ACCELERATED DEGRADATION OF SELECTED AZO DYES BY SOME MICROBIAL STRAINS

By

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IN THE NAME OF ALLAH
THE COMPASSIONATE,
THE MERCIFUL.
This study involves aerobic decolorization of three reactive azo dyes: REACTIVE ORANGE 16, REACTIVE BRILLIANT RED K2BP AND REACTIVE BLACK B by newly isolated microbial strains. After optimization of reaction conditions bacterial strain B1 gave 73%, 74% and 84% decolorization, Bacterial strain B2 gave 85%, 77% and 78% decolorization while fungal strain gave 90.5%, 94% and 90.4% decolorization of REACTIVE BLACK B, REACTIVE ORANGE 16 and REACTIVE BRILLIANT RED K2BP respectively. No cometabolic role of any organic substrate was found. Color removal rates went on decreasing by addition of heavy metals. Cobalt and Nickel showed greater inhibitions on % decoloristion than zinc copper and iron there was about 50% color reduction by adding 6mg ml\(^{-1}\) of all heavy metals. Hydroquinone was proved to be a good redox mediator enhancing decolorization rate but only at lower concentrations. Biodegradation analysis was monitored by UV-VIS SPECTROSCOPY, HPTLC and GC/MS. None of the formed dye metabolite was from carcinogenic, toxic banned amines.
DEDICATED

TO ALL THOSE WHO EVER RAISED THEIR HANDS TO PRAY FOR MY HAPPINESS AND SUCCESS
ACKNOWLEDGEMENT

All the praises and all the gratitude are just for **ALMIGHTY ALLAH**, before whom every Muslim bows down his forehead five times a day. Whose bounteous blessings with the required strength, perseverance and patience, blessed me with health, thoughts, supportive & encouraging family, talented teachers and helping friends to make some contribution to the already existing ocean of knowledge and in the accomplishment of this manuscript. I offer my humblest thanks to **HOLY PROPHET HAZRAT MUHAMMAD (Peace Be Upon Him)** who is forever a model of guidance and knowledge for humanity, whose moral and spiritual teachings enlightened my heart and mind and flourished my thoughts towards achieving high ideals of life.

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**SOFIA NOSHEEN**
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Chapter # I

INTRODUCTION

Worldwide over 10,000 different dyes and pigment are currently used in various industries like dyeing and printing and pharmaceutical industries (Revenkar and Lele, 2007). Among various dyes commercially in use azo dyes find the largest applications and uses (Carliell et al., 1995) as 60-70% of all dyes stuff in use and production fall in this group. Dyes are versatile chemicals which are consumed by a number of chemical industries like textile, printing, paper, food and cosmetics industries (Khalid et al., 2008). According to a statistical data survey (Stolz 2001; Pandey et al., 2007) one million tons of azo dyes are produced annually world wide. On the basis of chemical composition there are various classes of azo dyes; reactive dyes the most important of them. Most of the reactive dyes (80-95%) have azo group as chromogen (Zollinger, 1991 and Edward, 2000). Most important characteristics of reactive dyes is their ability to form covalent bond with fabric on which they are applied (Kirk Othmer, 1979).

Almost 10% of the dye is lost during dyeing process of fabric and thus comes in environment through wastewater discharges (O’Neil et al., 1999, Khalid et al., 2008 and Pearce et al., 2003). Now a days production rate as well usage ratio of azo yes has increased dramatically according to consumer’s demand. In effluent of textile industries amount of dye has increased to several folds. The main reason behind this great loss is very low rate of fixation of these dyes in fabric dyeing processes; almost 50% (Boer et al., 2004 and Easton, 1995). Colored industrial effluent is the most obvious indicator of water pollution. This release of residual azo dye deteriorates the water quality and is responsible for a number of toxic effects on ecosystems and biospheres. Presence of the dyes in aqueous ecosystems reduces the photosynthetic rate by decreasing the ratio of light penetrating into deeper layers of waters and thus negatively affecting the water quality which in turn have toxic effects on the flora and fauna of the ecosystem (Talarposhti et al., 2001; Bae and Freeman, 2007). The general structure of azo dyes comprises -N=N- substituted by variety of groups on aromatic ring making it a complex structure which is recalcitrant and which is not destroyed by conventional effluent.
treatment processes currently in use. It is well documented that majority of the azo dyes have mutagenic character (Chung and Cerniglia, 1992). The removal of color from wastewater is one of the major issues related to wastewater treatment of textile industries. There are a number of physical as well as chemical methods being used for the decolorization of textile effluents (Wang et al., 2005; Alinsafi et al., 2007; Khalid et al., 2008). Adsorption and coagulation are main principles used in physical methods. On the other hand chemical decomposition of dye molecules include some chemical reactions like oxidation processes (e.g. photochemical and photocatalytic processes). Sometimes electrolysis is also employed. Practical implementations of physical/chemical methods have several inherent drawbacks like high cost, requiring more energy and chemical uses. Moreover they are inefficient to remove azo dyes considered as recalcitrant and their organic metabolites, due to which huge amount of sludge is produced being one of the reason of secondary pollution (Zhang et al., 2004).

The conventional aerobic effluent treatment process is unable to efficiently remove azo dyes from wastewater as most of the azo dyes are substituted by electron withdrawing groups. These groups render the oxygenase enzyme present in microorganisms to attack on dye molecule (Nigam et al., 1996; Asad et al., 2007). Bioremediation of azo dye is a modern, advanced and attractive approach since it is cost effective and environment friendly. Moreover less amount of sludge is produced (Robinson et al., 2001; Chen et al., 2003; Kalyani et al., 2008). Many microorganisms belonging to different taxonomic groups of bacteria; gram positive and gram negative (Chen et al., 2003; Moosvi et al., 2005), fungi (Zhang et al., 1999), actinomycetes (Zhou and Zimmermann, 1993, Khehra et al., 2005) and algae (Dilek et al., 1999) have ability to decolorise azo dyes. Under anaerobic conditions, azo dye is cleaved in reductive manner which results in decolorization leading to color reduction. It is evident that as a result of this chemical reduction mediated by enzymes some very toxic and harmful nitrogen containing products are formed in wastewater. These include amino benzidine, amino benzene and amino naphthalene. These products are not further reduced by microorganisms and thus degradation is stopped (Khehra et al., 2005). However substitution of aromatic amines with sulfonic groups like amino benzene or amino naphthalene derivatives of sulfonic acids can undergo decomposition in aerobic
conditions (Tan et al 1999; Tan et al., 2005). The practical and effective implementation of aerobic treatment technology for color removal is largely dependent on existence, adaptability and survival of microbial population being used (Chen et al., 2003; Stolz, 2001; McMullan et al., 2001).

A new legislation of European community has restricted the use of colorants which can not be converted under any condition (Carliell et al., 1994). Textile industry is vital in Pakistani economy due to its large export to Europe and United States of America. Our textile finishing industries are being labeled high priority industries with respect to pollution. The development of an efficient and economically viable process for decolorization of colored wastewater (having sufficiently higher amounts of dyes) is most urgent demand of this time as it has great impacts on eco sustainability. The root cause of this problem is that almost 75% of traditional dye houses are present in such areas where continuous fresh supply of water is almost impossible or at least very difficult. Almost 0.8-0.15m$^3$ of water is generally used for the production of one kg fabric (finished).

Overall for the manufacture of 25 tons of finished fabric, 1000-3000m$^3$ of wastewater (having dyes, HCl, ammonia, chromium salts, soda ash, caustic soda, sodium sulphate etc.) is generated which is discharged by textile industry into surrounding local environment without treatment causing a serious negative effect. Quantitative restriction on textile quality standards and environment compliances requirement of buyers (particularly USA and European Union) has increased. Presently being implied in the form of ISO CERTIFICATION (ISO 14000 series). With further restriction they will be changed into ECO–LABEL products, a new ecological criteria being set by European Commission. Overall these regulations have very negative effect on exports of textile products of PAKISTAN. In order to comply with national and EU regulation (Colorless after 1:40 dilution) dye houses need to develop novel biological decolorization processes leading to more effective clear up of azo dyes.

Present project focused to investigate the feasibility of biological aerobic treatment for degradation of azo dyes. The target dyes for this research project were chosen from class of cotton reactive dyes extensively in use and were known to be problematic with both to treatability and toxicity (Fatemeh et al., 1990, Lars and Mallika, 1997).
OBJECTIVES

1. Isolation of dye decolorizing microorganisms from contaminated soil of an industrial estate.
2. To study feasibility of biodegradation of selected dyes with isolated microbial strains under optimized environmental conditions.
3. The study kinetic pattern of dye degradation.
4. To study effect of co-metabolism on decolorization of selected azo dyes in presence of additional organic and inorganic carbon and nitrogen sources.
5. To compare decolorization potential of isolates (both fungal and bacterial) with developed mixed microbial culture so as to find synergistic effect.
6. To study effect of electron redox mediators and heavy metals on biodecolorization.
7. To study various intermediates which will be formed during the dye decolorization.

DYES UNDER STUDY

Reactive Black B (Remazol Black 5) is a diazo type reactive dye having two vinyl sulfone reactive groups. Reactive orange 16 a water soluble reactive sulfonated mono azo dye with vinyl sulfone as reactive group and Reactive Brilliant Red K2BP; a water soluble reactive sulfonated monoazo dye with cyanuric chloride as reactive group.
REACTIVE BLACK B

NaO₃SOCH₂CH₂SO₃Na

= N=N

SO₃Na

O

O

REACTIVE ORANE 16

NaO₃SOCH₂CH₂SO₃Na

= N=N

SO₃Na

O

O

CH₃C-NH
REACTIVE BRILLIANT RED K2BP
Azo dyes are commonly used in several industries including textile, dyeing, printing and cosmetic industries. Due to their complex structure they are highly persistent in natural environments, which may lead to acute toxicity of ecosystem. In Pakistan textile factories are wide spread and discharge large quantity of effluents into water streams and soils. The treatment of textile wastewater is of great concern because of their toxicity as well as esthetic problems being created. The treatment of textile wastewater will remain a big problem until chemists and manufactures will design effective technology for such dyes having more efficient bonding with fabric. As a result there will be less loss of non bonded dye in textile effluents. In this chapter an attempt will be made to explain the current status of problem being created mainly by presence of huge amount of azo dyes in textile wastewater.

In order to comprehend the effluent problems facing the textile industry, various processes inside textile sector will be highlighted (in this chapter)which give rise to effluent requiring treatment. Efficient methodology for the degradation of fibre reactive azo dyes and their complete removal will also be explained. As many of these dyes have toxic effects on human health and ecosystems so all of these related issues will also be briefly discussed. Physiochemical factors effecting removal and decolorization of azo dyes will be described as well.

**Textile Industry**

In order to understand the effluent problems being faced by textile industry it is necessary to be familiar with the processes which result in effluent production. Figure 2.1 and 2.2 represent the main stages in the processing of natural fibres (Wool and Cotton) and synthetic fibres (Barnes *et al.*, 1992, Carliell, 1993). As effluents emanating from wet processing of cotton are main focus of this project so it will be described in more detail.
Figure 2.1. Flow chart indicating the process carried in textile industry
Figure 2.2. Main processes stream and respective pollution characterization
Dyeing

Most commonly used dyes for dyeing of cotton are fibre reactive azo dyes, direct dyes, sulphar and vat dyes (Carliell, 1993). Fibre reactive dyes are rapidly replacing direct dyes (Burkinshaw, 1990) and are usually the principle dyes used for coloring cotton. Different dye classes require specific dyeing procedures however a common factor is that water is required for all forms of dyeing, either as a solvent or transport medium and therefore effluent is generated by all dyeing processes. The volume and characteristics of the effluent are determined by the type of dyeing process and the class of dye being used (Carliell, 1993). Dyeing can either be performed in discontinuous batch dye machines or in continuous range. The volume of effluent having large amount of azo dyes which is produced in batch dyeing is much greater than being produced from fabric dyeing in continuous manner. Former requires at least four to ten times water rinses to produce acceptable quality goods. The total volume of effluent emanating from a dyeing process is determined by the liquor ratio, i.e. the volume of dye solution required to dye a kilogram of fabric. Liquor ratio values range from 8:1 to 20:1 with 10:1 being a convenient average (Buckley, 1992). The concentration of dye in the effluent is determined by the dye exhaustion properties, i.e. the proportion of dye that is fibre substantive and ranges from 95-98% for acid, basic and disperse dyes, though 60 to 80% for reactive dyes and 40-60% for the balance of dyes (Clariell, 1993; Buckley, 1992). After discharging a batch dye bath 1M of solution is usually retained per kg of goods i.e. 90% of the unreacted dyes and auxiliary chemicals are present in the first drop (exhausted dye bath) giving rise to a low volume, concentrated, highly colored form of effluent. Subsequent rinses become more dilute giving rise to large volumes of colored effluent with a low organic load.

Effluent coloration is of priority status all over the world in all textile industries. In order to understand the mechanism of dye degradation it is necessary to review in detail the chemical composition and binding of dye molecules to fabric.
The history of azo dyes goes back over hundred years. First dye named as mauve was synthesized accidentally by William Henry Perkin in 1856. He commercialized his accidental innovation and then a new process was developed on large scale for synthesis of that dye which lead to beginning of a new chemical industry (Zollinger, 1987). Almost 0.1 million dyes are registered or in other words are available in market on commercial lines. Annual production of dyes (from all classes) is estimated to be about one million ton (Selvam et al., 2003). Among all dyes, azo dye is the class of dye which is most important with respect to both application and production (Carliell et al., 1995) constituting 60-70% of all dyes stuffs produced. These dyes are widely used in a number of industries. Textile industries use dyes as largest consumers of azo dyes. However they are also present in small amount in effluents of food, paper, printing, leather and pharmaceutical industries.

**Azo Dyes**

Azo dyes have diversity in structure but their most important structural feature is presence of azo linkage i-e \(-\text{N=N-}\). This linkage may be present more than one time and thus mono azo dyes have one azo linkage while two in diazo and three in triazo respectively. These azo groups are connected on both sides with aromatics like benzene and naphthalene moiety. Sometimes aromatic heterocyclic units are also present being connected with azo groups. (Zollinger, 1991). Different shades of the same dye having various intensities of color are due to these aromatic side groups (McCurdy, 1992). Azo dyes containing sulfonate groups as substituent are called as sulphonated azo dyes. Azo groups in conjugation with aromatic substituents or enolizable groups make a complex structure which lead to huge expression of variation of colors in dyes (O’Neil et al., 2000 and Rajaguru et al., 2000).

Some common examples of azo dyes are shown in Figure 2.3.
YELLOW AZO DYE

METHYL ORANGE

SUDAN 4

CITRUS RED 2

BRILLIANT BLACK BN
TRYPAN BLUE

Figure 2.3. Chemical Structure of Azo Dyes Having Different Substituents.

The synthesis of azo dyes involves two main steps.

i) Diazotization  

ii) Coupling

An overview of azo dye synthesis is shown below:
The diazotization reaction must be carried out at 0-5°C to minimize the reaction with water to produce a phenol. Diazotization must be carried out as rapidly and possible with good stirring and cooling. The diazonium salts produced tend to be unsatable and are usually not isolated.

In diazotization reaction, nitrous acid is generated in situ from sodium nitrate and a mineral acid, usually hydrochloric acid according to the reaction.

\[ \text{HCl} + \text{NaNO}_2 \rightarrow \text{HNO}_2 \text{ (nitrous acid)} + \text{HCl}. \]

The mechanism of this reaction is:

\[ \text{NaNO}_2 + \text{H}_2\text{SO}_4 \xrightarrow{\text{H}_2\text{O}, 0^\circ} \text{H-O-N=O} + \text{NaHSO}_4 \]

\[ \text{H-O-N=O} + \text{H}_2\text{SO}_4 \xrightarrow{} \text{N=O} \text{ HSO}_4 + \text{H}_2\text{O} \]

The nirosonium ion promptly reacts with the lone pair of nitrogen leading to an N-nitrosoanilinium ion. The diazonium ion is a weak electrophile that will react with electron rich aromatics such as phenols and anlines to give electrophilic aromatic substitution reactions. As with other electron-rich substituents ortho and para substitution predominates. The sequence of steps is shown here:
As there is a large diversity in substituents or functional groups which can be present on dyes thus here is great variety of structurally variable dyes present in market which are used in industries (McCurdy, 1991).
Fibre Reactive Azo Dyes

There are different sub classes of azo dyes. The present project will focus only on reactive azo dyes. Larger proportion of reactive dyes (80-90%) have azo group as chromophoric group (Zollinger, 1991 and Edward, 2000). Reactive dyes are a class of dyes which have essentially a functional group having ability to make a bond (essentially a covalent bond) with fabric. A carbon or phosphorus atom present in dye molecule can bond to free hydroxyl groups common in cellulosic fabric (Kirk-Othmer, 1979). Mostly fibre reactive azo dyes are employed for dyeing cellulosic material so cotton finds largest application of these dyes.

