Chapter III

Molecular study on the decolorization and degradation of Reactive Black HFGR and Disperse Red F3BS
Azo Dyes

Dyes may be classified according to chemical structure or by their usage or application method. The former approach is adopted by practicing dye chemists who use terms such as azo dyes, anthraquinone dyes, and phthalocyanine dyes. The latter approach is used predominantly by the dye user, the dye technologist, who speaks of reactive dyes for cotton and disperses dyes for polyester. Very often, both terminologies are used, for example, an azo disperse dye for polyester and a phthalocyanine reactive dye for cotton. Azo dyes, which are aromatic compounds with one or more (–N=N–) groups, are the most important and largest class of synthetic dyes used in commercial applications (Vandevivere et al., 1998). They are considered as xenobiotic compounds that are very recalcitrant to biodegradation processes (Zollinger, 1991; Stolz, 2001). The textile industry accounts for two-thirds of the total dyestuff market. During the dyeing process, approximately 10–15% of the dyes used are released into the wastewater. The presence of these dyes in the aqueous ecosystem is the cause of serious environmental and health concerns (Fang et al., 2004; Asad et al., 2007).

Color is a physiological sensation associated with the wavelength of light striking the retina of the eye. The sensation of color is produced when light having a wavelength within the visible region of electromagnetic spectrum strikes the retina of the eye. The visible region of the spectrum extends from 4000 to 7500 Å in wavelength.
Otto Witt Theory of Color (1876)

An early theory of dyes first formulated by O. Witt provided a basis for understanding the reaction between color and structure of the molecule. According to the O. Witt color theory a dye is made up of two essential kinds of parts, Chromophores and Auxochromes. Chromophores are unsaturated groups. Presence of at least one such group is essential to produce a color in an organic compound and a molecule containing such a group is called as chromogen (Mansoor, 2008). Some most effective chromophores are

Thus for example nitrobenzene is pale yellow, azobenzene is orange-red, $p$-quinones are yellow and $o$-quinones are orange or red. Certain other unsaturated groups produce color only when several of them are present in a molecule and when they are conjugated. They are as follows
Thus though acetone is colorless, biacetyl is color. Certain groups, while not producing color themselves, are able to intensify the color when present in a molecule together with a chromophore. These are called auxochromes (Gr, *auxanein* = to increase). The most effective auxochromes are given below.

<table>
<thead>
<tr>
<th>-OH</th>
<th>-OR</th>
<th>-NH₂</th>
<th>-N–R</th>
<th>-NR₂</th>
</tr>
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<tbody>
<tr>
<td>Hydroxyl</td>
<td>Alkox</td>
<td>Amino</td>
<td>Alkylated</td>
<td>Amines</td>
</tr>
</tbody>
</table>

Thus nitrophenols and nitroanilines are more intensely colored than nitrobenzene and aniline and are deep yellow to orange. Further auxochromes are salt forming groups, *i.e.*, they are basic or acidic and makes the colored compound to attach itself to the fabric, so that it is fast to light, soap and water. Acidic auxochromes like –OH, --COOH and –SO₂H give acidic dyes and basic auxochromes like –NH₂, –NHR and –NR₂ gives basic dyes. Auxochromes like –SO₃H group has little value as auxochrome but it has a solublishing effect. The halogen atom also functions as auxochrome and the relative order of color intensifying effect is I>Br>Cl. It can be observed that all the auxochromic groups contain atoms with unshared pair of electrons.

**Valence Bond Approach to Color**

Like many other theories, the *Witt* theory has also been replaced by modern electronic theory. According to this theory, it is the resonance stabilization of excited states that is responsible for the absorption in the visible region. When ultraviolet or visible light
is absorbed by a molecule, an electron is excited, that is, it is promoted to an orbital of higher energy. The wavelength of light absorbed depends on the energy difference between the excited and ground states of the molecule. The smaller difference between the two states, the longer is the wavelength of the light absorbed.

The energy required to promote an electron depends upon the environment of the electron. Sigma (σ) bond electrons are firmly held and very high energy (or short wavelength) is necessary to promote electrons and may at times break the molecule and form free radical. Pi (π) electrons are less firmly held and require less energy (or longer wavelength) to excite. Electrons belonging to conjugated systems required even less energy (still longer length). Conjugation and resonance stabilize the excited state by sharing and delocalizing higher energy of the excited electron. As conjugation and resonance increases, the wavelength of light absorbed also increases and when the wavelength is long enough to be in the visible region, we observe color. This can be explained with the help of following example.

**Bathochromic Effect**

In this case benzene ring may be considered to be chromophore, while amino group and nitro group auxochromes. When they are conjugated, the longer resonance system decreases the energy gap between the ground state and excited state transitions, thus producing visible color. All these groups, which lengthen wavelength of absorption, are bathochrome groups. Thus displacements (or shift) to longer wavelength are known as
bathochromic effects or bathochromic shift and displacements to shorter wavelength are hypsochromic. Hypsochromes are groups which decrease resonance. This is done by forcing the pi (π) orbitals out of planarity. As the number of fused rings increases, the absorption in the visible region also increases e.g. naphthalene absorbs in blue region and is yellow. Pentacene absorbs in orange region and is blue. Graphite, which is a sheet of benzene rings is black, it absorbs all colors almost completely.

**Spectral Analysis – A Molecular Signature**

Different molecules absorb radiation of different wavelengths. An absorption spectrum is a function of the molecular constituency where each entity will absorb in the corresponding wavelength. For example, the absorption that is observed in the UV region for the carbonyl group in acetone is of the same wavelength as the absorption from the carbonyl group in diethyl ketone. The absorption of UV or visible radiation corresponds to the excitation of outer electrons. When an atom or molecule absorbs energy, electrons are promoted from their ground state to an excited state. Beside these changes, in a molecule, the atoms can rotate and vibrate with respect to each other. In dyes these atoms or molecules happen to be specific or characteristic constituent such as the functional groups where vibrations and rotations of these constituent atoms/groups have discrete energy levels, which can be considered as being packed on top of each electronic level. Hence, the spectral analysis in the UV, Visible and Infrared region represents the molecular characteristics of the dye component.
Colored wastewater is a consequence of batch processes both in the dye manufacturing industries and in the dye-consuming industries. Two per cent of dyes that are produced are discharged directly in aqueous effluent, and 10% are subsequently lost during the textile coloration process (Easton, 1995). The main reason for dye loss is the incomplete exhaustion of dyes on to the fibre. The amount of dye lost is dependent upon dyestuff type, the application route and the depth of shade required (Willmott, 1997). Colored wastewater is particularly associated with those reactive azo dyes that are used for dyeing cellulose fibres. These dyes make up approximately 30% of the total dye market (Kamilaki, 2000). Residual color is a problem with reactive dyes because, in current dyeing processes, as much as 50% of the dye is lost in the wastewater. These losses are due to the relatively low levels of dye-fibre fixation and to the presence of unreactive hydrolysed dye in the dye-bath. Dye hydrolysis occurs when the dye molecule reacts with water rather than with the hydroxyl groups of the cellulose. These problems are compounded by the high water solubility and characteristic brightness of the dyes.

