Treatment of Azo Dye Mixtures by Single Bacterium/Consortium and Evaluation of Phytotoxicity

INTRODUCTION

Industrial effluents show a complex composition that is extremely variable even within the same factory. Thus decolourization of real effluents requires an appropriate choice, perhaps a consortium of bacterial strains in conjunction with operative conditions. Real textile dye effluents contain not only dyes but also salts, sometimes at very high ionic strength and extreme pH values, chelating agents, precursors, by-products and surfactants. Thus, in spite of high efficiency of dye decolorization by some selected strain, decolorization of real industrial effluent is quite troublesome (Faraco and Pezzella, 2009).

Biological processes present eco-friendly and cost competitive alternatives to abiotic treatment. However, the presence of azo, nitro and sulfo groups make the dyes more resistant to microbial degradation and their residues accumulate in nature (Joe et al., 2008). Nevertheless there have been numerous attempts to develop biological processes for the treatment of textile effluents using bacteria, fungi and enzymes (Banta et al., 1996; McMulla et al., 2001; Wesenberg et al., 2003). White rot fungi that produce lignolytic enzymes, such as lignin peroxidase, manganese peroxides and laccase have been studied extensively because of their ability to degrade various organic compounds (Fu and Viraraghvan, 2001).

Decolorization of azo dyes normally begins with initial reduction cleavage of azo bond anaerobically, which results in colorless but toxic aromatic amines. This is
followed by complete degradation of aromatic amines strictly under aerobic conditions (Kothari, 2006). Therefore, anaerobic/aerobic processes are crucial for complete mineralization of azo dyes. However, not all bacteria have both anaerobic and aerobic properties. Usually consortia are routinely used for the degradation of azo dyes.

Recent research has exposed the survival of wide variety of organisms in mixed culture capable of decolorizing a wide range of dyes. The complexity of the microbial consortium enables them to act on a variety of pollutants. Microbial consortia are usually used without analyzing the constituent microbial populations for environmental remediation (Mohorcic et al., 2004). Bioremediation relies on the pollutant degrading capacities of naturally occurring microbial consortia in which bacteria play central role (Liu and Suffita, 1993; O’Neill et al., 2000). Several bacteria capable of dye decolorization either individually or in consortia, have been reported (Patil et al., 2008).

Many reports indicate that textile industry effluent have toxic effect on the germination rates and biomass concentration of several plant species which play important ecological functions such as providing the habitat for wildlife, protecting soil from erosion and providing huge bulk of organic matter that is significant to soil fertility (Wang, 1991). The toxicity of effluent is because of the presence of dye or its partially degraded product which are mutagenic or carcinogenic (Kalyuzhnyi and Sklyar, 2000). Therefore the treatment of textile industry becomes necessary prior to their final discharge to the environment (Kumar and Dastidar, 2009).

The present study deals with studies on the Reactive Balck 5, Black E, and Green B decolorization by individual bacterial strains as well as consortium. Assessment of the toxicity of Reactive Black 5, Black BT and Green B dyes as well as its degradative metabolites was carried out by phytotoxicity studies.
MATERIALS AND METHOD

Dyes
Five commercially available textile azo dyes Reactive Black 5, Green B, Black BT, Black E and FF sky blue were obtained from local textile dye manufacturing unit at GIDC Ahmedabad. The chemical structure of dyes used in this study is as per Figure 6.1.

Decolorization of mixed azo dyes
Decolorization of mixture of three azo dyes Reactive Black 5, Green B and Black E was investigated. Each of the dye was added with the concentration of 33 mg l$^{-1}$ with 100 mg l$^{-1}$ of total dye concentration in reaction mixture. In another set of experiment five azo dyes Reactive Black 5, Green B, Black E, Black BT and FF Sky Blue was added 20 mg l$^{-1}$ each dye with final concentration of 100 mg l$^{-1}$. Individual bacterial isolate or its consortium was offered for decolorization of dye mixture. Decolorization processes were monitored according to the method reported by Harazono and Nakamura (2005) with slight modification. Dye concentration was measured at selected different visible wavelengths (400, 450, 500, 550, 600, and 650 nm). \( \Sigma OD \) of mixed azo dyes was calculated as the sum of absorbance at each wavelength and color removal (%) was calculated as the extent of decrease from the initial value of \( \Sigma OD \). The reaction was also monitored at the absorbance maximum of each dye (Liu et al., 2007).