Fibre reactive azo dyes show a high wet fastness because of their stronger linkage with fabric in the form of covalent bond. If any dye undergo hydrolysis before making a bond to fibre it will be lost during washing processes (Loyd, 1992). Schematically, fibre reactive azo dyes can be viewed as follows

- The chromophoric groups are from azo, carbonyl or phthalocyanine class as shown in earlier discussion.
- The water solubilising group belongs to ionic class most commonly sulphonic salts which will exceptionally enhance the solubility of dye since reactive dyes
need high solubility for being applied to the fabrics. This shows that reactive dyes are not like acid dyes in chemical nature

- The bridging group makes a linkage between chromophore and functional group (also being called as reactive group). Most commonly used bridging groups are amino group or alkyl group. This is usually for convenience rather than for any specific purpose. It should be highly stable and soluble in water and exhibit a certain degree of flexibility

- There is only one part in dye that can react with fabric that is fibre reactive group

It serves to connect the dye with substrate via nucleophilic substitution or addition reactions (Kirk-Othmer, 1979). Mono-di- and triazinyl are common examples of reactive functional groups. The major fibre-reactive group falling in this class are six-membered heterocyclic, aromatic rings having halogens most commonly chlorides as substituents.

![Diagram of dye structure](image1)

The other type of dye class reacts via nucleophilic addition, the most common example the Remazol reactive dye. This dye type requires a base like hydroxide for its reaction with fabric. The mechanism for the reaction of such dye is given here under.

![Diagram of Remazol reactive dye mechanism](image2)
There is a reported list of at least 200 different reactive groups in patent literature (Zollinger, 1991). Some common examples of fibre reactive azo dyes can be seen in Figure 2.4.

According to a research report almost 10-15% of dye applied in dye bath is lost through wastewater during application (Vaidya and Datye, 1982). This loss of azo dye has been increased several times now as extent of use of these dyes has increased. Fixation rate of these dyes might be as low as 50 % (Easton, 1995). At present control of
water pollution is major area of concern for researchers. In the earlier reports it was considered that major problem related to wastewater is color rather than organic load as it causes aesthetic pollution as well and is first obvious indicator of water pollution. But it is well established fact that dyes which are responsible for this color cause serious damage to receiving water bodies other than aesthetic pollution (Nigam et al., 1996).

Out of total production of dyes two percent are discharged in aqueous effluent during manufacture and 10% are finally released during dyeing of fabric (Pearce et al., 2003). This study does not directly involve the problem of toxicity being created by release and decomposition of azo dyes in textile effluents however discussion and emphasis on this problem is necessary as dyes endangers the humans and other biocomponents of ecosystems. Azo dyes especially those having nitro group are found to have mutagenic (Chung and Cernillia, 1992). These nitro group lead to toxicity in methanogenic granular sludge (Donlon et al., 1997). Moreover some of these azo dyes can lead to production of toxic compounds as a result of degradation. Examples of such harmful moieties are 1,4-phenylenediamine, 1-amino-2-naphthol, benzidine and substituted benzidine, like o-toulidine (Chung et al., 1981, Reid et al., 1984, Rosenkranz and Klopman, 1989, Rosenkranz and Klopman, 1990).

![Chemical structures that after metabolic activation of azo dyes show toxicity (Tan, 2001)](image)

Figure 2.5. Chemical structures that after metabolic activation of azo dyes show toxicity (Tan, 2001)

It is well reported that azo dyes having sulphonic acid group have very low or negligible mutagenic toxicity when compared with dyes having no sulphonic groups as
because they have low electric charge and show less lipophilic character. This character prevents them from undergoing any metabolic activities (Chung and Cerniglia, 1992, Jung et al., 1992 and Levine, 1991). Many textile wastewaters have heavy metals as contaminants which can make complex with azo dyes present there. High concentration of salt is commonly used to force fibre reactive dyes out of solution and onto substrates (Zollinger, 1991). These compounds can result in high electrolyte and conductivity concentrations in textile effluent and thus toxicity problems (both chronic and acute) are evolved.

Table 2.1. List of aromatic amines and dye metabolic products being considered to be human carcinogens

<table>
<thead>
<tr>
<th>Aromatic Amine Group</th>
<th>Human Carcinogen Evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-naphthylamine</td>
<td>Slight/Mixed</td>
</tr>
<tr>
<td>2-naphthylamine</td>
<td>Good</td>
</tr>
<tr>
<td>Auramine</td>
<td>Slight</td>
</tr>
<tr>
<td>Benzidine</td>
<td>Good</td>
</tr>
<tr>
<td>Magenta</td>
<td>Slight</td>
</tr>
<tr>
<td>4-nitrobiphenyl</td>
<td>Slight/Mixed</td>
</tr>
<tr>
<td>4-Biphenylamine</td>
<td>Good</td>
</tr>
</tbody>
</table>

In last few years a lot of emphasis has been given to the control of pollution. Several new methods for color removal from colored industrial effluents are currently being developed. World regulations for industrial wastewater quality are continuously being updated and huge emphasis is given on application of effective effluent treatment system. Wastewater coming out from dyeing sections of fabric (wool, cotton and polyester) is colored due to presence of both soluble and insoluble dyes. Most common concentration of dyes in such solutions are above 1 mg l\(^{-1}\) and in some reports it is documented that amount of dye in textile wastewater could be as high as 300 mg l\(^{-1}\) (Cooper, 1995 and Laing, 1991).
A number of physical and chemical treatment methods have been used to decrease the color contents from wastewater. Here a critical review of most widely used physical and chemical methods of dye removal from dye-containing industrial effluents is given.

**CHEMICAL METHODS**

This is most widely applied method for decolorization of effluent by chemical means due to its simple nature. Chemical oxidation being mediated by oxidizing agents generally cause ring opening of aromatic part of dye and thus removal of dye from wastewater occurs (Raghavacharya, 1997).

a) $\text{H}_2\text{O}_2$-Fe (II) Salts (Fenton’s Reagent)

Fenton’s reagent is very suitable for such wastewaters where biological treatment cannot be applied as they show toxicity for living biomass (Slokar and LeMarechal, 1997). In order to remove dissolved dyes from textile effluents chemical separation is also used on industrial scale which involves bonding of dyes or sorption of dyes. This can be applied for successful removal of insoluble dyes, insoluble dyes will be removed as well (Pak and Chang, 1999).

b) Ozonation

Oxidation using ozone as an oxidizing agent has potential to decompose hydrocarbons having halogens and hydroxyl substituents or with no substituents (Lin and Lin, 1993, XU and Lebrun, 1999). The type of waste water (%dye content and COD level) is most important factor which determines the amount of ozone to be used for treatment (Ince and Gonec, 1997) leading to no toxic metabolite formation (Gahr et al., 1994). Ozonation leaves the effluent with no color and low COD suitable for direct discharge into main water channel (Xu and Lebrun, 1999).
c) **Photochemical Removal**

This method results in formation of carbon dioxide and water as a result of decomposition of dye molecule using UV –light along with hydrogen peroxide (Yang *et al.*, 1998, Peralto-Zamora *et al.*, 1999). During the course of reaction there is production of Hydroxyl free radicals as result of action of UV light on Hydrogen peroxide which in turn causes oxidation of organic substrates under consideration.

\[ \text{H}_2\text{O}_2 + \text{hv} \to 2\text{OH}^\circ \]

d) **Sodium Hypochloride (NaOCl)**

It involves destruction of amino group of dye molecule by chloride (Cl\(^+\)) of sodium hypochloride which in turn is responsible for azo bond cleavage (Bannat *et al.*, 1996).

e) **Cucurbituril**

It is a cyclic polymer obtained by condensation of glycoluril and formaldehyde (Karcher *et al.*, 1999). It forms complexes with azo compounds having aromatic side groups like enzyme substrate or host guest relationship (Mock, 1995). These complexes are responsible for dye adsorption and ultimate removal.

f) **Electrochemical Destruction**

It is an attractive and economically viable protocol for removal of dyes. It results in high color removal and destruction of a range of organic compounds including resistant pollutants (Ogutveren and Kparal, 1994 and Pelegrini *et al.*, 1999).
PHYSICAL METHODS

a) Adsorption

Among various physical methods adsorption is very popular because it is highly effective in treatment of such wastewaters having very stable pollutants generally not removed by common conventional treatment methods (Slokar and LeMarechal, 1997).

b) Activated Carbon

This is most commonly type of adsorbent (Nasser and El-Geundi, 1991) and shows very good potential to adsorb cationic, mordant and acid dyes largely. Its efficiency is slightly poor to remove disperse, direct, vat, pigment and reactive dyes (Raghavacharya, 1997 and Rao et al., 1994).

c) Peat

It is exceptionally good choice because of its cellular structure. It shows high potential to adsorb transition metals as well as organic compounds (polar in nature) from textile mill effluents (Pools and Mckay, 1976).

d) Wood Chips

They are effective only in removal of acid dyes.

e) Fly Ash and Coal (Mixture)

In this mixture, if we increase the concentration of fly ash, the efficiency rate increases as higher flash content increase the surface area available for adsorption (Gupta et al., 1990).
f) Silica Gel

A good adsorbent causing removal of only basic dyes.

Other Materials

The use of these compounds such as natural clay, corncobs, rice hulls, etc for removal of dyes is very advantageous because of their widespread availability and low cost (Nawar and Doma, 1989).

Membrane Filtration

This method has the potential to remove color from dye effluent continuously. It can also concentrate the dye molecule and finally lead to their separation (Mishra and Tripathy, 1993).

Ion Exchange

This method finds less application on commercial scale as it is restricted to type of charge present on dye molecule and thus most of dyes cannot be eliminated from wastewater by using ion exchange resins (Slokar and LeMarechal, 1997).

Irradiation

This type of wastewater treatment can be used only on laboratory scale for efficient removal of dyes and organic molecules like phenols (Hosono et al., 1993).

Electro kinetic Coagulation

It is a low cost effective method for color removal from effluents. It is commonly employed for direct dyes by using ferrous Sulphate and Ferric chloride (Gahr et al., 1994).
Table 2.2. Advantages and disadvantages of the current technologies for dye removal from industrial wastewaters

<table>
<thead>
<tr>
<th>Physical/Chemical Methods</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fenton’s reagent</td>
<td>High grade removal of both soluble and insoluble dyes</td>
<td>Huge Sludge being produced</td>
</tr>
<tr>
<td>Ozonation</td>
<td>Applied in gaseous state: no alteration of volume</td>
<td>Short half-life (20 min)</td>
</tr>
<tr>
<td>Photochemical</td>
<td>No sludge production</td>
<td>Generation of toxic chemical compounds as by-products</td>
</tr>
<tr>
<td>NaOCl</td>
<td>Cause azo-bond cleavage</td>
<td>Formation of aromatic amines</td>
</tr>
<tr>
<td>Cucurbituril</td>
<td>Good sorption potential a large variety of dyes</td>
<td>Economically unfavorable</td>
</tr>
<tr>
<td>Electrochemical destruction</td>
<td>Decomposition products are non toxic</td>
<td>High cost of electricity</td>
</tr>
<tr>
<td>Activated carbon</td>
<td>High grade potential to adsorb a wide variety of dyes</td>
<td>Very high cost</td>
</tr>
<tr>
<td>Peat</td>
<td>Efficient adsorbent</td>
<td>Less effective than activated carbon</td>
</tr>
<tr>
<td>Wood chips</td>
<td>Good sorption capacity for acid dyes</td>
<td>Requires long retention times</td>
</tr>
<tr>
<td>Silica gel</td>
<td>Applied successfully for basic dye removal</td>
<td>Less suitable on commercial scale due to side reactions</td>
</tr>
<tr>
<td>Membrane filtration</td>
<td>Good potential to Removes all dye types</td>
<td>Concentrated sludge generation</td>
</tr>
<tr>
<td>Ion exchange</td>
<td>Regeneration: no adsorbent loss</td>
<td>less effective</td>
</tr>
<tr>
<td>Irradiation</td>
<td>Effective oxidation at lab scale</td>
<td>Requires a lot of dissolved O₂</td>
</tr>
<tr>
<td>Electro kinetic coagulation</td>
<td>Economically feasible</td>
<td>High sludge production</td>
</tr>
</tbody>
</table>

**BIOLOGICAL METHODS**

Microorganisms can play a very significant role in decomposition and ultimate mineralization of biopolymers and xenobiotics like dyes (Lie et al., 1998). In literature many reviews are available which have described in detail the use of microorganisms (bacteria, fungi) for dye decolorization (Bannat et al., 1996, O’Neil et al., 1999, Coughlin et al., 1999, McMullan et al., 2001, Stolz 2001, Bhatt 2005, Asad et al., 2007, Pandey et
Biological treatment methods are cheap and offer the best alternative for physicochemical processes due to their cost effectiveness, production of less sludge and environmental benignity (Chen et al., 2003). Options for the biological treatment of textile dyeing wastewater may consist of single phase aerobic, anaerobic processes or multiphase systems combining both aerobic and anaerobic processes.

**Anaerobic Biodegradation of Azo Dyes**

Anaerobic treatment of azo dyes based on reduction in presence of microbial sludge can be an efficient and economic treatment method for removal of color from dye house effluent. This was initially studied with respect to mammalian metabolism, due to food colorants (Meyer, 1981). The ability of azobond to be reduced under anaerobic conditions (such as mammalian intestine) was cause for concern as the by-products of azo-fission (derivatives of amino benzene) was suspected carcinogens. Many researcher have well documented through their research that anaerobic bacterial strains could successfully reduce azo dyes finally causing cleavage of aromatic ring; a very important part of azo dyes (Chinwetkitvanich et al., 2000, Razo-flores et al., 1997; Loyd 1992, Ganesh, 1992, Brown and Hamburger, 1987, Brown and Laboureur, 1983). This reduction can change the nature of functional groups (chromophoric system) and a remarkable reduction of color of wastewater. Many aromatic rings (part of azo dyes) are resistant (some even show complete stability) to this reduction being carried by microorganisms (Razo-Flores et al., 1997).

The exact mechanism leading to color removal through reduction is not clearly reported uptill now. It is generally assumed that dye reduction may involve various mechanisms like enzymatic (Rifii et al., 1990) non-enzymatic (Gingell and Walker, 1971) mediated (Vander Zee et al., 2000), intracellular (Mechasner and Wuhrmann, 1982) extra cellular (Carliell et al., 1995) or in other way the mechanisms involved may be more than one of the above. It may be adsorption of dye on microbial cells as well (Knapp and Newby, 1999, Sani and Banerjee, 1999 and Asad et al., 2007).
Generally it is accepted that a rapid color removal of azo dyes under anaerobic conditions mainly involving reductive transformation of azo chromophoric system. Due to this breakage two aromatic amines are obtained. Aromatic ring substituted with amino group show no adsorption in UV region of spectrum. Thus anaerobic reduction of azo dye is defined only as decolorization of wastewater. Azo reduction of sulphonated azo dye can lead to formation of both of sulphonated as well as unsulphonated aromatic amines. In the study presented here azo disalicylate was used as test dye and its complete mineralization under methanogenic conditions is presented here. At first its reduction lead to formation of aromatic amine 5-aminosalicylic acid (5-ASA) while in the second step 5-ASA was completely mineralized in anaerobic environment (Razo-flores et al., 1997).

Figure 2.6. Example of reductive decomposition of sulphonated azo dye (Razo-flores et al., 1997)
Anaerobic decolorization of azo dyes by single species

Much of the experimental work involving anaerobic decolorization of dyes (predominantly azo dyes) has been conducted using mono cultures. Species of *Bacillus* and *Pseudomonas* were found to be active in anaerobic degradation of a number of dyes but other microorganisms such as those belonging to the genus *Aeromonas* and purple non-sulfur photosynthetic bacteria, have also been found to successfully decolorise a range of dyes (Carleiell *et al.*, 1995).