Due to their stability and to their xenobiotic nature, reactive azo dyes are not totally degraded by conventional wastewater treatment processes that involve light, chemicals or activated sludge (Leisinger et al., 1981). The dyes are therefore released into the environment, in the form of colored wastewater. This can lead to acute effects on exposed organisms due to the toxicity of the dyes, abnormal coloration and reduction in photosynthesis because of the absorbance of light that enters the water (Slokar and Le, 1998; Strickland and Perkins, 1995). Also, public perception of water quality is greatly influenced by the color. The presence of unnatural colors is aesthetically unpleasant and
tends to be associated with contamination (Waters, 1995). Removal of azo dyes in general involves adsorption to activated sludge and aerobic biodegradation occurs very slowly. Some azo dyes induce liver nodules in experimental animals and there is a higher incidence of bladder cancer, splenic sarcomas, hepatocarcinomas and nuclear anomalies in experimental animals and chromosomal aberrations in mammalian cells. Some azo dyes induce liver nodules in experimental animals and there is a higher incidence of bladder cancer in dye workers exposed to large quantities of azo dyes. In mammals, both hepatic and bacterial azoreductases reduce the azo compounds to their corresponding amines (Bragger et al., 1997). Bacterial azoreductases are more active than hepatic azoreductases in reducing azo dyes and are capable of converting some azo dyes to mutagenic and carcinogenic amines.

**Exclusive Study of Representative Dyes - Reactive HFGR & Disperse Red F3BS**

Azo dye Reactive Black HFGR (Chemical Index No. 20505, chemical formula \( \text{C}_{26}\text{H}_{21}\text{N}_{5}\text{Na}_{4}\text{O}_{19}\text{S}_{6}, \text{W} \ 991.82 \ \text{g} \ \text{mol}^{-1}, \text{and } \lambda_{\text{max}} \ 599 \ \text{nm}, \)) was selected as the model azo dye for the characterization of the breakdown process during decolorization using the best decolorizing-bacteria. The IUPAC name of the dye is Tetrasodium (6E) -4-amino-5-oxo-3-[4-(2-sulfonatoxy ethylsulfonyl) phenyl]diazenyl-6-[[4-(2-sulfonatoxy ethyl sulfonyl) phenyl] hydrazinylidene] naphthalene-2,7-disulfonate. The structure of the dye is represented in the Figure 3.1.
C$_{20}$H$_{22}$N$_6$O$_2$S is the molecular formula for the Disperse Red F3BS dye having the Chemical Index name Disperse Red 343. The structure of the dye is represented in Figure 3.2. The CAS no. of the dye is 99031-78-6 and has a molecular weight of 410.0 (g mol$^{-1}$).

Knowledge of biological decolorization of azo dyes is still inadequate and especially with reference to Reactive Black HFGR and Disperse Red F3BS. The azo dyes which are widely used in textile dyeing and is reported to be toxic too (Fatemeh et al., 1990; Lars and Mallika 1997). In this study, the optimal conditions for decolorization of Reactive Black HFGR and Disperse Red F3BS have been investigated. After the evaluation of two groups of dyes (such as Reactive and Disperse) for decolorization in earlier chapters, an attempt was
made to characterize the process of decolorization and degradation with the representative compounds such as Reactive Black HFGR and Disperse Red F3BS. For this, dyes were subjected to UV-Vis and IR spectral analysis after subsequent decolorization process by the best decolorizers of the respective dyes.
Materials and Methods

Dye(s)

All chemicals and reagents were of Analytical grade purchased from Himedia, Mumbai, India. The common name of all dyes has been used for convenience; the dyes were procured from the Textile Industry in Tirupur and Mettupalayam, Tamil Nadu, India. The dyes were Reactive Black HFGR and Disperse Red F3BS. Prior to the degradation study, the conditions were optimized for the decolorization by the best decolorizers.

Organism Chosen

Reactive Black HFGR

Based on the earlier study as described in Chapter I, the best decolorizers of Reactive Black HFGR such as the SK03, SK20 and SK21 were chosen for the optimization of decolorization conditions and degradation study thereof.

Disperse Red F3BS

Similarly, among the 46 bacteria isolated from the United Bleachers Pvt. Ltd., Mettuppalayam, Tamil Nadu, India, the JMC-UBL 02 was selected for decolorization
experiments and degradation studies. *JMC-UBL 02* was chosen based on the best decolorizing capacity of the isolate as described in Chapter II.

**Optimization of the Decolorization Conditions**

The Reactive Black HFGR and Disperse Red F3BS were decolorized by using best decolorizing bacterial isolate. Optimization of the decolorization was carried out at various pH, Temperature, shaking and static conditions and varying concentrations of the dye. All these decolorization study was performed in Nutrient Broth with triplicates and respective controls. Percentage of decolorization was determined as explained in Chapter I.

**Effect of Temperature**

Both the dyes were studied for their progress of decolorization in static conditions at various temperatures such as 20°C, 30°C, 40°C and 50°C. A loopful of log phase pure culture was inoculated into 250 ml Erlenmeyer flask containing 100 ml nutrient broth with the dye concentration of 100mg/l (Kalyani *et al.*, 2008). The pH was set at 7.4. Controls were maintained simultaneously and the optical density was measured at respective $\lambda_{max}$. 

Effect of Shaking and Static Conditions

Reactive Black HFGR and Disperse Red F3BS were similarly studied for its decolorization by respective isolates in the static and shaking conditions set at 37°C. Flasks were kept in shaker-Incubator set at 37°C and 150 rpm (Mohandas et al., 2008). The pH was set at 7.4. Controls were maintained simultaneously and the optical density was measured at respective $\lambda_{\text{max}}$.

Effect of pH

The chosen dyes were studied for its decolorization by respective best decolorizing isolates in Nutrient Broth as described above but setting different pH: 3 to 10. The flasks were maintained in triplicates and respective controls in 37°C and at static condition.

Effect of Concentration of the Dyes

Decolorization experiment was studied in Reactive Black HFGR and Disperse Red F3BS by the respective bacterial isolates under different concentrations of the Dyes. For this, 25, 50, 75 and 100 mg/l concentration of the respective dyes were used. The flasks were set at 7.4 pH and kept at 37°C in static condition. Controls were maintained simultaneously and the optical density was measured at their respective $\lambda_{\text{max}}$. 
Decolorization in Minimal media

The two dyes were subjected to similar decolorization experiments under Minimal media (Cappuccino and Sherman, 2004) following the optimal conditions (pH, Temperature, Static/Shaking and Dye concentration as known from the above studies).

