucrose, lactose and starch, yeast extract, peptone, malt extract, meat extract and urea, respectively [26]. 100 mg/l of the dye was used. Filter sterilized dye was added after sterilization of the medium and after inoculation of the isolate.

Repeated dye decolorization in fed batch process: Repetitive decolorizing ability of the cells was (repeated use) studied by addition of the dye (100 mg/l) at each cycle [27].

IV. Bacterial Strains and Culture Conditions

P. rettgeri strain HSL1 (Genebank acces-sion no. JX853768.1) isolated from textile effluent contaminated soil was selected based on its capacity to degrade textile azo dye. Other bacterium *Pseudomonas sp.* SUK1 (Genebank accession no. EF541140). previously isolated from textile dye contaminated site and known for textile effluent de-colorization was used to develop consortium (Kalyani et al., 2008, 2009). The stock cultures of both the bacterial strains were maintained at 4 ± 0.2 °C on nutrient medium slants containing 1 % textile effluent to retain

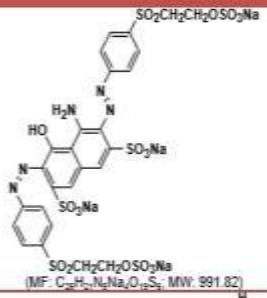
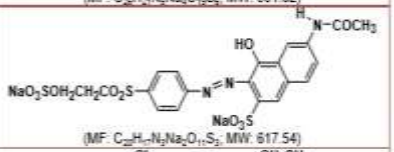
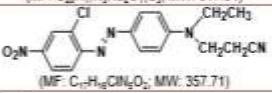
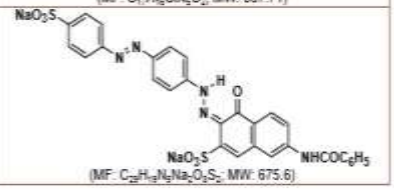
C.I. name and no., Common name and Abbr.	Wavelength λmax (nm)	Chemical structure, Molecular formula and Molecular weight
Reactive black 5 (C.I. 20505), Remazol black B	597	 (MF: $C_{26}H_{21}N_5Na_4O_{19}S_6$; MW: 991.82)
Reactive orange 16 (C.I. 17757), Remazol orange 3R	494	 (MF: $C_{20}H_{17}N_3Na_2O_{11}S_3$; MW: 617.54)
Disperse red 78 (C.I. 11226), Rubine GFL	530	 (MF: $C_{17}H_{16}ClN_5O_2$; MW: 357.71)
Direct red 81 (C.I. 28160), Direct red 5BL	508	 (MF: $C_{27}H_{19}N_5Na_2O_8S_2$; MW: 675.6)

Table 1: - The structural information of textile azo dyes used in this study

V. Pre-Enrichment Conditions

Both the bacterial cultures *P. rettgeri* strain HSL1 and *Pseudomonas sp.* SUK1 were individually grown in 250 ml Erlen-meyer flask containing 100 ml of nutrient broth having composition g L^{-1} of sodium chloride 5.0, beef extract 1.5, yeast extract 4.0 and peptic digest of animal tissue 5.0. The initial pH of the medium was adjusted to 7.0 with 0.1 M HCL and/or 0.1 M NaOH, autoclaved at 121 °C for 20 min, and sepa-rately inoculated with 100 µl of 24 h old ac-tive cultures of bacterial strains. The enrich-ment was carried out for 24 h at 30 ± 0.2 °C under microaerophilic conditions and then used as inoculum for further degradation studies.

Optimization of decolorization conditions Decolorization experiments were con-
ducted in 250 ml Erlenmeyer flask contain-ing 100 ml of individual pre-enriched *P. rettgeri* strain HSL1, *Pseudomonas sp.* SUK1 and equal amounts of both the cul-tures (50 ml of each) as bacterial consortium. The decolorization efficiency of individual cultures and bacterial consortium was investigated with 100 mg L^{-1} dyes concentration, initial broth pH of 7.0 (adjusted after pre-enrichment) and 30 ± 0.2 °C of incubation temperature under microaerophilic conditions. The process parameters optimization for all the azo dyes decolorization by bacterial consortium were performed in microaerophilic conditions by one parameter at a time approach viz. initial pH of pre-enriched broth (3 to 12), incubation temperature (20, 30, 37, 40, 50 ± 0.2 °C) and initial dye concentration (50 to 250 mg L^{-1}).