Decolorization of dyes with mixed populations of Microorganism in Anaerobic digester Sludge

Xenobiotics like azo dyes can be degraded successfully by mixed microbial cultures (Pearce *et al.*, 2003 and Knacknuss, 1996). Brown and Laboureur(1983) have reported a series of experiments to assess the primary biodegradability (decolorization) of dyestuffs under conditions of anaerobic digestion. The study was performed with 22 dyestuffs which were chosen as commercially important representatives of the major classes of dyestuffs. All dyestuffs evaluated were water soluble and direct red 7 was used as a positive control. The results showed that the structure of dye most importantly its chromophoric system needed more consideration for its treatability option rather than the mode of application of dye on fabric. The dyes having mono, dis and poly-azo groups showed a substantial degree of decolorization, although the dyes with anthraquinones chromophores showed more variation among the decolorization rate staring from scarcely degraded to substantial degradation. Other dye chromophores which were represented were stilbene (53% decolorization), triphenylmethane (no decolorization was measured as it was unstable in aqueous solution), phthalocyanine (36% decolorization), oxazine (62% decolorization), methane (35% decolorization) and nitro (62% decolorization) was initially reported but a colored metabolite was subsequently formed. The authors concluded that with the single exception of Acid Blue 80, an anthraquinone dye which showed only 7% decolorization, all dyestuffs tested showed a substantial degree of color removal and that the breakdown by dyestuffs in the environment is likely to be initiated under anaerobic conditions.
Kremer (1989) have reported anaerobic degradation of two monoazo dyes, acid red 88 and acid orange 7, inoculated with sludge from an anaerobic digester. The rate of decolorization of the azodyes was noted to be increased when supplemented carbon (other than dyes) was present. The addition of supplemental carbon was also found to have an effect on degradation products formed as result of azo reduction, although no mineralization of dyes was seen to occur.

Thus it can be concluded that azo dye reduction will occur readily when these dyes are incubated in an anaerobic treatment system in which supplemental labile carbon was also present. However the fates of the resultant dye metabolites in anaerobic treatment system remained uncertain.

AEROBIC BIODEGRADATION OF AZO DYES

An important property of textile dyes, which manufactures and users have striven for, is resistance to oxidation. A garment saturated with water or perspiration and well inoculated with microorganisms is an excellent culture medium. Only compounds resistant to biochemical/oxidative degradation under these conditions would be fast and could be considered as stable dyes (Straley, 1984). The above criterion clarifies why many commercial dyes are recalcitrant to oxidative microbial breakdown and therefore, why existing forms of aerobic wastewater treatment are not usually effective against dyehouse effluent.

Kulla (1981) reported the isolation of a bacteria having ability to use azo compounds for supply of nutrients like carbon and nitrogen and energy. A simple azo compound, 4-4’ dicarboxyazobenzene, was initially used to isolate a Flavobacterium species which could get carbon, nitrogen and energy solely from substrate. Adoption of this bacterium to utilize various derivatives of orange II (a commercially used textile dye) was then attempted in batch culture, however, no success was achieved and a chemostat system was developed to facilitate rapid selection of adopted microorganisms. The breakthrough to growth with orange II as supplier of essential nutrients of growth occurred after approximately 400 generations. Although a microorganism capable of mineralizing
the dye of choice was successfully developed, but the authors concluded that the fairly stringent substrate specificity of strain was not feasible for practical wastewater treatment.

Zimmermann et al. (1982) continued this research by isolating and characterizing orange II azoreductase, the enzyme could initiate degradation of azo dye by *Pseudomonas* KF44. The purpose of the study was to elucidate the substrate requirements of the enzyme. Keeping in view the important structural features following generalization were found to be important;

a) A hydroxyl group at 2\textsuperscript{nd} position of parent ring i.e naphthalene for the decolorization reaction

b) Hindrance of reaction occurred due to presence of any charged substituent in the neighborhood of azo linkage

c) Interaction of enzyme with dye as substrate could be lowered if another charged polar group is also present on dye molecule.

Kulla et al. (1993) also found that substitution of sulfonate dyes with carboxyl groups on orange I and II dyes led to disturbances of degradative pathway. However, the enzymes initiating degradation (Orange I & II azo reductase) had no preference for carboxylated or sulphonated dyes and it was proposed that sulphanilic acid, the product of the initial fission reaction was creating a blockage as it was further degraded and thus pathway came to an end. These results were considered to be partially significant as the sulphonated azo dyes are reported to be resistant to aerobic degradation, on the other hand are largely used in textile industry on commercial scale.

Heiss et al. (1992) has also reported the decolorization of azo dyes (with sulphonic acid) in aerobic environment. They investigated the cloning of DNA from *Rhodococcus* strain capable of decolorising azo dyes under aerobic conditions, to microorganisms previously unable to decolorise azo dyes. All the microorganisms capable of decolorizing the dyes could decolorise both test dyes (Orange II and Amido Black); and those microorganisms unable to decolorise the dyes could decolorise neither, suggesting that the similar enzyme system could be used for a large number of dyes
acting as substrate. This is in contrast with the work by Kulla (1981) who found that azo reducing enzymes were substrate specific. In addition Heiss et al. (1992) reported that none of the azoreducing microorganisms could get carbon and other nutrients from dyes.

A bacterial strain named as S5 was derived from Hydrogenophage palleronii S1. It showed extra potential to decolorise selected azo dye 4-carboxy-4'-sulfobenzene as well as its metabolic products i-e 4-aminobenzenesulphonic acid (4-AB) and 4-aminobenzoic acid. This strain used organic dye for provision of carbon and nitrogen (Blumel et al. 1998). Coughlin et al., (1999) has reported isolation of an active bacterial strain; M12 from a biofilm reactor. This strain successfully utilized Acid Orange 7 and 8 as source of inorganic nutrients. They also reported that Sphingomonas sp. Strain 1CX could successfully decolorise azo dyes substituted with sulphonic acid group or not like acid Orange 7, Acid Orange 8, Acid orange 10, Acid Red 4 and Acid red 88.

The aerobic biodegradation of many amino substituted aromatic compounds like aniline has been studied in detail. It is well reported that these compounds could be degraded in aerobic environment (Konopka 1993), carboxylated aromatic amines (Russ et al., 1994; Stolz et al. 1992), chlorinated aromatic amines (Loidl et al. 1990), and (substituted) benzidines (Baird et al. 1977). Tan et al., (2000) has reported the resistance of sulphonated substituted aromatic amines to degradation. These compounds could enter into wastewater system during pathway of degradation of sulphonated azo dyes.
AROMATIC SULPHONATES

Figure 2.7. Examples of chemical structures of sulphonated aromatic compounds (Tan et al., 2005)

One of the very important characteristics of sulphonic acid containing compounds is their high solubility in water and thus they are present abundantly in water systems subsequently deposited in sediments. Chemical industries like leather, textile, paper, printing and pharmaceuticals are larger consumers of sulphonated aromatic compounds and so are mainly responsible for release of these compounds (being xenobiotics) to wastewater systems. Desulphonation or in other words mineralization of these compounds in aerobic environment results in release of sulfur containing group from substrate. The first step in this desulphonation is formation of $\text{HSO}_3^{-1}$ which in the second step undergoes oxidation to give sulfate ($\text{SO}_4^{2-}$). This desulphonation of aromatic moiety may occur before ring opening after or during ring opening of aromatic part.
DESULPHONATION BEFORE RING CLEAVAGE WITH DIOXYGENASE AND MONOOXYGENASE

DESULPHONATION DURING RING CLEAVAGE
Water-soluble textile dye intermediates are usually highly sulphonated, a property which is known to increase the recalcitrant nature of these compounds by decreasing their ability to penetrate into microbial cells (Wuhrmann et al., 1980). In fact, naphthalene-sulphonic acids which are manufactured as pre-products for detergents and textile dyes have been classified as persistent xenobiotics (Luther and Soeder, 1991) due to the consistent recalcitrance of these compounds in both aerobic and anaerobic biological treatment systems.

Research involving the biodegradation of sulphonated aromatic compounds has shown that degradation of these compounds only occurs subsequent to the removal of the sulphonic acid group from the compound. The C-SO$_3$ bond is labialized by oxygenolytic cleavage (Brilon et al., 1981a) and is therefore a strictly aerobic reaction. Aerobic degradation of naphthalene-sulphonic acids by a *Pseudomonas* sp was reported by Brilon et al. (1981a, 198b). The sulphonic acid group was eliminated as hydrogen sulphite as a result of oxygenolytic cleavage of the C-SO$_3$ bond and the naphthalene compound was mineralized as the only source of carbon and energy. Successful biodegradation of sulphonated azo dyes has been reported by a number of scientists each using different type of microbial specie for treatment processes. (Feigel and Knackmuss,1993, Junker et al.,1994, Pasti-Grisby et al.,1996,Kandelbauer et al,2004 and Pandey et al 2007).
Biodegradation of reactive azo dyes present in textile wastewater is a complicated procedure due to versatility in structure of dyes present. A number of other environmental factors can also affect biodecolorization of reactive azo dyes. These include both dye and non-dye related parameters as reported in literature by several researchers e.g. temperature (Wong and Yuen 1996, Song et al. 2003, Keharia et al. 2007 and Seesuriyachan et al., 2007) pH (Vijay Kumar et al., 2006, Ghosh et al., 1993, Xu et al., 2006 and Chang and Lin, 2001). Other include amount of dissolved oxygen, redox mediators (chemical structure and concentration), type of microorganism used, cell permeability, amount of nitrate and supply of inorganic nutrients. Type of dye, its chemical structure, size and nature of substituents, concentration, chemical nature of first metabolites being formed and other chemicals present as dye auxiliaries can also greatly affect rate as well as extent of biodegradation both in aerobic as well as anaerobic environment. Thus the color removal is a complex process and no clear evidence has been yet observed for action of enzymes involved (Cheethan and Bucket, 1984).

Wuhrmann et al. (1980) studied the effect of pH on dye reduction process but a conclusive relation between dye and its reduction could not be established. They found that rate of dye decolorization could be enhanced by lowering pH of media. However this change is largely dependent on the type of dye. Efficiency of the treatment system i.e. working under aerobic conditions or anaerobic conditions is dependent on pH of wastewater. Grady et al., (1990) and Loyd (1992) observed that rate of effluent decolorization having Navy-106 was suppressed sharply by increasing pH during batch experiments which were conducted in anaerobic conditions. Later on a number of research reports have been described which conclusively show a relationship between pH and biotechnological approaches for the removal of dyes from wastewater. However optimal pH for maximum efficiency of process varied from case to case depending both on type of microorganisms and structure of dyes under investigation. (Miranda et al., 1997, Raghukumar et al., 1996, Fu and Viraghavan., 2001, Asad et al., 2007 and Chen et al., 2003).
Presence or absence of nitrate moiety and most importantly oxygen has a crucial role in extent of reduction of azo dye. It is discussed earlier that dye reduction particularly with azo chromophoric system is inversely related with presence of oxygen. Interestingly Wuhrmann et al., (1980) have reported that obligate aerobes can successfully decolorise azo compounds under temporary anoxic conditions. However azo dye removal was inhibited significantly by higher concentrations of nitrate or nitrite in mixed liquid culture of activated sludge. Zissi and Lyberatos (1996) found that *Bacillus subtilis* have ability to decolorise p-aminoazobenzene in anoxic environment.

Oxygen also plays a crucial role in decolorization of dyes. Uptill now dye decolorization has been studied in aerobic, anaerobic as well as sequential aerobic and anaerobic conditions. Success of each process varied greatly depending upon both the structure of azo dye and the type of microbial culture being employed (Moosvi *et al.*, 1999, Soares and Duran, 1998, Stolz, 2001, Chen *et al.*, 2003, Asgher *et al.*, 2006, Kalme *et al.*, 2007, Revenker and Lele, 2007 and Mane *et al.*, 2008).

Without presence of necessary redox mediators to get higher microbial respiration and growth, dye reduction may be decreased. Most commonly bacterial cultures do not have ability to show high growth rate with azo dye as the only supplier of nutrients most importantly carbon and nitrogen. So an additional easily biodegradable substrate may be required. Wuhrmann *et al.*, (1980) reported that in the absence of oxygen a dye with azo chromogen will act as the only oxidant present in media so reduction rate would be greatly affected by the rate of electron donor formation. Role of redox mediators in dye decolorization has been previously reported by Zimmermann *et al.*, 1982, 1984, Keck *et al.*, 1997, Keck *et al.*, 2002, Suzuki *et al.*, 2001 and Chen *et al.*, 2005. Recently a number of researchers have found that effectiveness of a redox mediator depends upon its concentration being applied, reaction conditions and redox potential as well (Rau *et al.*, 2002, Vander Zee *et al.*, 2003, Sadhasivam *et al.*, 2009 and Su *et al.*, 2009).
Figure 2.9. Proposed mechanism for reduction equivalent dependence of azo dye decomposition by BN 6 (Keck et al., 1997)

Rate of dye decolorization is also dependent on the type of microbial culture (single or mixed) being used. It is generally accepted that microorganisms growing under aerobic conditions do not have the potential to completely decolorise reactive azo dyes, but they can cause oxidation of dye metabolites. The opposite is the case with anaerobic microorganisms. (Knapp and Newby 1995, Steffensen and Alexender 1995, Chang et al., 2001, Hu et al., 2001 and Joshi et al., 2008)

Cell permeability is another very important factor not related with structure of dye but having a major role in dye decolorization mechanism and extent. Wuhrmann et al., (1980) studied the affects of dye absorption by the cell wall and found that adsorption of dye exhibited a high variability depending on the dye. Ganesh (1992) observed that very small amount of the dye added to a biological reactor would be leached after its placement in landfills. So it could be suggested that the dye was reduced after its adsorption to microbial cell wall effectively or that negligible amount of dye was practically adsorbed. Removal of dye by biomass based on adsorption or chemical
reactions mediated by loving cells has been studied extensively using various types of fungi and bacteria. (Yu and Wen, 2005, Knapp and Newby, 1995, Li and Xi, 2004, Asad et al., 2005, Khehra et al., 2005, Wang et al., 2005, and Joshi et al., 2008).

Chemical structure of azo dye have a prominent effect in the decolorization and biodegradation rate (Wuhrmann et al., 1980 and Mandez et al., 2005). Depending on the position of azo group in dye molecule and their number as well some azo dyes will be decomposed more easily and fastly than others. It is commonly accepted by the researchers that an increase in number of azo groups on dye molecule would lead to decrease in rate of dye reduction as it would require more time to reduce more groups. A very little reported information is available which could explain this factor directly. Brown and Laboureur (1983) observed that two poly-azo dyes showed very low decomposition when compared to dyes having four mono azo and six diazo linkages.

Fibre-reactive azo dyes often have side groups responsible for solubilization, along with reactive groups being nucleophilic in nature. Depending on the type of these substituents, biodegradation rate might be changed. According to Ganesh (1992), sorption of dye molecules is largely dependent on the type, chemical nature, number and the relative positions of various substituents in the dye molecule. Sulphonic side groups (water loving) decrease dye removal rate by increasing sorption which in greatly increased if hydroxyl, nitro and azo groups are also present in the dye molecule.

The formation of toxic by-products as a result of first decomposition can also suppress the rate of dye decomposition. Textile wastewater has high salt concentration which poses adverse negative effect on biodegradation. Dispersing and solubilizing agents may also cause inhibition in rate of dye reduction (Carliell, 1994).

In numerous studies (Wuhrmann et al., 1980 and O’Neill et al., 2000) the production of toxic dye metabolites from dye molecule is cited to cause a further depression in dye degradation. O’Neill et al. (2000) concluded from respiration-inhibition testing that biodegradation of simulated textile wastewater lead to generation of dye
metabolites which were found to be very harmful for growth and microbial activities of aerobic microorganisms.

Wurhmann et al. (1980) reported a decrease in degradation rates to the production and accumulation of toxic products in the growth culture. Growth inhibition of several microbial strains (both bacterial and fungal) to dye metabolites is largely cited in the literature and is generally concluded that growth and efficiency of microorganism is affected badly by production of toxic metabolites which is a key factor to be considered in effluent treatment having azo dyes (Zille et al., 2005, Oranuri and Ogugbue, 2005).