Analysis of the Decolorized Product - UV–Vis Spectral Analysis

The samples were collected before and after the decolorization processes (anoxic and aerated conditions) and filtered through 0.2 µm membrane filters. The filtrates were then scanned in the UV-Vis Spectrophotometer (Schimadzu UV-Vis 1800, Japan) within the range of 200 – 800nm. Appropriate blank was also subjected to the scanning process. The absorbance was noted at the respective characteristic peak area ($\lambda_{\text{max}}$) for the interpretation of results (Sagarika et al., 2006).

FTIR Analysis

The controls and samples were dried and mixed with KBr (1:20; 0.02 g of sample with KBr at a final weight of 0.4 g). The samples were then ground, desorbed at 60°C for 24 h and pressed to obtain IR-transparent pellets. The absorbance FT-IR spectra of the samples were recorded using FT-IR (Schimadzu, Japan) spectrometer. The spectra were
collected within a scanning range of 400–4000 cm\(^{-1}\). The FT-IR was first calibrated for background signal scanning with a control sample of pure KBr, and then the experimental sample was scanned. The FT-IR spectrum of the control was finally subtracted from the spectra of the non-degraded and degraded dyes.

**HPTLC**

Degradation of dyes was monitored on pre-coated TLC silica gel F\(_{254}\) TLC plates (10 cm x 10 cm aluminium sheets, Merck, Germany). For this, cell-free supernatant of the decolorized dyes of Reactive Black HFGR and Disperse Red F3BS were used. Broths were extracted twice with an equal volume of \(n\)-butanol to extract the residual dye and biotransformed products of Reactive Black HFGR and Disperse Red F3BS. The pooled extracts were concentrated on a rotary vacuum evaporator. A 15\(\mu\)l of the sample was spotted (as a band) on TLC plates using a microsyringe (HPTLC, Camag, Linomat 5 and Camag TLC Scanner3, Switzerland). The solvent system used was Methanol/Propanol/Water/Ammonia (3:6:1:4, by volume) and Butanol/Water/Methanol (4:5:1, by volume) for the Reactive Black HFGR and Disperse Red F3BS respectively. The dye chromatograms were observed by exposure to ultraviolet light (302 and 365 nm).
Results

Black HFGR- Absorption Spectrum

The absorption spectrums of the Reactive Black HFGR were studied from 250 nm to 900 nm in a double beam UV-Vis Spectrophotometer (Schimadzu UV-Vis 1800, Japan). From the optical density at 1 nm bandwidth, the absorption maximum was found to be at 598 nm. The absorption spectrum screen of the analysis software has been captured and presented.

Optimization of Decolorization conditions for Reactive Black HFGR in Nutrient Broth

Effect of Temperature on the Decolorization by SK03, SK20 and SK21

Reactive Black HFGR dye was studied for its decolorization ability at various temperatures such as 20, 30, 40 and 50°C. All the flasks were maintained in static conditions inside incubators set at respective temperatures. pH was set at 7.4 in all the flasks. Controls were maintained simultaneously and the optical density was measured at 600 nm at every 2 hours interval until 24 hours. Optimum temperature for SK03 was found to be 40°C with 57.8% (Fig.3.3). That of SK20 and SK21 demonstrated decolorization of up
to 57% and 97% (Fig. 3.4 & Fig.3.5), respectively at 40°C. Of these, SK21 showed the highest decolorization ability and 40°C was found to be the optimum temperature for the decolorization.

Effect of Shaking and Static Conditions in the Decolorization by SK03, SK20 and SK21

Reactive Black HFGR dye was studied for its decolorization by all the three isolates SK03, SK20 and SK21 in the static and shaking conditions set at 37°C. The pH was set at 7.4. Controls were maintained simultaneously and the optical density was measured at 600 nm at every 2 hours interval until 24 hours. For all the bacterial isolates, static condition demonstrated the highest decolorization. SK21 demonstrated highest decolorization with 95.5% (Fig. 3.8), whereas, SK03 and SK20 produced 55% and 53.7% (Fig. 3.6 and Fig. 3.7) respectively.

Effect of pH on the Decolorization by SK03, SK20 and SK21

Reactive Black HFGR was decolorized by all the three isolates SK03, SK20 and SK21 and followed for every 2 hours at various pH (such as 3 to 10). The inoculated flasks were kept at 37°C in the Incubated shaker under 150 rpm. Controls were maintained simultaneously and the optical density was measured at 600 nm at every 2 hours until 24 hours. SK03 showed highest decolorization ability of up to 54.2% at pH 6.0 (Fig. 3.9). SK20
showed maximum decolorization of 46% (Fig. 3.10) at pH 7.0 while that of SK21 demonstrated a maximum 57.4% decolorization at pH 8.0(Fig. 3.11).

**Effect of Concentration of the Dye on Decolorization by SK03, SK20 and SK21**

Reactive Black HFGR dye was studied for its decolorization by all the three isolates SK03, SK20 and SK21 at various concentration of the dye itself. For this, 25, 50, 75 and 100 mg/l concentration of the dye was used. The flasks were set at 7.4 pH and kept at 37°C in the Incubator-shaker under 150 rpm. Controls were maintained simultaneously and the optical density was measured at 600 nm at every hour until 24 hours. For SK03 75 mg/l dye concentration was optimum with 52% (Fig. 3.12) of decolorization while that of SK20 and SK21 showed 44.1% (Fig. 3. 13) at 100mg/l dye concentration and 91.1% (Fig. 3.14) at 50 mg/l dye concentration respectively.

**Decolorization of Black HFGR in Minimal Media under Optimized Conditions**

When Reactive black HFGR was studied for the optimization of decolorization in Nutrient media, SK21 was found to be the best decolorizing bacterial strain among the three tested and therefore, decolorization of the dye was performed in Minimal media using SK21 following the optimized conditions (pH 8.0, 40°C at 100mg/l of dye
concentration in static condition). In this, SK21 was found to decolorize the dye upto 97.67 ± 0.33 %. (Fig. 3.19).

**Disperse Red F3BS - Absorption Spectrum**

The absorption spectrum of Disperse red F3BS was recorded from 200 to 900 nm in a double beam UV-Vis spectrophotometer (Schimadzu UV-Vis1800, Japan). The spectrum of the dye is presented in Fig. 3.20

**Optimization of Decolorization conditions for Disperse Red F3BS in Nutrient Broth**

During the screening of the 46 isolates for the decolorization of the Disperse Red F3BS, JMC-UBL02 strain alone demonstrated significant decolorization (as described in Chapter II) and therefore, the strain JMC-UBL02 was chosen for the optimization of decolorization conditions.