Effect of pH

Bacterial cultures generally exhibit maximum decolorization at pH values near 7.0. Our culture exhibited decolorization activity in the range of pH 5–8. At pH 3 and 4 decolorization was 15 and 40% respectively. The isolate showed more or less constant decolorization between pH 5–8. Maximum decolorization (99.00%) was at pH 6 (Figure 2). *Bacillus spp*. ETL-2012 grows very well in the pH range of 5–9. *E. coli* and *Pseudomonas luteola* both exhibited best decolorization at pH 7.0 with constant decolorization rate up to pH 9.5 [14].

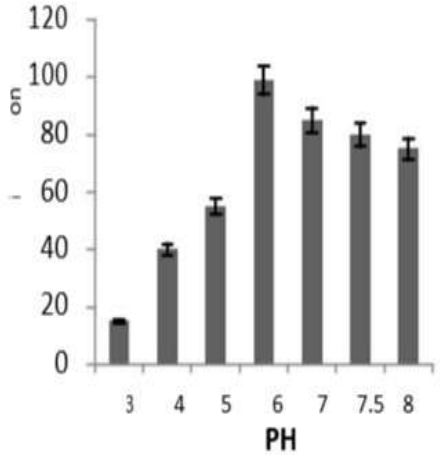


Figure 1 :- The effect of pH on degradation of azo dye

Effect of temperature

The rate of chemical reaction is the direct function of temperature. Bacteria require optimum temperature for growth. Since dye decolorization is metabolic process hence shift in temperature from optimum results into decrease in dye decolorization as high temperature causes thermal inactivation of proteins and possibly of such cell structures such as membrane. The isolate showed complete decolorization at 27, 37 and 40◦C but rapid decolorization i.e., within 12 h was observed at 37◦C (Figure 3). This could be due to a greater production of enzymes and optimal growth conditions of the isolate for its dye decolorizing ability. The decolorization at this optimum temperature may be owing to higher respiration and substrate metabolism. This also demonstrates that decolorization of the dye was through microbial reaction which relies on optimal temperature and not by adsorption. There was no decolorization at 45 and 50◦C. Hence pH and temperature optima for Remazol Black B were found to be pH 6 and 37◦C respectively.

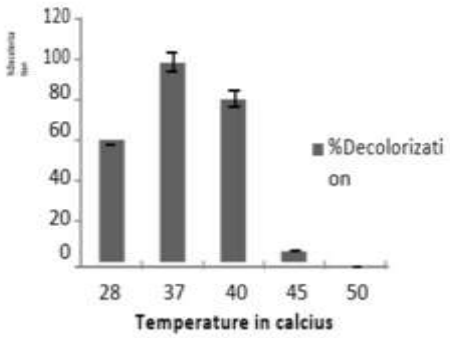


Figure 2:- Effect of Temperature on degradation of azo dye

Effect of initial dye concentration

Figure shows decolorizing ability of our culture increased with increase in dye concentration from 50 to 500 mg/l. The activity was lower at dye concentration 600 mg/l and above which decolorization was strongly inhibited at dye concentration 1000 mg/l (Figure 4). It has been proposed that dye concentration can influence the efficiency of microbial decolorization through a combination of factors including the toxicity imposed by dye at higher concentration. Thus, the isolate which could decolorize dye much above the reported dye concentration in wastewater, can be successfully employed for treatment of dye bearing industrial wastewater.