A final and crucial factor to be considered is the initial dye concentration in textile effluent. Seshardi and Bishop (1994) conducted a series of experiments to determine the correlation between influent dye concentrations on the color removal potential and finally concluded that higher amount of dye present in wastewater decreased rate of dye removal. Moreover this depression in rate of removal might be directly related to the formation of increased dye metabolite production at higher dye concentrations in wastewater. A negligible amount of toxic product formation was noted at lower concentration of dyes. Carliell et al., (1995) conducted a series of toxicity assays using C.I. Reactive Red 141 as tested dye and concluded that at higher concentrations greater than 100 mgL$^{-1}$, growth of microorganisms (anaerobic) was inhibited. It was proposed that prior adaptation of involved microorganisms elevated their potential to resistance at greater dye concentrations. Effect of dye concentration (acting as substrate) on rate of color removal by various bacterial and fungal strains have been studied by a number of researchers (Ogawa et al., 1981, Ogawa et al., 1986, Amaral et al., 2004, Khehra et al., 2005, Renganathan et al., 2006, Kalme et al., 2007 and Kalyani et al., 2008) and confirmed earlier findings that at higher concentration of dyes, cell growth might be inhibited leading to a lower rate of decolorization.
The following conclusions have been drawn from the literature presented in this chapter:

- Whole Bacterial and Fungal cells or isolated enzymes can be successfully employed for removal of dyes.

- Aerobic as well as anaerobic microorganisms have been adapted to degrade simple as well as complex azo dyes. The enzymes responsible for azo reduction are inducible, with the azo dye being the inducer compound and thus are highly dependent on the type and structure of dye under investigation.

- Decolorization of azo dyes under anaerobic conditions is a relatively simple and non-specific process that involves reduction of the azo bond and subsequent destruction the chromophore. The resulting metabolites might be more toxic than dye itself. So fate of dye metabolites in anaerobic conditions is uncertain.

- Aromatic amine metabolites formed as a result of anaerobic digestion could be degraded by aerobic activated sludge systems.

- A very little research has been done to study complete decolorization and degradation of reactive azo dyes under aerobic conditions.

- Sulphonation of dyes increases their recalcitrance in both aerobic and anaerobic systems, although, the ability of some aerobic microorganisms to oxygenolytically cleave the C-SO$_3$ bond and liberate sulphite allows the mineralization of these sulphonated aromatic compounds under aerobic conditions.

Above critical review explains briefly attempts made for treatment of wastewater. It is quite clear that biotechnological approaches are quite attractive and can be easily applied on industrial scale. Textile sector of PAKISTAN is one of the most promising sectors for economical growth. However the businessmen in PAKISTAN think that cleaning up of the environment is luxury and they cannot afford to pay for it. But now by the change in world trade scenario in the form of WTO, business and government have to
respond to environmental issues urgently so as to keep their export level high.

Keeping in view all of requirements present work was planned. It is an attempt in direction for use of biotechnology; the most suitable method for dye decolorization (as clear from above discussion). The results obtained would be used to start development of a comprehensive analytical approach on industrial level for investigation of reactive azo dye decolorization and complete degradation by microbial strains isolated from effluent channel.
MATERIALS AND METHODS

This research activity was carried out in the Department of Chemistry and Biochemistry and Institute of Soil and Environmental Sciences, University of Agriculture, Faisalabad.

3.1. Collection of Samples

Rhizosphere soil samples were collected from azo dye contaminated sites of a local textile Industry of Faisalabad using extensively dyes under study.

3.2. Chemicals and Culture Medium

Three azo dyes, namely Reactive Black B, Reactive Orange 16 (water soluble reactive sulfonated azo dyes with vinyl sulfone as reactive group); Reactive Brilliant Red K 2BP (water soluble reactive sulfonated azo dye with cyanuric chloride as reactive group) were purchased from Sigma-Aldrich CO., USA. Chemical compounds constituting MSM and used in all other experiments were also of analytical grade.

3.3. Growth Medium

Mineral salts medium (MSM) of following composition (gt⁻¹) Na₂HPO₄ (3.6), (NH₄)₂ SO₄ (1.0), KH₂PO₄ (1.6), Mg SO₄ (1.0), Fe (NH₄) citrate (0.01), CaCl₂. 2H₂O (0.10) and 10.0 ml of trace elements solution per liter was used for all studies. The trace element solution used was of following composition (mg l⁻¹): ZnSO₄. 7H₂O (10.0), MnCl₂. 4H₂O (3.0), CoCl₂. 6H₂O (1.0), NiCl₂. 6H₂O (2.0), NO₂MoO₄. 2H₂O (3.0), H₃BO₃ (3.0) and CuCl₂. 2H₂O (1.0). The final pH of the medium was adjusted to 7.0 ± 0.05. The MSM was supplemented with 0.1% (w/v) of yeast extract and glucose. Reactive dyes selected for this study were added to sterilize MSM from their respective filter sterilized stock solutions.(Khalid et al, 2008)
3.4. Isolation and screening of efficient azo dye decolorising bacterial and fungal strains

Bacterial and fungal strains were isolated from rhizosphere soil samples of effluent channel of a local textile industry where dyes under study were extensively used. Procedure used for isolation of microorganisms (Khalid et al, 2008) is given below.

Some of the isolates which showed a good growth on azo dyes under investigation were isolated from each inoculum. These isolates were then enriched in culture having both Minimal Salt media along with mixture of dyes (in equal amount) under study. No extra source of carbon and nitrogen supply was introduced. Final concentration of dyes in this culture was 100 mg l\(^{-1}\). In a 500ml Erlenmeyer flask 200 ml of MSM was inoculated along with dyes and 10 ml volumes of rhizosphere soil samples. These flasks were incubated at 25\(^{\circ}\)C for a time period of 48 hours under shaking conditions. Cell suspensions were taken from each incubated flask (after completion of time of incubation) and plated onto MSM agar media. These plates were further incubated for 24 hours at 30\(^{\circ}\)C. Microbial colonies which were formed on the agar medium were washed with sterile water. These washed colonies were resuspended into the flasks having fresh MSM broth. Finally it was spiked with mixture of three dyes under study. These cell suspensions were transferred again onto MSM agar plates having 0.1% yeast extract. Finally 90 actively dominant growing colonies each having different colony growth rate was selected. These were further purified by streaking twice on agar medium. The purified cultures were preserved at -20\(^{\circ}\)C in 15\% (w/v) glycerol to be used for further study.

Bacterial and fungal isolates obtained from above enrichment cultures were checked further for their potential to grow and multiply on agar plates having and utilizing selected reactive dyes as the only source responsible for provision of inorganic carbon and nitrogen. Before cultivating microbial cells on agar plates, microbial cells were centrifuged and washed with autoclaved MSM broth separately. Uninoculated controls were included in study to check for a biotic decolorisation. Experiments with each dye-bacteria and dye- fungi combinations were run in six replicates. This could permit measurements at different intervals.
Screening was carried out in liquid cultural medium and thus forty isolates (20 bacteria, 20 fungi) were selected on the basis of decolorization index. These isolates were further tested in agar plates inoculated with uniform cell densities. The bacterial and fungal isolates were first cultured in media MSM having 0.1% yeast extract (with out dye) for 24 hour at 30°C with shaking at 150 rpm. The live cells of isolates were harvested and were maintained to a uniform cell density (0.6 OD at 500 nm). The mean cell counts of these microbial cultures were between 10^8 and 10^9 cfu ml^-1. The cell density standardized decolorization experiment was performed using the same protocol as above.

3.5. **Decolorization of Reactive Azo Dyes in Liquid Media**

From above step most efficient bacterial and fungal strain were selected and were further examined for their decolorization potential in 100 ml Erlenmeyer flasks. Ninety milliliters of the sterilized MSM broth containing 100 mg dye l^-1 were added to autoclaved flasks supplemented with 0.4% yeast extract. It is well reported earlier that yeast extract could support growth of microorganisms; both bacteria and fungi (Sponza and Isik, 2002). In this research work, it was omitted in enrichment where the bacteria and fungi were screened for use of reactive azo dyes as a sole C and N source, but was added in dye decolorization experiments explained hereunder to have higher rates of decolorization for isolated strains. The media were inoculated with the respective bacterial strains by adding inocula of uniform cell density (OD_0.6). A cell density of approximately 10^7 cfu ml^-1 was maintained while ratio between medium to inoculum ratio was kept 50:1. The flasks were tightly sealed and were incubated under shaking conditions at 35°C. Uninoculated flasks (having azo dye, MSM and yeast extracts) were also incubated as control experiments. For each selected strain six flasks were used. Aliquots (1.5 ml) were taken periodically from different flasks alternatively to measure the extent of decolorization of the dyes. The results obtained are recorded as the average of three replications of various intervals.

3.6. **Decolorization Assay via UV-Vis Spectroscopy**

For the measurement of absorption in UV-VIS region, beam Spectrophotometers are most commonly used. Bear- Lambert law (given here under) is then applied to determine quantitative concentration of unknown analyte in given solution under examination.
\[ A = \log \left( \frac{i_o}{i} \right) = \varepsilon cl \]

A = absorbance of sample  
\( c \) = conc. of sample (moles/l)  
\( l \) = path length of sample solution (cm)  
\( \varepsilon \) = molar extinction coefficient

**Figure 3.1.** UV-VIS Spectrophotometer
Samples were withdrawn from serum vials. Supernatant obtained after centrifugation (10,000 rpm for 15 min) was run through spectrophotometer to check absorbance at $\lambda_{\text{max}}$ of selected dyes i.e. 597 nm for Reactive Black, 494 nm for Reactive orange 16, and 531 nm for Reactive Red K 2BP using UV-Vis spectrophotometer (Model Hewlett Packard 8452A). The uninoculated dye free medium was used as blank. All assays of dye decolorization were performed in triplicate.

Chromophoric system of reactive azo dye consists of azo bond present in conjugation with aromatic part of dye. If azo system of these dyes is chemically decomposed color reduction will occur as a result. This color removal can be recorded quantitatively by measuring OD at $\lambda_{\text{max}}$ of that specific dye. Negative controls were also run as uninoculated cultures in two sets; one with dye and the other without dye.
The decolorization efficiency of different isolates was expressed as:

\[
\text{Decolorization (\%)} = \frac{(I - F)}{I} \times 100
\]

Where

- \( I \) = Absorbance of media prior to incubation
- \( F \) = Absorbance of decolorized medium

The data obtained was checked statistically for standard error and standard deviation. (Among triplicate values)

### 3.6. Decolorization under Different Culture Condition

Biodegradation of any pollutant is highly affected by various environmental factors in any ecosystems. Most important of them are pH, temperature, oxygen, and time and substrate concentration etc. (Ganesh et al., 1994). This part of research work would be helpful in characterization of these factors which limit the growth and decolorization potential of newly isolated microbial strains for selected azo dyes.

Decolorization experiments were run under various culture conditions was done by varying one at a time and keeping others constant. (Kalme et al., 2007)

- **Dye concentration**  
  25 mg\text{l}^{-1} – 150 mg\text{l}^{-1}
- **pH**  
  4-8.5
- **Temperature**  
  25 – 50°C
- **Incubation Period**  
  0hr – 240 hours

- Static vs. shaking conditions: Microbial strains from preserved cultures were inoculated into liquid culture media having optimal dye concentration, pH, time of incubation and temperature. Half of the flasks were kept at static conditions after addition of dyes while half were shaken on rotatory shaker at 150 rpm for optimal time of incubation. % Decolorization was monitored in same way as described earlier. All assays were performed in triplicate uncultured controls were prepared parallel in all experiments.
3.7. **Effect of Various Amendments**

Biodegradation of reactive azo dyes is often limited and slow due to limited availability of carbon and nitrogen from complex structure of dyes. The addition of nutrients like carbon and nitrogen may increase the degradation of target organic compound. (Pritchard and Costa, 1991). On the other hand it can also exert no or inhibitory effect on biodegradation. Textile wastewater has generally a very complex chemistry having a number of organic as well as inorganic compounds; dyes being a major compound. So biodegradation in such environment having several metabolizable organic substances acting as substrates more commonly in circumstances when supply of one or more nutrient is a limiting factor is very interesting. Keeping in view the importance of carbon and nitrogen supply, this part of study was undertaken. It involved effect of Carbon and Nitrogen amendments.

3.7.1. **Effect of Added Carbon Sources**

After optimization of experimental conditions to get maximum decolorization for each dye individually, effect of various carbon and nitrogen sources (replacing yeast) on growth and extent of dye decolorization was studied by the protocol already explained.

1. Glucose (2, 6, 8, 10 g l\(^{-1}\)).
2. Sewage sludge (2, 6, 8, 10 g l\(^{-1}\)).
3. Starch (2, 6, 8, 10 g l\(^{-1}\)).

3.7.2. **Effect of Added Nitrogen Sources**

1. Ammonium Sulfate (2, 6, 8, 10 g l\(^{-1}\))
2. Urea (2, 6, 8, 10 g l\(^{-1}\))
3. Ammonium Nitrate (2, 6, 8, 10 g l\(^{-1}\))

No inoculum was added in control flask. All the samples were run in triplicate.

3.8. **Effect of Added Heavy Metals**

Textile effluent is often accompanied by a number of xenobiotics; heavy metals very important members of that group. Remediation of polluted sites both with organic (in the form of dyes) or inorganic (in the form of heavy metals) is a serious problem as both of them belong to two different groups of chemical compounds and thus must be treated with different treatments. But due to co-contamination treatment technology of
one is affected by the other (Roane et al., 2001). As present research work involves biodegradation of dyes as major objective so effect of heavy metals on the activity of microorganisms involved in study must be considered. Metals show interaction to soil biological systems by changing pH, ions bioavailability, and interacting with enzymes directly involved in biodegradation (Nies, 1999).

To study effect of heavy metals on decolorization of three reactive azo dyes under study following metals were selected.

1. Cobalt chloride (CoCl$_2$) 0-2.5 mg/ml$^{-1}$
2. Ferrous sulphate (FeSO$_4$) 0-2.5 mg/ml$^{-1}$
3. Copper Sulphate (CuSO$_4$) 0-2.5 mg/ml$^{-1}$
4. Zinc Sulphate (ZnSO$_4$) 0-2.5 mg/ml$^{-1}$
5. Nickel Chloride (NiCl$_2$) 0-2.5 mg/ml$^{-1}$

At optimal conditions the culture media contained salt of metals in various concentrations (given above) separately in each experiment. Without addition of any heavy metal experiments were performed for comparison. Dye decolorization was measured as described earlier.

3.9. Effect of Electron Donors and Acceptors

Dye decolorisation is generally accepted as oxidation reduction reaction. The first step in degradation of azo dyes by microbial strains grown either aerobically or anaerobically involves reduction of –N=N- bond. (Pandey et al., 2007). However often researchers have reported an enhanced biodegradation in the presence of redox mediators such as antharaquinone 2, 6 sulphonic acid in an anaerobic system (Vander Zee et al., 2000) and 1-hydroxy benztriazole (HBT) for increase of Laccasse activity (Sadhasivam et al., 2009). However a very little literature has been found about role of redox mediators in decolorization of reactive sulphonated azo dyes in aerobic conditions. Hence in present study the ability of newly selected bacterial and fungal strains was investigated in presence of various redox mediators. Redox mediators selected for this purpose are listed here under:

Redox mediators selected for this purpose are.

1. Mannitol (1 – 10 mmol l$^{-1}$)
2. Uric acid (1 – 10 mmol l$^{-1}$)
3. Hydroquinone (1 – 10 mmol l⁻¹)
4. EDTA (1 – 10 mmol l⁻¹)
5. Sodium benzoate (1 – 10 mmol l⁻¹)

A series of control experiments was also performed to check any degradation of selected reactive dyes in the absence of any added redox mediators (Hong et al., 2007).

![Benzene 1,4 diol](image1.png)

**BENZENE 1,4 DIOL**

![Uric acid](image2.png)

**URIC ACID**

![Sodium benzoate](image3.png)

**SODIUM BENZOATE**

![Mannitol](image4.png)

**MANNITOL**
3.10. Study of dye decolorization by Inactive Cells

Fresh culture media of each isolated and selected strain was grown individually. Half of them were autoclaved. Autoclaved cells (dead or inactive) as well as living cells were centrifuged separately at 10,000 rpm for 15 minutes. To determine if extra cellular biosorption of microbial cells were involved in process of decolorization, the supernatant and pallets of living and non living cells were incubated wit dyes and their UV-Vis absorption were used as a measure of decolorization activity (Khehra et al., 2005).

3.11. Effect of Synergism

Isolated laboratory microbial strains named as B2, B1and F1 showing best decolorization potential were choosen and consortium was developed. (Khehra et al., 2005). Different combinations of isolates were made to check presence of any concerted metabolic activity affecting positively or negatively the decolorization potential of isolated strains. Consortium was developed by mixing isolates in 1:1 ratio. The decolorization potential of different consortia thus developed was determined by using same protocol as described earlier.