**Effect of various Temperature on the Decolorization of Disperse Red F3BS**

For the optimization of temperature, nutrient broth containing 100mg/l of the dye was incubated at 20, 30, 40 and 50° C in static condition. From this, 50° C temperature was
found to be optimum demonstrating upto 90.40 ± 0.23 %. Interestingly decolorization was achieved to about 87.71 ± 0.41 % within 5 hours of incubation (Fig. 3.15).

**Effect of Shaking and Static Conditions on the Decolorization of Disperse Red F3BS**

Static condition demonstrated the rapid decolorization of dispersed red F3BS (approx 85%) by UBL 02 strain within 7 hours of inoculation, where as shaking condition could decolorize less than 10% within 7 hrs of inculcation. (Fig. 3.16).

**Effect of various pH on the Decolorization of Disperse Red F3BS**

As decolorization was achieved at a faster rate in static condition, further optimization experiments were carried out in static conditions alone. Among various pH tested (3 to 10), pH 8 was observed to be the optimum pH for maximum decolorization of the dye by JMC-UBL02 (83.40 ±0.51 5) followed by pH7 (82.50 ± 0.23 %) and pH 9 (81.50 ± 0.16 %). As observed for the temperature, this decolorization was achieved within 7 hours (Fig. 3.17.).
Effect of Various Concentration of Disperse Red F3BS

Different concentrations of the dye Disperse Red F3B such as 25, 50, 75 and 100 mg/l was performed to optimize the concentration of the dye. Results revealed that difference in dye concentration did not vary significantly. The average decolorization of the dye at 50, 75 and 100mg/l was found to be 87% and that of 25mg/l was 90%. As this experiment was also performed at static condition, decolorization was achieved within 7 hours of incubation (Fig. 3.18).

Effect of Minimal Media on the Decolorization of Disperse Red F3BS

The decolorization was performed using JMC-UBL02 in minimal media following the optimal conditions (Temperature = 50ºC; pH = 8, dye Concentration = 100 mg/l; Static Condition) as revealed by the above experiments. Results were not encouraging as the decolorization was not beyond 10% even after the incubation for 48 hours.

Analysis of the Degraded Product

Reactive Black HFGR

The biodegradation of the dye Reactive Black HFGR was monitored by UV-Vis spectrophotometric analysis. For the untreated dye, as shown in Figure 3.19, the solid line
represents the large absorbance peaks at 580 and 325 nm for the treated dyes. After biodecolorization in the microaerophilic condition, the absorbance peaks in the visible region disappeared i.e., the absorbance peak at 580 nm completely disappeared, indicating the complete decolorization and deformation of the structural conformation that was responsible for the color. In the UV – region, the peaks at 258 disappeared and new peaks at 362 nm appeared after aeration (following decolorization by the *Paenibacillus lautus* SK21).

**Disperse Red F3BS**

The biodecolorization of Disperse red F3BS was similarly monitored by UV-Vis spectrophotometric analysis before and after the treatment with *E. faecalis* JMC-UBL02 under microaerophilic condition. Before decolorization, the dye indicated peak absorbance’s at 544 and 520 nm in the visible region. These two peaks totally disappeared and remained flat after the biodecolorization process as an evidence for the removal of color due to the dye. In the UV region, peak at 288 disappeared and new peaks at 248 nm appeared after decolorization process (Fig. 3.20).

**FTIR Analysis of Reactive Black HFGR dye**

The FTIR spectra of Black HFGR and metabolites obtained after the decolorization suggests that biodegradation occurred. The FTIR spectrum of Black HFGR showed peaks
at 777/cm for S-O stretching vibrations, 648/cm for C-H stretching vibrations, 931/cm for C-N bending vibrations and 1037/cm for S-O stretching vibrations. These vibrations <1000/cm are said to be the finger print zone. The group frequency region showed specific peaks for the functional groups; peaks at 1386/cm for N=N stretching vibrations, 1541/cm for N-CH$_2$- bending vibrations, 1610/cm for the C-H bending vibrations, 2929/cm for C-H stretching vibrations (Figure 3.21).

On the other hand, the FTIR spectrum of metabolites obtained after (microaerophilic) decolorization (Figure 3.22) and aerated decolorization (Figure 3.23) of Black HFGR showed peaks at 1099/cm for N-H stretching vibrations, 1448/cm for C=C stretching vibrations, 1610/cm for C-C stretching vibrations, 2858/cm, 2873/cm and 2927/cm for CH$_2$ and CH stretching vibrations. After the decolorization of the dye, there is a characteristic doublet formation (two-bands) that appeared with peaks at 3421/cm and 3246/cm for N-H (symmetrical and asymmetrical) stretching vibrations. the absence of the peak at 1386/cm suggests that the azo bond was cleaved.

**FTIR Analysis of Disperse Red F3BS dye**

The considerable differences in FTIR spectrum of disperse red F3BS and degradation product indicate the biodegradation of the dye. The FTIR spectra of the native dye showed specific peaks in finger print region for substituted benzene ring which is supported by the peaks at 1022/cm, 1078/cm and 1097/cm for the –C-C-C bending vibrations and the peak at 1242/cm asymmetric SO3 stretching vibrations (Figure 3.24).
The group frequency region showed specific peaks for functional groups. The peak at 1654/cm for \(-N=N-\) stretching vibrations of azo group and the peaks at 2937/cm and 3421/cm for OH group. The FTIR spectrum of extracted product showed a peak at 1597/cm and 1570/cm which is in support of the loss of azo bond and formation of N-H (primary) amine (Figure 3.25).

**HPTLC Analysis of the Dyes**

The dye decolorization study was further supported by TLC analysis. Native dye and the break-down metabolites were separated by HPTLC and analyzed by winCATS Planar Chromatography Manager.

**Reactive Black HFGR**

Three tracks of samples such as the native, decolorized dye under anoxic and aerated states were developed. Under white light illumination, the color of the native dye was observed in track 1 while there were no colors seen in track 2 and 3 showing that the decolorized dye samples did not have any color of its own (Figure 3.26-A). To detect bands of the break-down metabolites, the plate was illuminated under 365 nm and the observations were recorded. Track 1 (Native form of Reactive Black HFGR) resolved into four bands with respective \(R_f\) values and Area Under Curve (AUC) as mentioned in Table 3.1. Decolorized dye under anoxic condition resolved into 5 bands representing five different metabolites with the complete disappearance of the bands corresponding to the
native dye. The \( R_f \) values and AUC are presented in Table 3.1. Decolorization under aerated condition also represented 5 different bands. However, these metabolites showed different \( R_f \) values demonstrating the fact that they were not identical (Table 3.1). The chromatogram under UV illumination (at 365nm) is presented in Figure 3.26-B; 28; 29; 30 & 31. Tracks 2 and 3 on the figure showed the presence of metabolites that is distinct from that of the native form. This is direct evidence that, Reactive Black HFGR dye has undergone structural changes rendering the dye colorless.