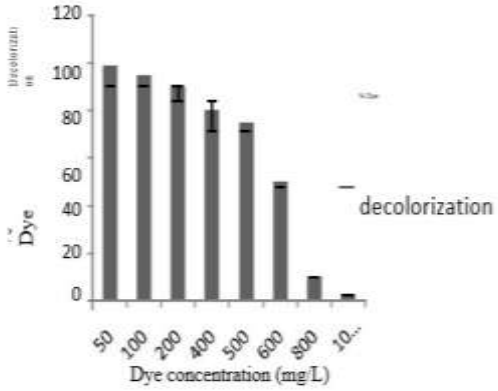


Figure 3 :- Effect of initial dye concentration

Effect of carbon and nitrogen sources on decolorization

While trying to enhance decolorization performance of Remazol Black B, extra carbon and nitrogen sources was supplied. Percentage decolorization (95%) (Figure 5) was maximum with purified substrate peptone while less decolorization with other supplements of carbon and nitrogen source. Similar result was also reported by for decolorization of C.I.Reactive Green 19A. [26]. The culture showed negligible decolorization in the presence of sucrose (25%) whereas moderate activity was shown in presence of glucose (55%), lactose (80%) and maximum decolorization was reported in presence of starch (95%). In addition, supplying urea as a nitrogen source exhibited less decolorizing ability. Similar results were reported by Saratale [26]. In contrast, addition of carbon sources seemed to be less effective to promote the decolorization probably due to the preference of the cells in assimilating the added carbon sources over using the dye compound as the carbon source [26]. Decolorization with repeated addition of dye aliquots. The repeated use of microorganisms is important in commercial point of view. This study was carried out to examine the ability of *Bacillus spp*. ETL-2012 to decolorize repeated addition of Remazol Black B dye aliquot (100 mg/l) at static condition. The isolate had an ability to decolorize 100% dye up to third dye aliquot addition and after that subsequent cycle required 10-12 h. The eventual cessation of decolorization was likely due to nutrient depletion [26,27]. Thus *Bacillus spp*. ETL-2012 showed the ability to decolorize repeated addition of dye aliquots which is noteworthy for its commercial applications.

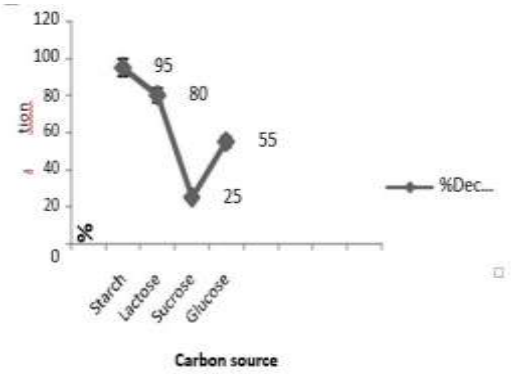


Figure 4 :- Effect of Carbon Source on degradation of azo dye

Dyes	Experimental conditions	Decolorization (%)	Amine conc. (mM)	TOC reduction (%)
RB 5	Microaerophilic (30 h)	98±1.5	0.26±0.02	63±2.0
	Aerobic (30 h)	12±2.0	n.d.	10±1.0
	Aerobic (15 h) to microaerophilic (15 h)	98±1.5	0.08±0.01	62±2.0
	Microaerophilic (15 h) to aerobic (15 h)	98±1.5	n.d.	62±2.0
RO 16	Microaerophilic (12 h)	99±1.0	0.18±0.02	68±2.0
	Aerobic (12 h)	20±2.0	n.d.	20±1.0
	Aerobic (6 h) to microaerophilic (6 h)	99±1.0	0.06±0.01	69±2.0
	Microaerophilic (6 h) to aerobic (6 h)	99±1.0	n.d.	68±2.0
DR 78	Microaerophilic (18 h)	98±1.5	0.20±0.02	72±2.0
	Aerobic (18 h)	22±2.0	n.d.	20±1.0
	Aerobic (9 h) to microaerophilic (9 h)	98±1.5	0.07±0.01	72±2.0
	Microaerophilic (9 h) to aerobic (9 h)	98±1.5	n.d.	72±2.0
DR 81	Microaerophilic (24 h)	99±1.0	0.23±0.02	65±2.0
	Aerobic (24 h)	21±2.0	n.d.	18±1.0
	Aerobic (12 h) to microaerophilic (12 h)	99±1.0	0.09±0.01	65±2.0
	Microaerophilic (12 h) to aerobic (12 h)	99±1.0	n.d.	64±2.0

n.d. = not detected
Values are mean of three experiments, ± Standard deviation for all the data

Table 2 :- Decolorization of azo dyes by bacterial consortium and formation of aromatic amines and TOC reduction under various experimental conditions

VI. Conclusion

Although decolorization is a challenging process to both the textile industry and the waste water treatment, the result of this findings and literature suggest a great potential for bacteria to be used to remove color from dye wastewaters. Interestingly, the bacterial species used in carrying out the decolorization of azo dye Remazol Black B in this study was isolated from the textile dye industry waste effluent. The bacterial strain *Bacillus spp.* ETL-2012 showed decolorizing activity through a degradation mechanism. This observation has established that the bacteria are adaptive in nature and can degrade contaminants. The ability of the strain to tolerate, decolorize azo dyes at high concentration gives it an advantage for treatment of textile industry waste waters. However, potential of the strain needs to be demonstrated for its application in treatment of real dye bearing waste waters using appropriate bioreactors.

Acknowledgment

The preferred spelling of the word “acknowledgment” in American English is without an “e” after the “g.” Use the singular heading even if you have many acknowledgments. Avoid expressions such as “One of us (S.B.A.) would like to thank ... .” Instead, write “F. A. Author thanks ... .” **Sponsor and financial support acknowledgments are placed in the unnumbered footnote on the first page**.

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