3.12. Biodegradation Analysis via HPTLC and UV-VIS Spectroscopy

Thin layer chromatography (TLC) is a type of chromatographic technique commonly used to isolate components of a mixture. Most commonly used material in TLC separation is glass but now aluminum foils are also frequently employed. These materials have a thin coating of cellulose, silica gel or aluminum oxide being used as adsorbent or in other words it acts as stationary phase. Sample is applied on it. Mobile phase consists of a single solvent or a mixture of solvents which is responsible for mobility of sample on plate. Separation of components of mixture sample is attained as
each component has its own mobility rate in different solvents and thus they ascend on TLC plates at different time intervals.

In order to get better separation in less resolution times a number of modifications have been made in thin layer chromatographic technique. Following is one of them. This method is named as HPTLC, or "high performance TLC". HPTLC (high performance TLC) plates are characterized by smaller particles (<10 µm), thinner layers (<150 µm) and smaller plates (<10 cm developing distance). Also, the particle size distribution of the sorbent is narrower than for conventional TLC layers. HPTLC plates give better separation as result of higher resolution of components per unit distance. As a result faster development is achieved and less amount of solvent is consumed. TLC silica gel (200 x 200 mm) 0.25 mm thickness plates supplied by Merck, Germany were used to determine type of dye degradation products. After complete decolorization by isolated strains, the supernatant was obtained by centrifuging culture media(same method as used in optimization experiments). Finally supernatant obtained was extracted using ethyl acetate (Supaka et al., 2004). Rotatory evaporator was used to evaporate the phase extracted with organic solvent. The concentrated extract were dissolved in 1ml methanol, and used for their analysis by Thin layer chromatography (TLC). 5 microlitre of the sample was spotted on HPTLC plates (silica gel plates of silica gel 60F254) supplied by Merck (Germany) using a micro syringe. (HPTLC, Cmag, Linomat5)(Moosvei et al, 2005). Three mobile phases were used in separate sets of experiments.

- n-propanol:methanol:ethyl acetate: water: glacial acetic acid (3:2:2:1:0.5)v/v (Kalyani et al., 2005)
- Hexane: ethyl acetate: methanol (5:3:2 ) v/v (Khehra et al.,2005)
- n-butanol:diethylamine: liquor ammonia: methanol (9:5:5:2)v/v (Bhatt et al.,2005)

The presence of any bands corresponding to aromatic compounds was checked under UV lamp (254 nm and 365 nm).

Same samples were run by UV-VIS spectrophotometer (Model Hewlett Packard 8452A) to get information about metabolites formation.
3.11. Biodegradation analysis Via Via GC/MS

A lot of information is available in literature about biodecolorization of azo dyes in aerobic, anaerobic as well as sequential aerobic/anaerobic by bacterial and fungal strains. But relatively less is known /reported about biodegradation and biotransformation products of azo dyes particularly reactive azo dyes. So one of the most important objective of present project was to study degradation metabolites formed as a result of these dyes with the selected microbial strains. For this purpose GC/MS analysis was performed.

GC/MS is stated to be the "gold standard" in scientific analysis

Gas chromatography-mass spectrometry (GC-MS) is a technique that combines the two most important analytical tools i-e gas chromatography and mass spectrometry. This very unique combination is widely used to separate as well as identify various components present in a complex mixture sample. Chromatographic component of this separation technique play a role in resolution of sample into components while the second part i-e Mass Spectrometry identifies the components by determining molecular weight. This technique finds wide applications in environment The GC works on the principle that a mixture will separate into individual components when heated. The heated gases working as mobile phase pass through a column along with helium or any other inert gas. Thus separated components coming out from the column opening enter directly into the MS. Mass spectrometry identifies these compounds on the basis of mass of separated compound. A “library” of known mass spectra, having data of a number of chemical compounds, is stored on a computer. Mass spectrometry is believed to be the only definitive and confirmative analytical tool.
Figure 3.4. General Scheme of GC/MS

![Diagram of GC/MS system]

Figure 3.5. GC/MS

![Image of GC/MS equipment]
The specifications for GC/MS instrument under use were:

- MS ENGINE (989B-Hawlett Packard)
- Integrated gas Chromatogram
- HPI Column
- Length: 30m
- Diameter: 0.25mm
- Non polar in nature
- Helium as carrier gas
- Flow rate (1.1 ml min\(^{-1}\))
- Temperature
- Injection temp: 300\(^\circ\)C
- Oven conditions maintained at 100\(^\circ\)C for 2 min
- Temp. increased to 250\(^\circ\)C with rate of 30\(^\circ\)C min\(^{-1}\)

The analysis was performed with a little modification in protocol: EPA8270. (Kumar et al., 2007 and Kalme et al., 2007).

100 ml of samples were withdrawn from flask (showing maximum decolorization) for identification of metabolites. Samples were centrifuged at 10,000 rpm. Ethyl acetate was used (in equal volume) to extract metabolites from supernatant. The organic extracts thus obtained were dried over anhydrous Na\(_2\)SO\(_4\) and was evaporated to dryness with the help of rotary evaporator. The dried sample was dissolved in methanol and GC/MS analysis. Mass spectras thus obtained were used to identify the chemical compounds being formed as metabolites after comparison with GC-mass spectrometric analysis of standard materials. Final identification and structure determination was done using NIST library.
RESULTS AND DISCUSSION

4.1. STUDY ONE

ISOLATION AND PURIFICATION OF BACTERIAL AND FUNGAL STRAINS FROM SOIL

Microorganisms with efficient decolorization potential of textile dyes particularly reactive azo dyes were isolated from soil collected from effluent channel of a local Textile Industry. It was expected that sites near textile industries contaminated with dyes harbor several microorganisms which are capable to coexist with higher toxic levels of pollution. These microorganisms adapt to the new polluted environment thus that they can play an important role in clearance of this environment through their growth and function.

Pure strains were isolated from the microbial consortium to find out the most promising strains with the higher degradative activity. The final aim was to assure that they do not produce toxic metabolites and are safe if used in bioremediation processes. Isolation of micro flora from soil samples were carried out by enrichment culture technique using all the three dyes under study as sole source of carbon and energy. Screening experiments assessed the potential of two hundred bacterial and fungal isolates from enrichment cultures for decolorizing three different reactive azo dyes under shaking conditions in liquid medium. Out of these strains, twenty most efficient bacterial and ten fungal strains were isolated, purified. Results are shown in Fig 4.1.1-4.1.5. All of these strains was assayed to decolorize the three commercial azo dyes both in liquid and solid medium. Role of bacterial, fungal, yeast and algal biomass in dye decolorization is well known. Several researchers have well documented this fact that microorganisms can even mineralize xenobiotic, azo dyes under certain environmental factors (Banat et al., 1996, O’Neil et al., 2000, McMullan et al., 2001, Stolz, 2001 and Pandey et al., 2007). A number of bacteria having ability to degrade azo dyes have been isolated from sites
**FIG 4.1.1:** DECOLORISATION POTENTIAL OF VARIOUS FUNGAL STRAINS FOR MIXED DYES

**FIG 4.1.2:** DECOLORISATION POTENTIAL OF VARIOUS BACTERIAL STRAINS FOR MIXED DYES
Figure 4.1.3. Decolorization of Reactive black B in liquid culture by Bacterial and Fungal strains
Figure 4.1.4: Decolorization of Reactive Orange 16 in liquid culture by Bacterial and Fungal strains
Figure 4.1.5. Decolorization of Reactive Brilliant Red K2BP in liquid culture by Bacterial and Fungal strains

Further study presented here was carried out with whole bacterial and fungal cells (newly isolated) rather than isolated enzymes. Use of whole bacterial and fungal cells for color removal is more advantageous because isolation and purification of enzyme is a costly process. Moreover whole cells can work more efficiently in severe and harsh environments provided during biodecolorization. Cheetham and Bucket, (1984) have reported that color removal is a complex long process which involves working of a number of enzyme in a proper sequence. It would be very hard and difficult to reorganize and construct this pathway by use of extracted individual enzymes. Thus a definite variability is expected when different microbial strains would be used for color removal of a single dye.

Finally two bacterial and one fungal strain was selected (giving maximum decolorization for dyes under study) namely B₁, B₂ and F₁ for further study. This study also demonstrated that selected microbial strains were capable of removing the color of azo dyes from solid agar medium. The clear zones of the dye’s color around bacterial and fungal colonies were visible on agar plates (data not shown), which further confirmed the ability of selected microbial strains to remove dyes from solid medium. In a number of previous reports it has been shown that bacterial dye decolorization was due to adsorption (Knapp and Newbye, 1999; and Asad, et al., 2007). But appearances of clear zone or white colonies on dye decolorized agar plates obtained in present study clearly suggest that dye decolorization was an enzymatic process rather than adsorption of dyes. This finding is also in consistent with findings of Joe et al., (2008) who reported enzymatic color removal by bacterial strain.

Among the various strains a great difference was recorded for color removal with reference to dye as well as bacterial and fungal isolate. Previous literature makes it clear that dye decolorization is very much dependent on substrate specificity of an enzyme
azoreductase both for pure or mixed culture (Padmavathy, et al., 2003). Enzyme activity show greater changes with changes of structure of dyes (type and position of constituent on aromatic ring) (Zimmermann, et al., 1982). Dye decolorization on the surface of a solid medium by the addition of a bacterial culture has also been reported by (Barragan et al., 2007 and Khalid et al., 2008).

**STUDY TWO:**

### 4.2. OPTIMIZATION OF ENVIRONMENTAL FACTORS FOR MAXIMUM DECOLORIZATION OF SELECTED DYES

#### Effect of Dye Concentration

Dye tolerance is a very important factor to be considered for industrial application. Figure 4.2.1-4.2.3 show the dependence of decolorization rate of Reactive Black B, Reactive Orange 16 and Reactive Brilliant Red K-2BP respectively on dye concentration provided as a sole source of C and N. When initial dye concentration was increased from 0 -100 mg l⁻¹ the % decolorization went on increasing for Reactive Orange 16 and Reactive Black B reaching a maximum value of 94.8% (with F₁) and 89.5 % (with F₁) respectively. However in the case of Reactive Brilliant Red 2KBP maximum decolorization i.e. 94.2% (with F₁) was achieved at a concentration of 100 mg l⁻¹. By further increase in dye amount there was a sharp decrease in decolorization ability in all three dyes with all three microbial strains. If we compare the three strains among themselves for their decolorization ability fungal strain (F₁) showed better potential than other two bacterial strains as maximum decolorization was achieved with it in all three dyes. Among the other two strains B₂ gave better results than B₁. At higher level of concentration a decrease in decolorization potential can be due to substrate inhibition effect as a result of saturation in enzymatic system. It may also be suggested that for these selected microbial strains inhibition of enzyme production occur for dye conc. greater than 750 mg l⁻¹. The dyes understudy possess sulfonic groups, these groups may act as detergents and surfactants which can inhibit the growth of microorganisms (Wuhrmann, et al., 1980).
Fig 4.2.1: Effect of substrate conc. on % decolorisation of Reactive Black-B

Fig 4.2.2: Effect of substrate Conc on % decolorisation of Reactive Orange 16.
Dyes at higher concentration can interfere with synthesis of nucleic acids (Ogawa, et al., 1981) or they can inhibit the microbial cell growth (Ogawa, et al., 1986). The depression of dye decolorization at higher dye concentration (1250 mg l\(^{-1}\)) has also been reported earlier by (Kalyani, et al., 2008; Renganathan, et al., 2006; Moosvi, et al., 2005; Kalmne, et al., 2007 and Khehra, et al., 2005). Chen, et al., (2003) reported 90% of color removal of Red RBN by A. hydrophilr at a dye concentration of 3000 mg l\(^{-1}\) in anoxic culture. Amaral, et al., (2004) also reported 100 mg l\(^{-1}\) as optimum dye concentration (Ractive Black B) for decolorization by white rot fungi.

**Effect of pH**

All microorganisms possess a pH optimum for growth. It is necessary to regulate the pH of biotechnological processes. In the experiments with different pH, the initial dye concentration was fixed at 100 mg l\(^{-1}\) for Reactive Black B and Reactive Orange 16 while it was kept 75 mg l\(^{-1}\) for Reactive Brilliant Red K2BP and the variation of
percentage decolorization with respect to change in pH, keeping all the other conditions constant was studied. At acidic pH 4 there was less than 15% decolorization with all three isolates and three dyes. As pH was increased more towards neutral side the percent decolorization went on increasing and reached to a maximum level at pH 7 for bacterial strain B1 & B2. However fungal strains F1 showed a slight variation and showed highest efficiency at slightly acidic pH i.e. 6.5 will all three reactive dyes under study. As pH was further raised above 7.0 to more alkaline side up to 8.5 there was a decrease in decolorization for all microbial isolates used for biodecolorization of Reactive Black B, Reactive Brilliant Red K2BP and Reactive Orange 16. For Reactive Black b 23.68% color removal was achieved at 8.5 pH with B2, 23% with B1 and 34.73% with F1. For reactive Orange 16 41.5%, 32% and 39% dye decolorization was recorded at pH 8.5 with B2, B1 and F1 respectively. In the case of Reactive Brilliant Red K2BP, 30.2%, 32%, and 14.67% decolorization was observed for B2, B1 and F1 respectively at pH 8.5. From the figures (4.2.4-4.2.6) it is quite clear that bacterial strains showed more sensitivity towards change in pH as compared to applied fungal strain.

The pH tolerance range observed in present study is similar to that reported by Asad, et al., (2007) using halophilic and halotolerant bacteria and Joe et al., (2008) using Clostridium biofermentans SL 186. However Chen, et al., (2003) and Moosvi, et al., (2005) have reported a narrow pH working range. An optimum pH of 7-9 has been recorded by Song, et al., (2003) for biodecolorization of Red dye II by growing cells of Rhodobacter spaeroides. Klebsiella pneumoniae RS-13 completely degraded Methyl Red between 6.0 and 8.0 while Alcaligens liquefaciens S-1 completely degraded Methyl Red at pH 6.5 (Wong and Yuen, 1998). Mali, et al., (1999) found that pH between 6.0 and 8.0 was optimum for decolorization of triphenylmethane and azo dyes by Pseudomonas sp. Vijakumar, et al., (2006) found a pH range of 5.5 – 7.0 for 100% decolorization of acid blue 193(100 mg l\(^{-1}\)) by cladosporium cladosporiodes.

It is a well established fact that fungus grow at relatively low pH which normally ranges from 4-5 (Fu and Viraraghavan, 2001) while bacterial cultures generally show maximum dye decolorization at a neutral pH values i-e near 7.0 (Bhatt, et al., 2005).

The difference in pH effects for azo dye decolorizing bacteria and fungus may be due to difference in genetic determinants responsible for decolorization (e.g. The origin of azoreductase) or in bacterial physiology (e.g. mechanism for transport of dye molecule). Infact, the researchers have found that the molecular weight of azo reductase varies from species to species (Zimmermann, et al., 1984 and Ghosh, et al., 1993 and Xu, et al., 2006).

**Fig 4.2.4: Effect of pH % decolorization of Reactive Black-B**
Fig 4.2.5: Effect of pH on % decolorization of Reactive Orange 16.
Effect of Temperature of Incubation

Temperature is the most critical parameter for dye decolorization. Growth of microorganism is a cumulative activity of a large number of reactions mediated by enzymes. Therefore a direct relationship is observed in between rate of microbial growth and these enzymatic reactions. These enzymatic reactions are thus directly influenced by temperature. In most of cases growth increases with increase in temperature but it represses sharply and abruptly at extreme upper and lower limits of temperature.

In order to determine the effect of temperature on decolorization using three isolated microbial strains for three reactive azo dyes under study a series of experiments were conducted. Results are shown in Fig 4.2.7-4.2.9 and are discussed here under.

The selected bacterial and fungal strains showed highest potential when incubated at 30°C in the case of Reactive Black B and Reactive orange 16. There was a sharp decrease in decolorization by further rising temperature from 30°C to 35°C. With Reactive Brilliant Red K-2BP fungal strain showed maximum decolorization at 35°C while two bacterial strains showed better results at 30°C. To further check the effect of
temperature on rate of decolorization temperature was raised up to 50°C with all three strains and all dyes but continuous depression in color removal was recorded. In the case of Reactive Black minimum decolorization was achieved with B1 i-e 7%, for Reactive Red 14% (with B1) and for Reactive Orange 17.3% (with B2) at 50°C. Fungal strain F1 showed better tolerance and decolorization efficiency even at higher temperatures than the other two bacterial strains. It gave 42.5%, 35% and 19% decolorization for Reactive Black 5, Reactive Red K2BP and Reactive Orange 16 respectively at 50°C.