**Disperse Red F3BS**

HPTLC analysis of the Disperse Red F3BS and the decolorized form of the dye clearly indicated its breakdown. The native form of the dye resolved into 8 distinct bands with \( R_f \) values 0.18, 0.26, 0.32, 0.36, 0.52, 0.61, 0.96 and 0.98. The decolorized sample of the dye in Track 2 showed complete disappearance of the bands corresponding to the dye with concomitant appearance of two new bands with \( R_f \) values 0.29 and 0.62 (Table 3.2; Figure 3.27; 32; 33 & 34) indicating the conversion of dye molecule into some other metabolites during the treatment process.
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*Table 3.1: Analysis report of the win CATS Planar Chromatography Manager for the separation of Reactive Black HFGR dye and decolorized products by HPTLC*
<table>
<thead>
<tr>
<th>Track</th>
<th>Peak</th>
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<th>AUC</th>
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*Table 3.2: Analysis report of the win CATS Planar Chromatography Manager for the separation of Disperse Red F3BS dye and decolorized products by HPTLC*
Figure 3.3. Effect of temperature on the decolourization of Black HFGR by SK03

Figure 3.4. Effect of temperature on the decolourization of Black HFGR by SK20
Figure 3.5. Effect of temperature on the decolourization of Black HFGR by SK21

Figure 3.6. Effect of shaking and static conditions on the decolourization of Black HFGR by SK03
Figure 3.7. Effect of shaking and static conditions on the decolourization of Black HFGR by SK20

Figure 3.8. Effect of shaking and static conditions on the decolourization of Black HFGR by SK21
Figure 3.9. Effect of various pH on the decolourization of Black HFGR by SK03

Figure 3.10. Effect of various pH on the decolourization of Black HFGR by SK20
Figure 3.11. Effect of various pH on the decolourization of Black HFGR by SK21

Figure 3.12. Effect of various dye concentrations on the decolourization of Black HFGR by SK03
Figure 3.13. Effect of various dye concentrations on the decolourization of Black HFGR by SK20

Figure 3.14. Effect of various dye concentrations on the decolourization of Black HFGR by SK21
Figure 3.15. Effect of various temperatures on decolourization of disperse red dye F3BS by UBL-02

Figure 3.16. Effect of shaking and static condition on decolourization of Disperse Red F3BS by UBL-02
Figure 3.17. Effect of various pH on the decolourization of Disperse Red F3B by UBL-02

Figure 3.18. Effect of various dye concentrations on decolourization of Disperse Red F3B by UBL-2
Figure 3.19: Graph representing the absorption spectrum of the Reactive Black HFGR dye before and after decolorization followed by aeration (for oxidation of the dye); Solid line – spectrum of the dye; dashed line – spectrum of the decolorized dye; dotted line – spectrum of the decolorized and aerated dye.
Figure 3.20: Graph representing the absorption spectrum of Disperse Red F3BS following optimum conditions in minimal media; Solid line – dye before decolorization; dotted line – dye after decolorization.
Figure 3.21: FTIR spectrum of Reactive Black HFGR before decolorization.
Figure 3.22: FTIR spectrum of Reactive Black HFGF after (micro-aerophilic) decolorization.
Figure 3.23: FTIR spectrum of Reactive Black HFGR after decolorization (aerated condition after micro-aerophilic decolorization process)
Figure 3.24: FTIR spectrum of Disperse Red F3BS before decolorization.
Figure 3.25: FTIR spectrum of Disperse Red F3BS after decolorization.
Figure 3.26: Chromatogram of Reactive Black HFGR, under white light (A) and UV illumination (B).

Figure 3.27: Chromatogram of Disperse Red F3BS, under white light (A) and UV illumination (B).
Figure 3.28: HPTLC 3D (a) and 2D (b) display scanned analysis of native form and decolorization products of Reactive Black HFGR.
Figure 3.29: HPTLC display scanned analysis of the native form of the dye – R. Black HFGR on Track 1 with 4 peaks.

Figure 3.30: HPTLC analysis of the (microaerophilic) decolorization products of R. Black HFGR on Track 2 with 5 peaks.

Figure 3.31: HPTLC analysis of the (aerated) decolorization products of R. Black HFGR on Track 3 with 5 peaks.
Figure 3.32: HPTLC 3D (a) and 2D (b) display scanned analysis of the native form and decolorization products of Disperse Red F3BS.
Figure 3.33: HPTLC display scanned analysis of the native form of the dye Disperse Red F3BS on Track 1 with 8 peaks.

Figure 3.34: HPTLC display scanned analysis of the decolorized products of Disperse Red F3BS on Track 2.
Scheme 1: Tentative mechanism for anaerobic decolorization Reactive Black HFGR and metabolite formation.
Discussion

Knowledge of biological decolorization of textile dyes are still inadequate especially with reference to azo group of dyes. The bacterial reduction of the azo bond is usually nonspecific and bacterial decolorization is normally faster. Research on bacterial strains that are able to decolorize azo dyes under aerobic (Xenophylus azovorans KF46F, Bacillus strain, Kerstersia sp. strain VKY1 and Staphylococcus sp.) and anaerobic conditions (Sphingomonas xenophaga BN6, Eubacterium sp., Clostridium sp., Butyrvibrio sp. or Bacteroides sp.) have been extensively reported (Rafii et al., 1990; Kudlich et al., 1997; Suzuki et al., 2001; Blumel et al., 2002; Olukanni et al., 2006; Dos Santos et al., 2007; Vijaykumar et al., 2007; Hsueh and Chen, 2008; Lin and Leu, 2008).

Ecosystems are dynamic environments with variable abiotic conditions, like pH, temperature, presence of oxygen, metals, salts, etc. Microorganisms, which have a key role in the global C, N, and S cycles, are affected by changes in these parameters, and consequently, their decomposing activities are also affected. Thus, while evaluating the potential of different microorganisms for degrading particular organic xenobiotics, the effects of these parameters are to be taken into account. Optimization of such abiotic conditions will greatly help in the development of industrial-scale bioreactors for bioremediation.
Generally, fungi and yeasts show better decolorization and biodegradation activities at acidic or neutral pH while bacteria at neutral or basic pH. Nozaki et al. (2008) studied the decolorization of 27 different dyes, including monoazo, diazo, phthalocyanine, and triphenylmethane dyes, using 21 different basidiomycetes. They found that the optimum pH for the decolorization of the dyes was 3.0–5.0. The *Paenibacillus lautus* SK03 demonstrated a significant decolorization only at pH higher than 7. This is in accordance with Wang et al. (2009) who studied decolorization of Reactive Black 5 by a bacterial strain *Enterobacter* sp. EC3. According to their results, *Enterobacter* sp. EC3 showed a high decolorization rate at pH 7.0 after 108 h of incubation. Similar decolorization efficiency was observed from pH 8.0–12.0 in 120 h, whereas the rate of color removal was much lower at acidic conditions (pH 4.0 and 6.0). This could be due to the fact that the optimum pH for the growth of *Enterobacter* sp. EC3 was neutral. Saratale et al. (2009a) studied decolorization and biodegradation of Scarlet R by a microbial consortium- GR consisting of two bacterial strains, *Proteus vulgaris* NCIM-2027 and *Micrococcus glutamicus* NCIM-2168. They found that percent decolorization of Scarlet R at pH 5, 6, 7, and 8 was 62, 82, 100, and 100 after 24, 24, 14, and 36 h, respectively, by *P. vulgaris* while 55, 65, 100, and 100 after 24, 24, 20, and 48 h, respectively, by *M. glutamicus*. From these results, it is clear that the favorable pH for dye decolorization by these bacterial strains was 7–8 with optimum pH being 7 (Hazrat, 2010).