Development of bacterial consortium
Selected organisms namely; JTP-5, *Alcaligenes faecalis* JTP-07, JTP-13, *Lysinibacillus fusiformis* JTP-23, and JTP-30 were selected to develop consortia for decolorization. To develop a consortium, bacterial isolates were grown individually overnight and added in equal proportion to get 5% inoculum in reaction mixture. Bacterial isolates were mixed in different combinations and their ability to decolorize Reactive Black 5 and Black BT was studied. Biomass was determined as mentioned in chapter 4.
Dye decolorization experiments

Decolorization of azo dyes was studied under static and shaking culture conditions at 30°C in 250 ml Erlen-meyer flasks containing 100 CMB medium. Azo dyes either single or mixture were added in the reaction mixture with final concentration of 100 mg l⁻¹. Aliquots (3 ml) from each reaction flasks were withdrawn at regular intervals as indicated in the legends to respective figures and centrifuged at 6000 rpm for 15 min. The cell-free supernatant was analyzed for residual dye content.

Decolorization of industrial effluents

Industrial effluent containing a mixture of various textile dyes was obtained from a drainage line taking effluents to Common Effluent Treatment Plant in Jetpur (Figure 1.5). The effluent was centrifuged at 12,000 x g for 10 min to remove insoluble materials (Joe et al., 2008). This supernatant was used in place of water for preparation of CMB. After adjusting to pH 8 with 1 N HCl, the medium was sterilized by passing through 0.45 um membrane filter. 250 ml EM flask containing 100 ml medium was inoculated with 5% overnight grown culture of Lysinibacillus fusiformis JTP-23 and incubated at 30°C under static – shaking sequential culture condition.

Analysis of decolorization

The content of residual dye and percent decolorization was determined as described in chapter 2. Spectral analysis of the samples was performed using UV-Vis Spectrophotometer (Simadzu UV-Vis 1800, Japan). Samples withdrawn at 0 h were used as control.

Phytotoxicity study

The effect of original dye and its degradative metabolite on germination and early seedling growth of two plants; Triticum aestivum and Phaseolus mungo was evaluated. The degradation metabolites of Remazol Black 5, Green B, and Black BT were extracted in ethyl acetate were dried and dissolved in water to form the final concentration of 1000 ppm. The dye solutions were also prepared with concentration of 1000 ppm for phytotoxicity studies.
The seeds were germinated in sterile 10 cm petri dishes, layered with sterile filter paper. Seeds were sterilized as described earlier (Somasegaran and Hoben, 1985) before transferring to the surface of the paper in petri dish. The phytotoxicity study was carried out at room temperature (32 ± 2°C) in relation to *Triticum aestivum* and *Phaseolus mungo* seeds (10 seeds per plate) by watering separately 5 ml samples of dye and its degradation product per day. Seeds germinated in water irrigated petri dish were used as a control. Length of plumule (shoot), radicle (root) and germination (%) were recorded after every alternate day.
Figure 6.1: Chemical structures of azo dyes used in this study.
RESULT AND DISCUSSION

Decolorization of mixed azo dyes
As shown in the Figure 6.2, bacterial isolates JTP-05, JTP-13, and *Lysinibacillus fusiformis* JTP-23 were able to degrade mixture of azo dyes to different extent. It is clear from the data that all the three dyes (RB5, GB and BE, each at 33 mg l⁻¹) were decolorized simultaneously. Percentage of color removal by JTP-05, JTP-13, and *Lysinibacillus fusiformis* JTP-23 was 58, 42, and 92% at 24 h incubation and on extension of incubation, it reached up to 78, 49 and 92%, respectively. In order to see the combined effect of all three organisms on decolorization of mixture of azo dyes, a consortium was prepared and it was found to give complete removal of dye mixture in 24 h incubation.

As *Lysinibacillus fusiformis* JTP-23 removed more than 90% color within 24 h incubation, it was tested with mixture of five different azo dyes (RB5, GB, BE, BBT and FFSB, each 20mg l⁻¹). Results indicated that as the complexity of the dye mixture increased, there was decrease (86%) in dye decolorization efficiency. Biomass synthesis was also affected in the presence of five dye mixture, though the final dye concentration was the same (Figure 6.3).

Decolorization by bacterial consortium
Bacterial isolates used to develop consortium consisted of both type of organisms JTP-05, JTP-13, JTP-30, and *Lysinibacillus fusiformis* JTP-23 (four were Gm +ve) and *Alcaligenes faecalis* JTP-7, JTP-30 (two were Gm-ve) and was found to degrade the individual and mixture of azo dye by cometabolism. The degradation of individual dye Reactive Black 5 and Black BT (concentration 100 mg l⁻¹) using different mixture of organisms were carried out under static culture and shaking culture condition.

When combination of two bacterial isolates (JTP-23 and JTP-5, JTP-23 and JTP-7, JTP-23 and JTP-13, JTP-23 and JTP-30) was employed, decolorization achieved was more than 99% with no remarkable difference in decolorization
pattern under static condition (Figure 6.4), while less decolorization was achieved 65, 11, 84, and 63% respectively under shaking culture condition (Figure 6.5).