The results are accordance with those of Wong and Yuen, (1996) who reported that decolorization of methyl red was good between 23-37 °C and completely inhibit at 45 °C. Song, et al., (2003) has also reported maximum decolorization of Red dye II (200 mg dye l⁻¹) at 35-40°C by *Rhodobacter sphaeroides*. Bhatt, et al., (2005) reported an increase in color removal efficiency of NBAR12 up till 40°C with maximum activity and thereafter further increase caused a depression of decolorization potential. Keharia, et al., (2007) has suggested the use of mesophilic range of temperature because to maintain a high temperature at large scale would not be economically favorable and color removal rate is generally too low with psychrophilic range. Similarly Seesuriyachan, et al., (2007) have reported 35°C to be optimum for maximum decolorization, however a decrease occurred at 45°C and at 55°C very small color removal was recorded by using TISTR 1500 (*Lactococcus casei*).

The decrease of decolorization at higher temperature might be due to denaturation and deactivation of enzyme activities involved in decolorization. At higher temperature, enzyme activities are reduced as they are denatured. So microbial growth is drastically affected by change in temperature. Moreover the loss of color removal potential of microorganisms at higher temperature could be due to loss of cell viability (Chang et al, 2001)
**Fig 4.2.7:** Effect of incubation temperature on % decolorization of Reactive Black-B

**Fig 4.2.8:** Effect of incubation temp on % decolorization of Reactive Orange 16.
Effect of Time of Incubation

Results of time courses studies on decolorization of selected dyes are presented in Figure 4.2.10-4.2.12. For Reactive Black B less than 25% decolorization occurred for the first 2 days. It went on increasing slowly reaching a maximum value of 75%, 87% and 90.5% after 8 days of incubation for B₁, B₂ and F₁, respectively. Similarly, maximum decolorization was achieved for Reactive Orange 16 after 8 days of incubation i.e. 95.2% for F₁, 74% for B₁ and 77% by B₂. But maximum decolorization of Reactive Brilliant Red occurred slightly earlier i.e. within 6 days further incubating the dyes with microbial strains until ten days showed a decreasing trend for all three dyes. For Reactive Black B only 68.3% (with B₁), 83% (with B₂) and 87.2% (with F₁) color removal was recorded after 10 days of incubation at optimum conditions. After ten days of incubations B₁, B₂ and F₁ gave only 70%, 73%, and 74.9% decolorization of Reactive Orange 16 respectively. In the case of Reactive Red K2BP 67%, (with B₂), 73% (with B₁) and 80% (with F₁) decolorization was observed. All of these values are much lower than
maximum clearly indicating negative effect of prolonged time of incubation on
decolorization of selected dyes.

Asgher, et al. (2006) reported 100% decolorization of Drimarene Orange K-GL
after 8 days of incubation with *P. chrysosporium* culture. Decolorization of Direct Blue-6
(100 mg l⁻¹) has been reported by Kalme, *et al.*, (2007) in 72 hours using *P. demolyticum*.
Moosvi, *et al.*, (2005) recorded maximum decolorization of RV5 (100 mg l⁻¹) in 37 hrs.
Various strains of bacteria and fungi show different response at different times of
incubation which might also be contributed to difference in enzymatic system,
adaptability of particular strain to particular dye as a change in chemical structure of
substrate greatly effect the decolorization potential and rate.

![Fig 4.2.10: Effect of incubation time on % decolorization of Reactive Black-B](image)

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**Fig 4.2.10:: Effect of incubation time on %
decolorization of Reactive Black-B**
Fig 4.2.11: Effect of incubation time on % decolorization of Reactive Orange 16.
**Shaking Vs Static Condition**

Effect of oxygen on cell growth and dye decolorization is one of the most critical factors to be considered. To check the effect of aeration on decolorization of three reactive dyes two different sets of experiments were set. In one set of experiments, flasks were incubated at 30°C, 44 hours, pH, 7.0, 100 mg l$^{-1}$ of dye (for Reactive Orange 16 and Reactive Black B) and 35°C, 96 hours pH, 7.0, and 75 mg l$^{-1}$ (for Reactive Red K2BP) for bacterial strain B$_1$ and B$_2$ while changing pH 7.5 for F$_1$ under static conditions. While in other set of experiments all the conditions were same as described above however flasks were incubated under constant shaking conditions means aerobic environment.

After optimum time samples were withdrawn from flasks and the rate of decolorization was recorded by spectrophotometer. A comparison was made between shaking vs static condition for decolorization potential of dyes with all three strains. Results obtained are shown Figure 4.2.13-4.2.15. It is quite clear from the data obtained that under shaking conditions better decolorization could be achieved with all three dyes.
and three microbial strains used for this investigation. The decolorization decreased to half when experimental conditions were changed from shaking to static keeping all the factors same. Only 25.3%, 24.6% and 45.2% color removal was recorded for Reactive Black B with B2, B1 and F1 under static conditions. For reactive Orange 16 23%, 25% and 41% of dye was decolorized with B2, B1 and f1 respectively. Similarly 40%, 31.4% and 27.4% of dye (Reactive Brilliant Red) was decolorized under static conditions by B2, B1 and F1 respectively.

Earlier reports revealed that shaking might enhance the competition between the azo compounds and oxygen for reduced electron carriers under aerobic conditions (Chang and Lin, 2001 and Kalme, et al., 2007) and thus decreased the decolorization process (Khalid et al., 2008). Moosvi, et al., (1999) observed no inhibition effect of shaking on decolorization process. Yesilada, et al., (1995) reported no much difference in color removal achieved by static and shaking cultures. However Soares and Duran, (1998) reported essential role of agitation for achievement of a high level of decolorization rate by T. villosa which is in harmony with our findings. Similarly Coughlin et al,(1993)have reported degradation of an azo dye AO7 by an obligate aerobic sphignomaonas sp using dye as sole source of carbon and nitrogen.
FIG 4.2.13: Effect of shaking on decolorization of Reactive Black-B

FIG 4.2.14: Effect of shaking on % decolorization of Reactive orange 16
However the results reported in present work are in contrast with those reported by Chen, *et al.*, (2003) who observed a good growth of *A. hydrophila* in aerobic and agitating culture but very poor color removal in aerobic conditions.

In present study higher decolorization by agitation could be due to physiological state of microbial colonies and increased mass transfer between cells and medium. In stationary cultures formation of mat at the surface results in restriction of oxygen transfer to the cells beneath the surface and in the medium resulting in oxygen limitation, which inhibits the oxidative enzymes and prevent decolorization (Swamy and Ramsay, 1999) leading to low rates of decolorizations. These results are in agreement with that of Knapp, *et al.*, (1995) and Revanker and Lele, (2007).

In shaking conditions no adsorption of dyes on biomass was seen but decolorization of dyes was observed. Mane, *et al.*, (2008) has reported no decolorization of Navy-Blue Rx
at static conditions but the actinomycets grown as well kept for color removal at shaking condition showed 95.33 % decolorization of dyes. Oxidative biodegradation takes place upon action of enzymes such as peroxidases (Kandelbauer et al. 2004). which require essential presence of oxygen. Thus it can be concluded that presence of oxygen for the selected aerobic microflora under study played an essential role for better decolorization of dyes under study.

4.3. STUDY THREE

KINETICS OF DYE DECOLORIZATION

In order to determine the maximum decolorization and maximum tolerance to concentration of dye shown by isolated bacterial and fungal strains in shaking conditions, experiments with various initial dye concentration at different time of incubations. The shapes of the curves thus obtained give knowledge about metabolism of given dyes with selected microbial cultures under optimized conditions. Information like this is very important because it tells us about concentration of dye at particular time and thus its amount at particular future time can be predicted. This all information collectively can help in assessment about dye that whether it would be eliminated or will persist before its transportation to a point of exposure to life (including animals, plants and humans).

When the graphs were plotted between dye declorisation and time course of incubation, after reaching a maximum value there was a sharp decrease in color removal which on further addition of substrate remain unaffected. This type of behavior can be best explained by Monod Kinetic Model. It can best explain biodegradation of organic compounds acting as substrate for microorganisms which are growing by using this organic substrate as a source of carbon and energy. For its explanation it is assumed that when concentration of provided organic substrate is low, the growth rate of growing microflora is correspondingly lower. However with increase in supply of carbon source (here dye) growth increases. After reaching to maximum, at some higher level of substrate, growth rate does not increase further.
This is explained mathematically as:

\[ \mu = \frac{\mu_{\text{max}} + S}{k_S + S} \]

Where \( \mu \) = specific growth rate
\( \mu_{\text{max}} \) = maximum specific growth rate
\( S \) = substrate (dye) concentration
\( k_S \) = a constant (substrate conc.) at which the rate of growth is half the maximum.

The value of \( K_S \) shows the affinity of substrate with microorganism, greater the value, lower the affinity.

Gmport et al (1990) Hsuch and Chen (2007) have reported that “simple monod kinetic model applied only to the initial velocity of an enzyme catalysed reaction. That is to the velocity when no appreciable amount of product (e.g. dye decolorization intermediates) has accumulated. This point apparently suggested that monod kinetic model had better predications to decolorization of all days at some initial period of time due to negligible metabolite formulation. A general mechanism for cell catalyzed kinetics of such reaction consists of four reaction steps given below.

The k1 step: X+S \( \xrightarrow{K_1} \) XS
k2 step: XS \( \xrightarrow{K_2} \) X+S
k3 step: XS \( \xrightarrow{K_3} \) X+P
And k4 step: XS \( \xrightarrow{k_4} \) X+P

Where
X= bacterial or fungal cells
S= dye acting as substrate
XS= Cell substrate complex
P= dye decolorization metabolites

P may be two three or even more chemical compounds which depends upon structure of dye.

Specific decolorization rate of dyes with different selected strains under study are shown in Fig 4.3.1-4.3.9. From the graphs it could be concluded that bacterial strain B1 has
lower $K_s$ i-e 40 where $K_s$ is constant representing substrate concentration at which rate of growth is half of the maximum rate $K_s$ for $B_2$ is 60 where $K_s$ of $F_1$ is 40. Lower the value of $K_s$ higher would be its affinity for substrate. For Reactive Orange 16 again $B_1$ and $F_1$ showed every good affinities with $K_s$ value of 30 and 33 respectively while $B_2$ showed a poor affinity with $K_s = 47$. All the three strains showed very good affinities for Reactive Brilliant Red 2KBP. $K_s$ value of $B_1$ was 15, for $B_2$ 15, for $F_1$ 20, that’s why maximum decolorization of Reactive Brilliant Red 2KBP was achieved at farther rates than for Reactive black B and Reactive Orange 16. This fact is reflected in our previous findings where maximum decolorization of Reactive red was achieved earlier as compared to other two dyes under study.
Fig 4.3.1: Dependence of specific decolorization rate to the concentration of Reactive Black-B using B1
Fig 4.3.2: Dependence of specific decolorization rate to the concentration of Reactive Black-B using B-2 in shaking culture
Fig 4.3.3: Dependence of specific decolorization rate to the concentration of Reactive Black-B using F-1 in shaking culture
Fig 4.3.4: Dependence of specific decolorization rate to the concentration of Reactive Orange-16 using B-1 in shaking culture
Fig 4.3.5: Dependence of specific decolorization rate to the concentration of Reactive Orange-16 using B-2 in shaking culture.
Fig 4.3.6: Dependence of specific decolorization rate to the concentration of Reactive Orange-16 using F1 in shaking culture

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Graph showing the dependence of specific decolorization rate to the concentration of Reactive Orange-16 using F1 in shaking culture. The x-axis represents the dye level (mg/ml^-1) ranging from 20 to 100, while the y-axis shows the degradation (%) ranging from 0 to 80.

Key points:
- **Ks** is marked on the graph, indicating a significant point in the degradation curve.
- The maximum degradation rate is highlighted at **μ_dye, max.**
Fig 4.3.7: Dependence of specific decolorization rate to the concentration of reactive Brithant Red K-2BP using bacterial strain (B1) in shaking culture
Fig 4.3.8: Dependence of specific decolorization rate to the concentration of reactive Brilliant Red K-2BP using bacterial strain (B2) in shaking culture
Dependence of specific decolorization rate to the concentration of Reactive Brilliant Red K-2BP using fungal strain in shaking culture
4.4. STUDY FOUR

Effect of Added Carbon Sources on Decolorization Rate of Selected Dyes

Figure 4.4.1-4.4.9 show the effect of different selected carbon sources on decolorization of reactive azo dyes under study.

**Reactive Black-B**

Without any added carbon source, B₁ showed 74% B₂, 87%, and F₁ 90% decolorization at optimized conditions. By adding 2 mg ml⁻¹ of glucose as additional carbon source there was a slight increase in % decolorization reaching up to 93% (F₁), 89% (B₂), 78.2% (B₁) for Reactive Black-B. By further increase i.e. 4 mg ml⁻¹ of glucose there was a sharp decrease in color removal which continued up to 10 mg ml⁻¹ addition of glucose.

By adding starch (2mg ml⁻¹) as additional carbon source, there was no increase in decolorization potential of any bacterial and fungal strain. Starch was added up to 10 mg ml⁻¹ but there was a continuous decrease in rate of decolorization.

When sewage sludge was added along with the dye as additional source of carbon, an increase occurred in decolorization of Reactive Black-B. However, when amount of sewage sludge was further increased, no positive effect could be observed on percentage decolorization of Reactive Black-B.

**Reactive Orange-16**

By adding 2mg ml⁻¹ of glucose rate of decorization slightly increased from 77.2% to 79.5% for B₂, 74% to 76% for B₁ and 95% to 96.5% for F₁. By increasing further the amount of added glucose to mg ml⁻¹, decolorization potential of two bacterial strains increased to 85.9% (B₂) and 78.4% (B₁). However for fungal strain F₁ it was decreased to 86.9 at 4 mg ml⁻¹.

By further increase in concentration of glucose as additional source of carbon, color reduction of reactive Orange 16 decreased and only 40% with B₂, 23% with B₁ and 3% with F₁ was recorded at 10 mg ml⁻¹ of glucose.

By using starch as an additional source of carbon along with tested dye (Reactive Orange 16), at first there was a very small increase but hen a continuous decrease of decolorization potential occurred for all three microbial strains for bacterial strain B₂ 78.9% decolorization was achieved at 2 mg ml⁻¹ addition of starch, 86% with B₁ and 94% with F₁. However further addition of starch lead to depletion in decolorization potential. At 10 mg ml⁻¹ addition only 30.1% decolorization was recorded for B₂, 28.9% for B₁ and 35% for F₁.
Fig 4.4.1: Effect of glucose on % decolorization of Reactive Black-B

Conc(mg ml\(^{-1}\))

% Decolorization

Fig 4.4.2: Effect of starch on % decolorization of Reactive Black B
Fig 4.4.3: Effect of sewage sludge on the % decolorization of Reactive Black-B

Fig 4.4.4: Effect of glucose on % decolorization of Reactive Orange 16
Fig 4.4.5: Effect of starch on % decolorization of Reactive Orange 16.

Fig 4.4.6: Effect of sewage sludge on % decolorization of Reactive Orange 16.
Fig 4.4.7: Effect of glucose on % decolorization of Reactive Brilliant Red 2KBP

Fig 4.4.8: Effect of starch on % decolorization of Reactive Brilliant Red 2KBP
The third additional carbon source used in this study was sewage sludge. It was only effective to a small extent at very lower concentration. Only 2% (B₂) increase was observed when 2 mg ml⁻¹ of sewage sludge was added (from 76.8% to 78.9%). Similarly with B₁ only 2% increase occurred however, with F₁ there was decrease in decolorization potential of reactive Orange 16. There was a constant decrease in color reduction with increase in addition of sewage for all microbial strains. At 10 mg ml⁻¹ addition bacterial strain B₂ showed 24% decolorization, B₁ gave 39% and fungal strain F₁ showed only 44% color reduction of reactive Orange 16. Overall in the case of reactive Orange 16 none of tested additional carbon sources showed any remarkable increase in decolorization potential of either fungal or bacterial strains.

**Reactive Brilliant Red K-2BP**

When 2mg ml⁻¹ of glucose was added decolorization of Reactive Brilliant Red increased showing a maximum value of 95% for F₁, 80% for B₂ and 88% for B₁. By increasing the amount of glucose added, % decolorization went on decreasing reaching to a value of 30% (F₁), 34% (B₂), 23% (B₁) at 10 mg ml⁻¹ of glucose. These values are almost half of the maximum decolorization efficiency.
In second set of experiments starch (2 mg ml⁻¹ – 10 mg ml⁻¹) was used as additional source of ‘C’ along with Reactive Red. In start at lower levels of starch (2 mg ml⁻¹) decolorization potential of all three strains increased but then a sharp decrease occurred reaching up to 35% (F₁), 30% (B₂) and 30% (B₁).