Temperature is an important environmental factor, and the biodegradation activities of microorganisms are affected by changes in temperature. It is a common observation that dead bodies of animals are decomposed faster in summer than in winter.
It is because, in summer, the warmer environment is favorable for the growth and multiplication of the decomposers (mostly soil bacteria and fungi). However, this relationship is not linear beyond a certain temperature optimum temperature for the growth and reproduction of the concerned microorganisms. Beyond the optimum temperature, the degradation activities of the microorganisms decrease because of slower growth and reproduction rate and deactivation of enzymes responsible for degradation. Thus, the biodegradation performance of microorganisms will be best at the optimum temperature needed for their growth, reproduction, and activities. Different fungi have different optimum growth temperatures, with most of them growing at 25–35°C (Fu and Viraraghavan 2001). According to Shedbalkar et al. (2008), the optimum temperature for decolorization of Cotton Blue by *P. ochrochloron* MTCC 517 was 25°C. Jadhav et al. (2008) studied decolorization of Methyl Red by *G. geotrichum* (yeast) at different temperatures, i.e., 5°C, 30°C, and 50°C. The optimum temperature for decolorization was found to be 30°C. According to Saratale et al. (2009a), when the decolorization of Navy Blue HER by *T. beigelii* (yeast) was studied at various temperatures (30–50°C), faster decolorization was observed at 37°C within 24 h incubation. Wang et al. (2009) studied decolorization of Reactive Black 5 by a bacterial strain *Enterobacter* sp. EC3. They found that, with an increase in temperature from 22°C to 37°C, the decolorization rate increased and a further increase in temperature to 42°C drastically affected decolorization activity of *Enterobacter* sp. EC3. The optimum temperature for decolorization was found to be 37°C.

It has been reported that the significant suppression of decolorization activity at 42°C might be due to the loss of cell viability or deactivation of the enzymes responsible for
decolorization at 42°C. Saratale et al. (2009a) studied the effect of temperature on decolorization of Scarlet R by a microbial consortium consisting of two bacterial strains, *P. vulgaris* and *M. glutamicus*. They found that the percent decolorization of Scarlet R at 30, 37, 40, 45, and 50°C was 100, 100, 90, 82, and 45 after 24, 14, 24, 36, and 48 h, respectively, by *P. vulgaris* while 100, 100, 94, 70, and 50 after 36, 20, 30, 36, and 48 h, respectively, by *M. glutamicus*. These results show that favorable temperature range for decolorization by these strains was 30–37°C, with optimum temperature being 37°C. All the above-mentioned studies indicate that microorganisms degrade synthetic dyes best in the range of 25–37°C. In contrast to this, there is marked increase in the decolorization of the dyes with the increase in temperature up to 50°C in case of Disperse Red F3BS in the present work.

The effect of initial dye concentration on microbial decolorization of synthetic dyes is well studied. The decolorization of dye decreases with increasing dye concentration. It is clear that, by increasing the initial dye concentration, the decolorization is decreased considerably. This decrease in decolorization with increase in initial dye concentration is attributed to the toxicity of the dyes to the growing microbial cells at higher dye concentrations. According to Gopinath et al. (2009), during the biodegradation of Congo Red by a strain of *Bacillus* sp., obtained from tannery industry effluent, the increase in initial dye concentration decreased the decolorization rate, and at high concentrations (1,500 and 2,000 mg L\(^{-1}\)), inhibition was observed. According to Jirasripongpun et al. (2007), *Enterobacter* sp. was unable to grow in higher dye concentration, as it was dead when dye concentrations of 50 and 100 mg L\(^{-1}\) of Reactive Red 195 were used to test its
decolorizing activity. The dye was considered toxic to the cells at high dye concentrations. Similarly, the results of Parshetti et al. (2006) have indicated toxicity of malachite green to *Kocuria rosea* MTCC 1532 at higher dye concentration. Eichlerova et al. (2006) studied synthetic dye decolorization capacity of a white-rot fungus *Dichomitus squalens*. They found that Malachite Green and Crystal Violet caused strong growth reduction even at small concentrations (50 mg L$^{-1}$). Malachite Green at a concentration of 100 mg L$^{-1}$ inhibited the growth of *D. squalens* completely. Due to toxicity of the dyes to the microbial cells, the production of microbial biomass remains lower at higher dye concentrations. In accordance to this, the present study also reveals a similar trend, where there is higher percent of decolorization when the dye is at lower concentrations such as 25mg.L$^{-1}$ and 50mg.L$^{-1}$. However, former decreases as the concentration increases. This indicates the stress to the decolorizing bacteria and therefore its toxic nature at higher concentrations.

Based on the available literature, it can be concluded that the microbial decolorization of azo dyes is more effective under anaerobic conditions. On the other hand, these conditions lead to aromatic amine formation, and these are mutagenic and toxic to humans (Chung and Stevens, 1993; Do et al., 2002; Pinheiro et al., 2004) requiring a subsequent oxidative (aerobic) stage for their degradation. In this context, the combined anaerobic/aerobic biological treatments of textile dye effluents using microbial consortia are common in the literature (Chang and Lin, 2000; Van der Zee and Villaverde, 2005; Lodato et al., 2007). However, few commercial or industrial applications have been developed using a single adaptable microorganism in a sequential anaerobic/aerobic treatment (Lsik and Sponza, 2003; Lsik and Sponza, 2004a; Supaka et al., 2004). Moreover,
the available literature on the sequential microaerophilic/aerobic treatment with a single microorganism is extremely limited (Sandhya et al., 2004; Xu et al., 2007). Apparently there is a need to develop novel biological decolorization processes leading to the more effective clean up of azo dyes using a single microorganism that is efficient under both anaerobic/microaerophilic and aerobic conditions. Thus the main objective of this study was to observe the degradation of the azo dyes (Reactive Black HFGF and Disperse Red F3BS) in a successive microaerophilic/aerobic process using a single strain, thus *Paenibacillus lautus* SK21 strain for Black HFGF and *Enterococcus faecalis* JMC-UBL02 strain for Disperse Red F3BS. SK21 is the only isolate among the 24 bacteria which demonstrated the capacity to decolorize Reactive Black HFGF to a great extent (upto 98 %). Although this bacterium has shown greater dye-degradation ability as compared to other bacteria, there is no available literature on dye decolorization with *Paenibacillus lautus*.