Combination of three bacterial isolates did not showed any improvement in decolorization pattern. Decolorization of Reactive Black 5 in case of consortium (JTP-23, JTP-5, and JTP-13) and (JTP-23, JTP-13 and JTP-30) was more than 99% under static culture condition, while 83 and 76% under shaking culture condition, respectively.

In next set of experiment, Reactive Black 5 dye was replaced by Black BT and the consortiums used as above. Results of these experiments are depicted in Figure 6.6 and 6.7. There are instances when the dye decolorization was inhibited at certain stage due to the accumulation of products formed upon decolorization (Kothari, 2006). Such results were observed when bacterial isolate *Alcaligenes faecalis* JTP-07 was present in consortium. It can be attributed to the different mechanisms used by members of consortium leading to the formation of products which stop favoring decolorization. This effect becomes nullified if there is the presence of a bacterial strain which prevents accumulation of such degradation products and thereby helps the consortium to carry out extensive degradation.
Figure 6.2: Decolorization of mixture of RB5, GB and BE by bacterial isolate a, JTP-05; b, JTP-13; c, JTP-23; and d, consortium of these three bacterial isolates.

Figure 6.3: Decolorization of mixture of five dyes (RB5, GB, BE, BBT & FFSB) by Lysinibacillus fusiformis JTP-23 under static culture condition.
Figure 6.4: Decolorization of Reactive Black 5 by different consortiums under static culture condition.

Figure 6.5.: Decolorization of Reactive Black 5 by different consortiums under shaking culture condition.
Figure 6.6: Decolorization of Black BT by different consortiums under static culture condition.

Figure 6.7: Decolorization of Black BT by different consortiums under shaking culture condition.
Decolorization of industrial effluent

We examined the ability of *Lysinibacillus fusiformis* JTP-23 to decolorize industrial effluent. After 48 hrs of static-shaking sequential treatment of dye, we observed significant decolorization of textile effluent which was otherwise dark greenish colored (Figure 6.8). Absorbance from 400 to 700 nm was almost completely abolished and absorbance was reduced in UV region. On the other hand, a new peak appeared in the UV range at 280 nm. This may be the result of the complex mixture of dyes found in industrial effluents; the new peak likely represents the by products of other dyes used in the factor. These results indicated that *Lysinibacillus fusiformis* JTP-23 can be used in an anaerobic-aerobic two stage system or as a component of a bacterial consortium for the treatment of textile dyes.

![UV-Visible spectral analysis of textile effluent before and after treatment with Lysinibacillus fusiformis JTP-23](image)

**Phytotoxicity study**

Seed germination and plant growth bioassay are the most common technique used to evaluate the phytotoxicity (Kapanen and Itavaara, 2001). Thus, it was of primary aim to assess the phytotoxicity of the dye and its metabolites after degradation by bacterial consortium (JTP-05, JTP-13 and *Lysinibacillus fusiformis* JTP-23). Germination of both seeds (*Triticum aestivum* and *Phaseolus mungo*) was less with Reactive Black 5 treatment as compared to its degradation metabolites and plain water (Figure 6.9). The length of plumule
and radicle were significantly affected by Reactive Black 5 than its degradative metabolites (Table 6.1), indicating less toxic nature of degradation metabolites as compared to dye.

Degradation metabolites, extracted on decolorization under static condition at 20 h incubation without agitation treatment were identified as Extracted metabolites - 1. On the other hand, degradation metabolites extracted after static-shaking sequential treatments were identified as Extracted metabolites - 2. Toxicity in terms of germination and growth of seeds irrigated with Extracted metabolites - 1 (color less) was less than native dye compound. This colorless compound was still toxic to plant, which may be because of the presence of aromatic amines generated during reductive cleavage of azo bond under oxygen limiting condition (Puvneshwari et al., 2006).

Table 6.2 explains results of Green B phytotoxicity study. Germination percentage of seeds (Triticum aestivum) in plates irrigated with dye and extracted metabolites were compared with water control and found to be 46.67% and 96.67%, indicating toxic nature of the dye (Figure 6.10). Phaseolus mungo also showed similar toxicity of Green B with severely affected plumule and radicle growth. Toxicity of Black BT on Triticum aestivum and Phaseolus mungo was per summarized in the table 6.2 (Figure 6.11).

Toxicity study or some textile dyes on germination and early seedling growth of four plant; clover, wheat, lettuce and tomato had been studied by Moawad and Wafaa, 2003. Similar results were reported about Reactive Blue 59 toxicity on Triticum aestivum and Phaseolus mungo (Patil and Shedbalkar, 2008). So, phytotoxicity studies revealed biodegradation of Reactive Black 5, Green B, and Black BT by bacterial consortium resulted in the detoxification of dye.
Table 6.1: Phytotoxicity study of Reactive Black 5 and its degradation metabolites on seed germination and growth. a, *Triticum aestivum*; b, *Phaseolus mungo*.