Similar trends were recorded for sewage sludge. An increase occurred from 91% to 93%, 76% to 79%, and 84% to 85.5% for F₁, B₂ and B₁ respectively by adding 2 mg ml⁻¹ of sewage sludge. Then there was a sharp decrease in rate of decolorization. However further increase could not cause any increase in color removal ability of any strain.

**DISCUSSION**

It can be summarized from above results that all the three microbial strains were able to grow on chosen carbon source including glucose, starch and sewage sludge. However decolorization potential varied for each case differently. At start when concentration of additional carbon source was low the decolorization ability showed an average increasing trend for all dyes however with constant further increase in supply of additional carbon source, a decrease occurred. With addition of carbon sources other than dye as nutrient sources microbial growth was not inhibited (data not shown). This clearly indicates that these carbon sources have no toxic effects. Microbial counts were higher in liquid media with additional carbon nutrients (data not shown) Similar to the findings of Kirchman (1990) and Laird *et al.* (1990) who reported an increase in bacterial growth by organic additions. But on the other hand extent of % decolorization of dyes was not concurrently improved. Decrease in decolorization potential may be as a result of accumulation of simple carbon compound acting as other carbon sources or co metabolic substrate in media. Similar trend has been reported by other authors Adosinda *et al.*, 2001 and Swamy and Ramsay, 1999. A wide range of carbon sources could be used for increase in rate of dye transformation-glucose and acetic acid (Panswad and Luangdilok, 2000), ethanol (Tan *et al.*, 2000) and hydrolyzed starch. In most cases where rot fungi, was used as decolorizing micro flora glucose or other carbon sources provided a necessary substrate for cell growth and enzyme production. In such case the enzyme production occurred during their secondary metabolism and it was induced only by limited nutrient level, Wesenberg *et al.* (2003) has reported the dye degradation after
those enzyme productions. At this point our findings are different both for bacteria and fungi as a decrease in decolorization was observed by increase in supply of carbon source. However these are in accordance of Xu et al. (2006) who also reported a decrease in decolorization of anthraquinone dye by *shawanella discolorations* S12 when glucose, sucrose and dextrin were provided as additional carbon sources. Yang et al. (2004) reported that starch was unable to support the yeast growth and decolorization of Reactive Black-B.

Song et al. (2003) recorded an accelerated decolorization of Red dye II with additional carbon source including peptone, glucose, sucrose and malic acid. Khehra et al. (2005) determined effect of glucose on decolorization ability of MSM for dye AR-88. Glucose was found to be essential for decolorization and only 20% of decolorization occurred in its absence. The variability among the earlier reported studies and findings of foregoing research might be due to difference in microbial characteristic and enzymes responsible for color removal. It can be concluded that in start metabolism of glucose or starch resulted production of nucleotides (NADH & FADH) which in turn lead to increase decolorization efficiency (Khehra et al., 2005). These nucleotides in reduced forms are recorded to act as Redox mediators However at higher concentration the situation is different. For example metabolism of glucose causes the production and accumulation of some organic acids in liquid culture (Chen et al., 2003), which in turn can lead to an acidic pH of culture media. As in earlier part of study (already discussed) it was found that none of strains under study worked efficiently in very high acidic media. So this low pH might be one of the factors for lower decolorization potential at higher levels of additional carbon sources. In foregoing study sewage sludge showed a low increase in decolorization ability, this might be due the fact that it contains large amount of other pollutants including heavy metals which could be responsible for greater inhibitory effect at higher concentration. Moreover, sewage sludge contains more than one organic contaminants. Dyes being complex in structure cannot be degraded very easily, so presence of other organic substance in sewage sludge having more simpler structure are an attractive substrates for microbial strains understudy which could increase microbial growth. At higher growth rates there would be a competition between
inorganic nutrients present in media which may lead to a decreased rate of decolorization of target dyes.

### 4.5. STUDY FIVE

**Effect of Added Nitrogen Sources on Decolorization of Selected Dyes**

Potential of micro flora to use organic xenobiotics as substrate resulting in their degradation is highly affected by nutrient supply. For application of biotreatment on large scale for textile wastewater a complete knowledge about nutrient requirements of microorganism under investigation should be available. This part of investigation was undertaken to examine the effect of nitrogen; a very important nutrient for growth on decolorization ability of Reactive Sulphonated azo dyes under study. Three nitrogen sources selected were Urea, Ammonium Nitrate and Ammonium Sulphate. The results obtained are presented in Figure 4.5.1-4.5.9.

**Reactive Black B**

The results obtained are shown in Figure 4.5.1-4.5.3. With urea as additional organic nutrient supply there was a constant decrease in decolorization potential at all concentration for both bacterial as well as fungal strains. With B₂ (Bacterial strain) 86.6% dye decolorization could be recorded with out any addition which went on decreasing finally reaching to 30.6% at 10 g l⁻¹ of urea. Bacterial strain B₁ showed 20% reduction in color removal at 2 g l⁻¹ of urea. Further addition lead to a continuous but very slow decrease. At 10 g l⁻¹ 37% of dye was decolorized. Fungal strain F₁ showed a gradual slow decrease in its decolorization ability by addition of urea. At optimal condition 88.9% of dye was decolorized while at 10 g l⁻¹ of urea only 40.31% of dye was decolorized (almost 50% reduction in decolorization potential).

With Ammonium Nitrate bacterial strain (B₂) showed on different response than B₁ (Bacterial strain) and F₁ (fungal strain). There was an increase in color removal of Reactive Black B at 2 g l⁻¹ addition from 75.6% (0 g l⁻¹) to 79% with B₁. With F₁ 93% (2 g l⁻¹) of dye decolorization was recorded as compared to 91% (0 g l⁻¹). By increasing amount of Ammonium Nitrate from 4 g l⁻¹ to 6 g l⁻¹ a slow decrease was recorded for B₁ (Figure 4.5.2). However a reduction of 20% could be observed at 8 g l⁻¹. At 10 g l⁻¹ 42.7% of color removal was achieved with B₁.
With $F_1$ a sharp decrease in decolorization ability occurred while increasing concentration of Ammonium Nitrate from $2 \text{ g l}^{-1}$ to $4 \text{ g l}^{-1}$. Only 54% of color removal occurred at $10 \text{ g l}^{-1}$ of Ammonium Nitrate. Bacterial strain $B_2$ showed a continuous decrease in color removal of Reactive Black B by adding Ammonium Nitrate. At $10 \text{ g l}^{-1}$ 32% dye could be decolorized which was 50% less than that achieved with no addition.

Ammonium Sulphate increased decolorization potential of all three microbial strains to a very small extent at $2 \text{ g l}^{-1}$ addition. However further increase caused a depletion of color removal. Fungal strain showed lowest tolerance and 60% decrease in color removal rate was recorded at $10 \text{ g l}^{-1}$ addition. Bacterial strain $B_2$ gave 38.4% decolorisation while $B_1$ gave 28.9% decolorization at $10 \text{ g l}^{-1}$ of addition (Figure 4.5.3).

**Fig 4.5.1: Effect of urea on % decolorization on of Reactive Black-B**
FIG 4.5.2: Effect of Ammonium Nitrate on % decolorization of Reactive Black-B

% Decolorization

conc (mg/ml)

B1
B2
F1
Reactive Orange 16

In the case of reactive Orange 16, addition of urea caused a very sharp decrease in decolorization potential of all microbial strains even at a very low concentration i.e. 2 g\text{l}^{-1} (Fig 4.5.4). Decolorization rate decreased to 30% for B$_2$, 10% for B$_1$ and 30% for F$_1$ at initial addition (2 g\text{l}^{-1}). By increasing amount of added urea, color removal extent decreased and finally at 10 g\text{l}^{-1} addition 35.42% (B$_2$), 41.8% (B$_1$) and 43.21% (F$_1$) of dye was decolorized. With Ammonium Nitrate (Figure 4.5.5) there was no increase in color removal rate at all concentration. A continuous decrease was observed and 25% of dye was decolorized by B$_2$, 33% by B$_1$ and 50% by F$_1$ at 10 g\text{l}^{-1} addition. Among the various strains, bacterial strain B$_2$ showed maximum sensitivity towards additional inorganic nitrogen source showing a fifty percent decrease in decolorization potential.

The results for effect of Ammonium Sulphate and % decolorization of Reactive Orange16 are shown in Figure 4.5.6. Ammonium Sulphate showed an increasing, trend in color removal at 2 g\text{l}^{-1} for both bacterial strains. With B$_2$ 5% increase occurred and 82. % dye was decolorized while with B$_1$ 14% increase occurred and 88% of dye was decolorized at 2 g\text{l}^{-1} addition. However no more increase could be observed. With 45% decrease, 30.6% of color removal occurred at 10 g\text{l}^{-1} for B$_2$ while at same concentration
B₁ showed 40% decrease in decolorization potential with 36.7% color removal. Fungal strain showed a different response. A 20% decrease was recorded at 2 g l⁻¹ addition of ammonium sulphate with further addition a continuous but slow decrease was observed in dye decolorization. Finally at 10 g l⁻¹ of added ammonium nitrate 30.7% of dye was decolourised.

**Fig 4.5.4: Effect of urea on % decolorization of Reactive Orange 16.**
Fig 4.5.5: Effect of Ammonium Nitrate on
% decolorization of Reactive Orange 16
Reactive Brilliant Red K 2BP

With bacterial strain $B_2$ 76% color removal (maximum value) was achieved at optimal growth additions with no urea addition. By adding a very small amount of urea i.e. 2 g/l, 10% decrease in decolorization potential was observed and 68.5% dye decolorization was noted. With further increase in amount of added urea, decolorization ability of $B_2$ went on decreasing. At 10 g/l addition almost 50% reduction was recorded and 30.7% color removal occurred. Same was the case with other bacterial strain $B_1$. At first addition of urea (2 g/l), 20% reduction occurred in decolorization which went on decreasing with gradual increase in addition of urea as organic nitrogen source. With 50% reduction only 41.2% decolorization of Reactive Brilliant Red 2KBP could be achieved at 10 g/l of urea.
However data (Figure 4.5.7) shows that fungal strain F\textsubscript{1} responded more sensitively to urea amendment. At first a sharp decrease in color removal occurred i.e. 30\% less at 2 g l\textsuperscript{-1} of urea. With further increase in concentration of urea, a slow depression in color removal occurred. At 10 g l\textsuperscript{-1} of urea 30.2\% color removal of Reactive Brilliant Red 2KBP was noted. With Ammonium Nitrate bacterial strain B\textsubscript{2} behaved differently than bacterial strain B\textsubscript{1} and fungal strain F\textsubscript{1}. By adding 2 g l\textsuperscript{-1} of Ammonium Nitrate color removal increased from 77\% (with no addition) to 78\%. By increasing amount of added inorganic nitrogen source in the form of Ammonium Nitrate decrease in decolorization ability occurred and at 10 g l\textsuperscript{-1}, 40.4\% of Reactive Brilliant Red was decolorized with B\textsubscript{2}. The other unidentified bacterial strain B\textsubscript{1} showed a decrease in color removal ability by addition of extra nitrogen source. With almost 50\% reduction in decolorization potential only 30\% of dye was biodecolorized at 10 g l\textsuperscript{-1} addition of Ammonium Nitrate. As compared to bacterial strains, fungal strain F\textsubscript{1} showed less sensitivity. It gave 92.4\% decolorization of Reactive Brilliant Red 2KBP which slowly went on decreasing with increasing amount of Ammonium Nitrate. At 10 g l\textsuperscript{-1} of Ammonium Nitrate, fungal strain could decolorize only 52.7\% of dye.

**Fig 4.5.7:** Effect of urea on \% decolorization of Reactive Brilliant Red 2KBP
Fig 4.5.8: Effect of Ammonium Nitrate on % decolorization of Reactive Brilliant Red 2KBP

Fig 4.5.9: Effect of Ammonium Sulphate on % decolorization of Reactive Brilliant Red 2KBP
The third nitrogen amendment was in the form of Ammonium Sulfate, results are shown in Figure 4.5.9. Both of the bacterial strains i.e. B₂ and B₁ showed a positive response at initial concentration of Ammonium Sulphate. At 2 g l⁻¹ addition 80.2% and 88.2% of dye decolorization were recorded for B₂ and B₁ respectively as compared to 77% and 84% (with no addition). No increase observed at higher concentrations and only 29.5% color removal occurred at 10 g l⁻¹ for B₂. While for B₁ a sharp decrease was observed at 10 g l⁻¹ and only 15.2% dye decolorization could be noted. As far as fungal strain was concerned no enhancement in decolorization was observed with Ammonium Sulfate. At initial lower addition, slow decrease occurred in decolorization of dye however a decrease of 30% occurred while moving from 2 g l⁻¹ of Ammonium Sulfate to 10 g l⁻¹. Only 37.8% of Reactive Brilliant Red K2BP could be decolorized at this addition.

Overall by looking into above results urea showed inhibitory effect with all three microbial strains under study for three reactive azo dyes. Ammonium Nitrate showed a non-significant increase (at initial one) for bacterial strain however no enhancement in decolorization ability of fungal strain. Ammonium Sulfate gave a slow increase in color removal for all three dyes as well as microbial strains at initial additions. However a strong inhibition in decolorization occurred at higher levels for all additional nitrogen sources under investigation. These results are in harmony with those reported by Zhang et al. (1999) who observed a decrease in decolorization rate by addition of NH₄⁺. Similarly Tatarko and Bumpus (1998) have reported inhibition of decolorization of Congo red by addition of supplemental nitrogen. However Chen et al. (2003) reported an increase in decolorization of mixture of dyes both in presence of organic as well as inorganic nitrogen sources which is in contrast to our findings.

Sanghi et al. (2006) has also reported that inorganic nitrogen sources were inefficient to stimulate dye decolorization. Lignin degradation by P. chrysosporium increased at low doses of ammonium nitrate and asparagines but at higher concentration an inhibition was recorded (Reid, 1983).

The addition of inorganic nutrients does not always enhance degradation of organic compounds because there are many other factors which may decrease microbial activity (Steffensen and Alexander, 1995).
Urea when dissolved in liquid culture causes a shift of pH more towards acidic side. As microbial strains under study gave maximum decolorization at neutral pH so there activity in terms of growth as well as enzymatic activity would be decreased at acidic pH leading to a decrease in decolorization ability. In the case of Ammonium Nitrate the decrease in decolorization might be due to presence of nitrate. Firstly as dye decolorization is an oxidation reduction reaction in which azo dyes serves as electron acceptor, nitrate also serves as electron acceptor so its presence might slow down process of color removal (Carliell et al., 1995; Panswad and Luangdilok, 2000). The other reason might be simple structure of ammonium nitrate as compared to complex structure of reactive azo dyes under investigation. Due to presence of these simple inorganic nitrogen sources, microorganism preferably metabolized them rather than amines or azo dyes present in media. This lead to inhibition of decolorization of dyes at higher level addition of inorganic nitrate. Hu (1998) have also reported a better and higher mineralization of azo dye in nitrogen limited cultures. The third source used was Ammonium Sulphate. Sulphate could inhibit decolorization of dyes (Ganesh et al., 1994; Oranusi and Ogugbue, 2005). Dye decolorization can be achieved better in sulphate limited media. As desulphonation of sulphonic acid groups present on dyes understudy would provide sufficient amount of sulphar.

4.6. STUDY SIX

EFFECT OF HEAVY METALS ON DECOLORIZATION OF DYES UNDER OPTIMAL ENVIRONMENTAL CONDITIONS

Although many heavy metals are necessary for microbial growth and are required in very small amounts however, if their concentration is increased to higher levels they can become toxic (Kamel et al., 1989). It has also been well proven that some heavy metals which do not have any role in functioning of microorganisms are highly toxic even at very small concentrations (Coker and Ekundayo, 1998).

Synthetic wastewater is characterized often by presence of mixture of dyes as well as highly persistent inorganic ions e.g. Cu, Hg, Cr. (Lin and Peng, 1994). This is one of the serious problems for treatment biotechnology. Studies have found that metal affect the growth, structure, and chemical activities of microorganisms (Younni et al., 2002).
Different microbes react differently. Heavy metals like cadmium, copper, or mercury are known to be toxic for both white rot fungi (Baldrian and Gabriel, 1997; Baldrian et al., 2000) and soil micro flora (Baldrian et al., 2000). The presence of these substances in environment can negatively influence the effectiveness of bioremediation technologies. Dye decolorization is always performed using enzymes in microbial cells, which can be permanently inhibited by heavy metal compounds (Xu et al., 2006).