Reactive Black HFGF is widely used in textile dyeing and is reported to be toxic too (Fatemeh et al., 1990; Lars and Mallika, 1997; Sagarika et al., 2006). There has not been any exclusive report on the decolorization or degradation of Disperse Red F3BS. In this study optimal conditions for decolorization of these two dyes have been reported. Another interesting finding is, the decolorization is faster for both these dyes and complete near to 98% only in static conditions indicating microaerophilic nature of the process involved. Azo Reductase is a key enzyme responsible for this reductive azo-dye degradation in bacterial species (Franciscon et al., 2009). Dye degradation was performed under microaerophilic conditions until no residual color was observed. The medium was subsequently aerated by revolutionary motion in a shaker to promote oxidation of the
aromatic amines formed by the reductive break-down of the azo bond into non-toxic metabolites. The degradation products were also characterized using UV–Vis, FT-IR and TLC techniques (Kalyani et al., 2008). The results in the foregoing sections suggest that decolorization of Reactive Black HFGR and Disperse Red F3BS occurs during microaerophilic reaction set in by the isolates (P. lautus SK21, and E. faecalis JMC-UBL02) obtained from the acclimatized dumping ground of the sludge within the textile industry.

The presence of oxygen normally inhibits the azo bond reduction activity, since aerobic respiration may dominate use of the NADH, thus impeding electron transfer from NADH to the azo bonds (Chang and Lin, 2001). The advantage of the anaerobic reduction of azo dyes is that oxygen depletion is easily accomplished in microaerophilic cultures thus enabling anaerobic, facultative anaerobic and microaerophilic bacteria to reduce azo dyes. The reaction takes place at neutral pH values and is extremely unspecific (Stolz, 2001). However, the precise mechanism of anaerobic azo-reduction is still not totally understood. It was recently suggested that microbial anaerobic azoreduction was linked to the electron transport chain, and that dissimilatory azoreduction was a form of microbial anaerobic respiration (Hong et al., 2007). In addition, different models for the nonspecific reduction of azo dyes by bacteria, which do not require transport of the azo dyes or reduced flavins through the cell membrane, or that describe the extracellular reduction of azo dyes by anaerobic bacteria, were recently suggested (Maier et al., 2004). These results suggested that azo dye reduction was a strain-specific mechanism that could be performed by an azoreductase enzyme or by a more complex metabolic pathway. Thus, due to the lack of information about the metabolism of P. lautus SK21, and E. faecalis JMC-UBL02, the usual
true time dependant kinetic determinations of the azoreductase activity using the azo dye as substrate were not performed, and the azo reduction mechanism in *P. lautus* SK21, and *E. faecalis* JMC-UBL02 will be the subject of a future specific study.

The supplementary sources of N in the growth and decolorization media may affect the microbial decolorization of synthetic dyes. The amount of nitrogen present in the media affects dye decolorization by altering the enzyme production by fungi; for several fungal species, the ligninolytic enzyme activity is suppressed rather than stimulated by high nutrient N concentrations (25–60 mM) (Kaushik and Malik 2009). This view is also supported by Hu (1998), who states that decolorization and mineralization of azo dyes have been reported to be enhanced in nitrogen-limited than in nitrogen-sufficient cultures.

Tatarko and Bumpus (1998) also reported that the addition of supplemental nitrogen only inhibited decolorization of Congo Red in plates containing high amounts of nutrient nitrogen.

Though Reactive Black HFGR could be decolorized by the *P. lautus* SK21 strain completely, *E. faecalis* JMC-UBL02 could not decolorize the Disperse Red F3BS in the absence of Yeast Extract. This was evident from the experimental part where, decolorization was observed in nutrient broth (with Yeast extract) while not so with minimal media. In such case, the decolorization of the Disperse red F3BS probably occurred in a sequential microaerophilic / aerated process in the presence of yeast extract as the source of the electron donors NAD and NADH. In the absence of yeast extract, a partial decolorization (<50%) was achieved after 48 h. It is known that the decolorization rate of
azo dyes is increased by using redox mediators such as the water-soluble flavins (FADH$_2$, FMNH$_2$), NADH or NADPH, which speed up the reaction rate by shuttling electrons from the biological oxidation of primary electron donors or from bulk electron donors to the electron-accepting azo dyes (Dos Santos et al., 2004; Chang et al., 2001a; Chung and Stevens, 1993; Gingell and Walker, 1971). This probably suggests that *E. faecalis* JMC-UBL02 requires yeast extract as a redox mediator to attain efficient dye decolorization of Disperse Red F3BS. Yeast extract, a powder supplement consisting of protein, free amino nitrogen, B vitamins, minerals, nucleotides and other yeast cell components, has been the most commonly used nitrogen source for dye decolorization processes by almost all the investigators around the world (Robert et al., 1998). Many pure cultures like *Pseudomonas luteola*, *Klebsiella pneumoniae* and *Aeromonas hydrophila* have exhibited effective decolorization of different dyes in the presence of yeast extract (Hu, 1998; Chang et al., 2001a; Wong and Yuen, 1996; Chen et al., 2003; Khehra et al., 2005). Moreover, a recent study showed that a combination of the variables including glucose and yeast extract resulted in more than 90% decolorization of the azo dye Direct Black 22 (Mohana et al., 2008).

The chemical structures of the dyes greatly influence their decolorization rates, and the decolorization efficiency is limited to several azo dye structures (Chivukula and Renganathan, 1995). Dyes with simple structures and low molecular weights usually exhibit higher rates of color removal, whereas color removal is more difficult with highly substituted, high molecular weight dyes (Chen et al., 2003; Pearce et al., 2003). For this reason, Disperse Red F3B, which is a monoazo, showed a short decolorization time
(7 hours) and the highly substituted diazo Reactive Black HFGR showed longer decolorization times (24 hours) and this finding is consistent with earlier findings (Franciscon et al., 2009). It has been reported that the turnover rate of monoazo dyes increased with increasing dye concentration, whereas the turnover rate of the diazo and triazo dyes remained constant as the dye concentration increased (Hu, 2001). Moreover, the azo compounds with a hydroxyl or amino groups were more likely to be degraded than those with methyl, methoxy, sulfo or nitro groups (Nigam et al., 1996a, b).