<table>
<thead>
<tr>
<th>Parameter studied</th>
<th><em>Triticum aestivum</em></th>
<th>Water</th>
<th>Reactive Black 5</th>
<th>Extracted metabolites-1</th>
<th>Extracted metabolites-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germination (%)</td>
<td>100</td>
<td>36.67</td>
<td>56.67</td>
<td>86.67</td>
<td></td>
</tr>
<tr>
<td>Plumule (cm)</td>
<td>5.55</td>
<td>1.55</td>
<td>2.30</td>
<td>4.98</td>
<td></td>
</tr>
<tr>
<td>Radicle (cm)</td>
<td>8.77</td>
<td>2.36</td>
<td>5.34</td>
<td>8.16</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter studied</th>
<th><em>Phaseolus mungo</em></th>
<th>Water</th>
<th>Reactive Black 5</th>
<th>Extracted metabolites - 1</th>
<th>Extracted metabolites – 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germination (%)</td>
<td>100</td>
<td>66.67</td>
<td>ND</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Plumule (cm)</td>
<td>4.47</td>
<td>1.19</td>
<td>ND</td>
<td>3.95</td>
<td></td>
</tr>
<tr>
<td>Radicle (cm)</td>
<td>2.26</td>
<td>0.20</td>
<td>ND</td>
<td>2.19</td>
<td></td>
</tr>
</tbody>
</table>

ND, Not determined. Mentioned values in the table are mean of ten germinated seeds of three sets.

Figure 6.9: Germination of seeds irrigated with water control, RB5 dye control and degraded metabolites respectively from left to right. a, *Triticum aestivum*; b, *Phaseolus mungo*. 

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Figure 6.10: Germination percentage of *Triticum aestivum* seeds irrigated with different samples.

Table 6.2: Effect of dye and its degradation metabolites on germination and seedlings of *Triticum aestivum* and *Phaseolus mungo*. a, Green B dye; b, Black BT dye

<table>
<thead>
<tr>
<th>Parameter studied</th>
<th><em>Triticum aestivum</em></th>
<th><em>Phaseolus mungo</em></th>
<th><em>Phaseolus mungo</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
<td>Green B</td>
<td>Extracted metabolites</td>
</tr>
<tr>
<td>-------------------</td>
<td>--------</td>
<td>---------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>Germination(%)</td>
<td>100</td>
<td>46.67</td>
<td>96.67</td>
</tr>
<tr>
<td>Plumule (cm)</td>
<td>5.56</td>
<td>0.24</td>
<td>4.60</td>
</tr>
<tr>
<td>Radicle (cm)</td>
<td>8.77</td>
<td>1.74</td>
<td>8.17</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter studied</th>
<th><em>Triticum aestivum</em></th>
<th><em>Phaseolus mungo</em></th>
<th><em>Phaseolus mungo</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
<td>Black BT</td>
<td>Extracted metabolites</td>
</tr>
<tr>
<td>Germination(%)</td>
<td>100</td>
<td>40</td>
<td>96.67</td>
</tr>
<tr>
<td>Plumule (cm)</td>
<td>4.81</td>
<td>0.82</td>
<td>4.72</td>
</tr>
<tr>
<td>Radicle (cm)</td>
<td>8.17</td>
<td>1.60</td>
<td>8.31</td>
</tr>
</tbody>
</table>
Figure 6.11: Germination of seeds irrigated with water control, Green B dye control and degraded metabolites respectively from left to right. a, *Triticum aestivum*; b, *Phaseolus mungo*.

Figure 6.12: Germination of seeds irrigated with water control, Black BT dye control and degraded metabolites respectively from left to right. a, *Triticum aestivum*; b, *Phaseolus mungo*. 
CONCLUSION

Bioremediation of environmental pollutants relies on the pollutant degrading capabilities of naturally occurring microbial consortia in which bacteria play central role. Microbial consortia are used without analyzing the constituent microbial populations for environmental remediation and the complexity of microbial consortium enables them to act on a variety of pollutants (Watanable and Baker, 2000).

Under aerobic condition, the azo dyes are non-degradable by most bacteria. However, there are reports on the decolorization of azo dye Fast red by *Bacillus subtilis* HM under aerobic condition (Mona *et al*., 2008). Microbial species of bacteria, actinomycetes and fungi are capable of removing azo dyes via biotransformation, biodegradation or liberalization (Jack *et al*., 1992) and the effectiveness of microbial treatment depends on the survival, adaptability and activity of the selected organisms (Kothari *et al*., 2005).

Employing static-shaking sequential treatment along with designer consortium was successful in not only decolorization of dyes but extensive degradation of the dyes was achieved. This result was supported by sharp reduction in toxicity of degradation metabolites on the germination and early seedling growth in *Triticum aestivum* and *Phaseolus mungo*, when compared with original dye compound.