Usually, the presence of sulfonates in reactive dye structures results in low levels of color removal. However, this is not applicable to direct dyes that usually exhibit high levels of color removal independent of the number of sulfonate groups in the dye structure, reinforcing the idea that steric hindrance and the number of azo bonds are responsible for the different decolorization times (Hitz et al., 1978). It has also been reported that a correlation between the enzyme redox potential and its activity towards the substrates could influence the decolorization rate (Call and Mucke, 1997; Xu et al., 1996). In this context, the decolorization times obtained in the present work were in agreement with those of Zille et al. (2004), who found a linear relationship between the cathodic peak potentials and the time of maximum decolorization for several azo dyes. Thus the ability of the bio-agents to degrade azo dyes depends on the structural characteristics of the dye, the temperature and the pH of the medium, the presence of intermediates and the difference between the redox potentials of the biocatalyst and the dye.
The study of the products of biodegradation of synthetic dyes is important in order to know about the environmental fate of these pollutants. It is very important to analyze the treated water with regard to the dye content as well as intermediates, especially aromatic amines, since some are considered carcinogenic (Forss and Welander 2009). The removal of color from dye-containing wastewater may be the first and a major concern (Sun et al., 2009), but the aim of biodegradation of dyes is not only to remove the color but also to eliminate or substantially decrease the toxicity of the dyes (detoxification). Detailed characterization of the intermediates and metabolites produced during biodegradation must be done to ensure the safety of the decolorized wastewater (Kaushik and Malik 2009; Couto 2009). Various basic and advanced instrumental techniques of chromatography and spectroscopy can be used to isolate and characterize the products of biodegradation of dyes and thus have an insight into the mechanism of biodegradation. To date, very few reports are available on the intermediates or the products of biodegradation of triphenylmethane dyes (Chen et al., 2008). The bacterial metabolism of azo dyes is initiated in most cases by a reductive cleavage of the azo bond, which results in the formation of colorless aromatic amines (Khalid et al., 2008).

Relatively simple techniques of UV-visible spectrophotometry and Thin Layer Chromatography (TLC) can be used to know whether decolorization has occurred through adsorption of dye particles on the microbial cell surface or through breakdown of the dye structure by the living microbial systems or both. In adsorption, examination of the absorption spectrum will reveal that all peaks decrease approximately in proportion to each other, but if the dye removal is attributed to biodegradation, either the major visible
light absorbance peak will completely disappear or a new peak will appear (Chen et al., 2003; Saratale et al., 2009). In addition, in adsorption, cells may become deeply colored because of adsorbing dyes, whereas those retaining their original colors are accompanied by the occurrence of biodegradation (Sun et al., 2009). Similarly, by comparing the Rf values of the biodegradation products with those of the original dye and known standards on TLC, some useful information can be obtained about the nature of the biodegradation products. More advanced techniques like Gas Chromatography-Mass Spectrometry (GC-MS), High Performance Liquid Chromatography (HPLC) and nuclear magnetic resonance (NMR) spectroscopy can be used to get more authentic and detailed information about the products of biodegradation and also about the stepwise mechanism of biodegradation. The decolorization reaction was accompanied by the formation of a metabolite that showed up at 267 nm in UV–VIS spectra.

Biodegradation of Reactive Black HFGR may be expected to comprise two main steps: the reductive cleavage of the azo bond under anaerobic condition and the subsequent aerobic mineralization of metabolites formed in anaerobic process. These observations may be explained with the help of the tentative mechanism shown in the Scheme 1. In the Scheme 1, cleavage of azo bond (–N=N–) in Reactive Black HFGR molecule is expected to result in permanent decolorization of the Reactive Black HFGR dye solutions. Aromatic amines are produced as a result of complete cleavage of –N=N– bond. Prior to this, partial reduction of –N=N– via a two electron– two proton reduction process into –HN–NH– functionality may also occur (Mohanty et al., 2003). This –HN–NH– intermediate appears to be air sensitive and is oxidized back to azo group. This is evidenced through the
regeneration of color in the decolorized effluents when exposed to air. Probably, hydrogen peroxide is the byproduct of this oxidation reaction, though it was not attempted in the present study to detect peroxide. Some previous studies suggest the formation of peroxide in similar reaction (Gonzalez-Flecha and Demple 1995; Imlay 2003).

The formation of amine product in anaerobic process also is substantiated by FT-IR and UV-VIS data. The new peak at 258 and 248 nm in UV-VIS spectra (Figures 3.19, 3.20) is suggestive of the formation of amine intermediates. FT-IR data further confirms this observation (Figure 3.21 to 3.25). The FT-IR indicates the transformation in the structure of the molecule. These metabolites have been traced to amine compounds. Moreover, only a few bacterial strains were decolorizing type. The decolorization reaction was accompanied by the formation of a metabolite that showed up at 267 nm in UV-VIS spectra (McMullan et al., 2001). This indicates that amines are formed as decolorization metabolites. It was observed in the present study that time required for attaining >90% decolorization efficiency increased as the concentration of dye increased. Isik and Sponza (2003, 2004a, b) have also reported that decolorization rate was low at high organic loading under anaerobic conditions. However, approximately 25–33% decrease in treatment time can be obtained upon addition of glucose. Under anaerobic condition, azo dyes are readily cleaved via a four electron reduction at the azo linkage generating aromatic amines. The electrons are provided by electron donating carbon sources such as glucose, starch, volatile fatty acids (VFA) etc. Therefore, use of glucose as co-substrate resulted in reduction of treatment time for attaining > 90% decolorization.
It is found in the present study that only a few bacteria present in the textile activated sludge were responsible for anaerobic decolorization of Reactive Black HFGR and Disperse Red F3BS. This is also relevant as per the reports of Sagarika et al., (2006). To conclude, the potent strains SK21 and JMC-UBL02 isolated from the dumping grounds of the two textile industries as mentioned in Chapters I and II were identified as 16SrDNA as *P. lautus* SK21, and *E. faecalis* JMC-UBL02. Both the dyes were totally and rapidly decolorized under microaerophilic conditions, with some differences in decolorization times depending on the structure, as confirmed by the UV-Vis analysis. Decolorization of Disperse Red F3BS was strongly dependent on the presence of nitrogen source (Yeast Extract) in the medium indicating the need for additional vitamin and nitrogen sources. Whereas Black HFGR, showed better decolorization in minimal media as well. The formations of the amines in the microaerophilic stage and their disappearance in the aerophilic stage were confirmed by the direct measurements and FT-IR analysis.

This methodology using a single microorganism in a sequential microaerophilic/aerobic process were shown to be very effective in decolorization of both the dyes. By changing the agitation in a single reactor with a single bacterium, it is possible not only to decolorize the dyes but also to achieve a good degree of mineralization and low toxicity with low running and maintenance cost.