This part of present research work involves the investigation about effects of various metallic compounds on decolorization of tested dyes. The results obtained are shown in Figure4.6.1-4.6.15. From data it is clear that all metals in all concentrations inhibited decolorization. However, the extent of decolorization is highly variable, for bacterial as well as fungal strain. Detailed description of results with reference to each metal individually is given here under.

**Effect of Cobalt Chloride**

In the case of Reactive Black B, by addition of cobalt, bacterial strains B₂ and B₁ showed less inhibition as compared to fungal strain. For B₂ maximum decolorization was achieved with no added metal i.e. 85.5% which remained almost half (45%) by a very little amount of cobalt chloride (0.5 gl⁻¹). Minimum decolorization was 15% at 2.5 gl⁻¹ addition of cobalt. With bacterial strain B₁ no sharp decrease in decolorization potential occurred at first (decreased to 64% from 75%). A constant slow decrease occurred further reaching a minimum value of 19.84% at 2.5 gl⁻¹ of cobalt sulphate. Fungal strain F₁ showed greater inhibition as compared to bacterial isolates. At first by adding 0.5 gl⁻¹ of metal % decolorization of Reactive Black B remained 69% from 90.12% (no addition). By increasing the addition, decolorization went on decreasing. Almost negligible i.e. only 7% of decolorization was observed at 2.5 gl⁻¹ of cobalt sulphate. With Reactive Orange 16, a slow but constant decrease occurred in decolorization potential of bacterial strain B₂. With bacterial isolate a sharp decrease was noticed at start but with further increase a very slow inhibition of decolorization potential was noticed. Only 15% of decolorization was achieved at 2.5 gl⁻¹ addition of cobalt. However, just like reactive Black 5, fungal strain F₁ showed greater sensitivity to cobalt addition using reactive Orange 16 as carbon source. Its decolonization potential decreased from 95.2% to only 9.8% from 0 gl⁻¹ to 2.5 gl⁻¹ addition of cobalt.
With Reactive Brilliant Red 2KBP decolorization potential of all three tested strains (bacterial and fungal) decreased with increase in concentration of added heavy metal i.e. cobalt. With bacterial strain B1 a three fold decreased occurred by 2.5 g l\(^{-1}\).

*Fig 4.6.1::Effect of cobalt on % decolorization of Reactive Black-B*
Fig 4.6.2: Effect of Cobalt on % decolorization of Reactive Orange 16.

Fig 4.6.3: Effect of cobalt on % decolorization of Reactive Brilliant Red 2KBP
addition and only 27.2% of dye color removal was attained. With B₂ (bacterial strain) % decolorization reached to half of its maximum value at highest level of addition (2.5 g l⁻¹ of Cobalt chloride) i.e. 35.1% from 77.1%. With F₁ 92.4% decolorization of Reactive Brilliant Red 2KBP occurred at optimal conditions, however, it went on decreasing by addition of cobalt and only 20.2% decolorization could be achieved at 2.5 g l⁻¹ addition of cobalt.

**Effect of Ferrous Chloride**

With addition of ferrous chloride decolorization potential of three microbial strains decreased. It ranged from 85.3% - 35% with adding 0 – 2.5 g l⁻¹ of FeCl₂ for B₂, 78% - 39.12% (0 – 2.5 g l⁻¹ of FeCl₂) for B₁ and 89.9% - 40% for 0 – 0.25 g l⁻¹ of FeCl₂ with fungal strain F₁. In all three cases a rapid decrease was observed at first addition (0.5 g l⁻¹) of heavy metal: iron (FeCl₂). Then a slow decrease occurred with further addition. However, overall from the data (Figure 4.6.4-4.6.6) it is quite obvious that FeCl₃ has less inhibitory effect on decolorization potential of all three selected microbial strains for Reactive Black B.

Similar trend was recorded when FeCl₂ was added in same amount for bacterial and fungal decolorization of Reactive Orange 16 for bacterial strains B₁ and B₂. % decolorization reached to a minimum value of 40.7% and 39.7 at 2.5 g l⁻¹ addition of FeSO₄ from maximum value of 74.7% and 77.1% respectively where no heavy metal was added and conditions were optimal. These decolorization potential values are almost half than the maximum values. With fungal strain F₁ slightly higher inhibition (94.7% - 35.1%) occurred as compared to B₁ and B₂. From the data it is obvious that FeCl₂ decreased decolorization ability of microbial strains to greater extent for Reactive Orange 16 than the Cobalt did.

In the case of Reactive Brilliant Red 2KBP, FeCl₂ strongly inhibited decolorization ability of fungal strain F₁. Its activity decreased by 20% by just adding 0.5 g l⁻¹ of iron and at 2.5 g l⁻¹ addition of heavy metal only 28% of decolorization of dye could be achieved. With B₂ minimum decolorization was 32.4 (2.5 g l⁻¹ of FeCl₂) while with B₁, 25.4% decolorization of dye occurred at 2.5 g l⁻¹ which was three times less than achieved with no addition of heavy metal.
Fig 4.6.4: Effect of Iron on % decolorization of Reactive Black-B
**Fi4.6.5:** Effect of Iron on % decolorization of Reactive Orange 16

![Graph showing the effect of iron on % decolorization of Reactive Orange 16](image1)

**Fig 4.6.6:** Effect of iron on % decolorization of Reactive Brilliant Red 2KBP

![Graph showing the effect of iron on % decolorization of Reactive Brilliant Red 2KBP](image2)
**Effect of Zinc Sulphate**

Zinc sulphate has inhibited potential of both bacterial as well as fungal strain for decolorization of Reactive Black B. However, maximum inhibitory effect was observed with fungal strain whose potential decreased four folds with 2.5 gl⁻¹ addition of zinc sulphate reaching to a minimum value of 22.12%. Among the two bacterial strains, B₁ showed more inhibition for decolorization of tested dye than B₂ for B₁. 36.5% decolorization was achieved at 2.5 gl⁻¹ addition of zinc sulphate and with B₂ 35.25% decolorization was recorded while 78% and 85.38% decolorization was obtained with no addition with these strains respectively.

In the case of Reactive Orange 16, F₁ showed higher decrease than B₂ and B₁. At first by adding 0.5 gl⁻¹ of zinc sulphate at optimal conditions, 20% decrease occurred for B₂, 8% for B₁ and 31% for F₁ as compared with decolorization with no addition. Further increase in amount of added metal, there was a gradual but slow decrease in decolorization potential showing 32.6%, 40.1% and 37.21% color removal for B₂, B₁ and F₁ respectively at 2.5 gl⁻¹ addition of zinc sulphate.

With Reactive Brilliant Red 2KBP, 2.5 folds decrease was observed with maximum amount of added metal i.e. 2.5 gl⁻¹ for B₁. For B₂ a very low inhibition occurred at first with 0.5 gl⁻¹ addition of metal. It slowly went on decreasing with increasing amount of added zinc sulphate and only 32.5% decolorization of dye was achieved at 2.5 gl⁻¹ of addition. For fungal strain a sharp decrease was observed at first but thereafter a slow constant decrease was noted for decolorization potential of Reactive Brilliant Red and finally only 40.2% of color removal occurred at maximum addition of zinc sulphate.
Fig 4.6.7: Effect of zinc on % decolorization of Reactive Black-B
Fig 4.6.8: Effect of Zinc on % decolorization of Reactive Orange 16

Fig 4.6.9: Effect of zinc on % decolorization of Reactive Brilliant Red 2KBP
Effect of Copper Sulphate

For Reactive Black B by adding, 2.5 gl⁻¹ of copper sulphate only 20% decolorization was noted with B₂, 18.2% with B₁ and 13.5% with F₁. Among the various strains, fungal strain showed maximum inhibition for decolorization of Reactive Black B in presence of added copper sulphate. With 0.5 gl⁻¹ addition of metal decolorization extent remained half i.e. 45% from original 90.1%.

Copper sulphate showed more inhibition on decolorization ability of tested microbial strains for Reactive Orange 16. There was a rapid decrease in decolorization of dye when 0.5 gl⁻¹ of copper sulphate was added i.e. 34.5% (B₂), 45.2% (B₁) and 63.7% (F₁) from maximum potential 76.8%, 73.9% and 94.7% for B₂, B₁ and F₁ respectively. Finally only 9.5%, 16.2 and 27.2% of dye decolorization was achieved with 2.5 gl⁻¹ addition of copper sulphate with B₂, B₁ and F₁ respectively. Similar decreasing trend was observed for Reactive Brilliant Red 2KBP between metal concentration and % decolorization. Maximum inhibitory effect occurred with B₁ whose ability decreased to four folds by adding 2.5 gl⁻¹ of copper sulphate For B₂, two folds decrease and for F₁ 2.5 folds decrease in % decolorization of dye occurred with 2.5 gl⁻¹ (maximum addition) of copper sulphate.

From the data (Figure 4.6.13-4.6.15) it is obvious that there is a decreasing relationship between addition of Nickel chloride and decolorization potential of dyes under study. For Reactive Black seven folds decrease occurred for B₂, four folds for B₁ and eight folds decrease was noted for F₁ by adding 2.5 gl⁻¹ of Nickel in the form of its chloride salt. With first addition of heavy metal i.e. 0.5 gl⁻¹ % decolorization decreased to 53.8% from 85.8%, 51% from 78% and 61% from 89.7% for B₂, B₁ and F₁ respectively.

In the case of the Reactive Orange 16, 58.3% decolorization was recorded with lowest addition (0.5 gl⁻¹) of Nickel for B₂. and finally at 2.5 gl⁻¹, 19.21% color removal occurred. With B₁ 74% decolorization was achieved with no addition however by adding 0.5 gl⁻¹ of metal there was a rapid decrease and only 50.5% color removal was achieved. Finally 10.2% of dye was decolorized at 2.5 gl⁻¹ addition of nickel chloride with B₂. With F₁ which gave 95% decolorization of dye at optimal conditions only 10. Decolorization was recorded at 2.5 gl⁻¹ addition of metal. Which are nine folds less than maximum value. In the case of Reactive Brilliant Red a slow decrease occurred at start with bacterial strain B₂ but with continuous addition of metal, decolorization of dye went on decreasing and only 20.7% of decolorization was recorded at 2.5 gl⁻¹ addition of nickel. For B₁ a sharp decrease occurred at start i.e. from 84.5% to 61.2% with 0.5 gl⁻¹ of nickel.
Fig 4.6.10: Effect of copper on % decolorization of Reactive Black-B

![Graph showing the effect of copper concentration on % decolorization of Reactive Black-B. The graph plots % decolorization against copper concentration (mg/ml) with three lines representing different samples (B1, B2, F1).]
**Fig 4.6.11: Effect of copper on % decolorization of Reactive Orange 16**
Fig 4.6.12: Effect of Copper on % decolorization of Reactive Brilliant Red

2 KBP

%Decolorization

conc(mgml⁻¹)

B2
B1
F1
Fig 4.6.13: Effect of Nickel on % decolorization of Reactive Black-B
Fig 4.6.14 Effect of Nickel on % decolorization of Reactive Orange 16.

Fig 4.6.15: Effect of Nickel on % decolorization of Reactive Brilliant Red 2 KBP
Finally at $2.5 \text{ gl}^{-1}$ of nickel chloride 15.63% of decolorization was observed. Maximum inhibition was observed with fungal strain F$_1$, which gave 93% decolorization (at zero addition under optimal conditions) and only 14.9% decolorization at $2.5 \text{ gl}^{-1}$ addition of nickel.

If we compare inhibitory effect of various metals than it can be concluded from that cobalt and nickel showed greater inhibitions on % decolorization of all dyes for all tested bacterial and fungal strain than zinc, copper and iron. Among the strains, fungal strain showed greatest inhibition in decolorization potential with adding tested five metals (Co, Cu, Zn, Ni & Fe) for all three reactive azo dyes under study than the bacterial strain. However as presented earlier in the data the extent of decolorization inhibition was different for three dyes at different level of heavy metal amendments. Inhibition by heavy metals depend on concentration, and availability of heavy metals (Amor et al; 2001). Heavy metals might inhibit activities of enzymes which in turn could result in a decrease of dye decolorization (Gold et al; 1988 and Couto et al; 2000).

In a previous study Hiroki (1992) reported the effects of heavy metals (Cd, Zn, Cu) contamination on soil microbial population. According to his results degree of tolerance to heavy metals appeared to be: fungi > bacteria > actinomycetes. These results are not in harmony with our findings where bacterial strains showed more tolerance than fungal strains. The variation of extent of decolorization potential of various metals in present study might be attributed to this fact that influence of heavy metals on activities of soil micro flora largely depends upon the toxicity of that metal to bacterial growth as well as the solubility of salt of heavy metals in soil (Hattori, 1992).

Hatvani and Mecs (2003) investigated effects of nine heavy metals on dye decolorization and enzyme activity of *Lentinula edodes*. Metals included cadmium acetate, cobalt sulphate, cuprous chloride, ferrous chloride, manganese sulphate, nickel chloride, head nitrate and zinc sulphate in different concentrations. Each of the heavy metal inhibited dye decolorization (poly R-478) to a greater extent than growth. Co and Mn inhibited the decolorization completely, which is in accordance to our findings where cobalt was strongest inhibitor along with Nickel for dye decolorization of selected sulphonated azo dyes. Gold et al. (1988) and Couto et al. (2000) have suggested that
MnP was involved in degradation of dyes. Heavy metals inhibited growth of MnP (Hatvani and Mecs, 2003) so it might have probably inhibited dye decolorization.

Effect of heavy metals on biodegradation of dibenzofuran in liquid medium by *sphingomonas wittichii* RW₁ has been reported by Hong *et al.* (2007). It was found that 10 mg/L of cadmium, mercury and copper affected the growth of RW₁ with dibenzofuran. Moreover, ability of cells to degrade dibenzofuran in liquid media was decreased.

Thus it can be concluded that tolerance of bacterial and fungal strains to presence of heavy metals is very low. The mechanism involved in inhibition of biodegradation of dyes by adding metals vary greatly. Composition of the system under study, substrate structure and both physiological and ecological components of system might be involved in the whole process. (Romero *et al.*, 2006)It is generally considered that heavy metals mainly inhibit enzymatic reactions through either their complexing with substrate or blocking with complex enzyme-substrate and thus efficiency of dye decolorization is limited (Speir *et al.*, 1995; Smejkalova *et al.*, 2003 and Murugesan *et al.*, 2009). Thus presence of heavy metals in textile wastewater can interfere with dye decolorization even at very lower concentrations.

Increasing amounts of heavy metals inhibit dehydrogenase activities in bacteria (Smejkalova *et al.*, 2003) which could play an important role in dye degradation which this might be one of the major reason for inhibitory effects on dye decolorization observed in present study. Inorganic pollutants like heavy metals in their ionic forms like Cu⁺², Fe⁺² etc can interfere with remediation processes through their interactions with enzymes which are involved in biodegradation of xenobiotics (e.g. specific oxygenase) or in general they inhibit metabolism as they bind to sulphhydral groups of enzymes (Nies, 1999). Another factor which could not be neglected is depletion of dissolved oxygen of media. It might be consumed during conversion of metal to metal oxide. Thus oxidation potential of different metals may effect growth of microbial strains involved in study to different extent as reflected in obtained results.

Copper induces the transcription and activity of laccasse, a copper containing enzyme present mostly in white role fungi (Baldrain *et al.*, 2000), but in our study inhibitory effect of copper is observed. So it might be assumed that fungal strain used in present study does not involve laccasse as a major enzyme responsible for dye
decolorization further effect of heavy metals is more pronounced in liquid culture (as in present study) than reported earlier in solid media i.e. soil (Baldrain et al., 2000). Contamination of dye waste water with heavy metals play a very important role in activities of extra and intracellular enzymes. Bioremediation of industrial wastewater is an emerging technology and very attractive current methods. It is very essential and crucial to select such microbial strains which could tolerate the toxic and inhibitory effects of heavy metals.

4.7. STUDY SEVEN

EFFECT OF REDOX MEDIATORS ON DECOLORIZATION OF DYSES UNDER OPTIMAL ENVIRONMENTAL CONDITIONS

Azo reduction is proposed to occur through reduction of dye as terminal as accepter in microbial electron transport chain (Lourenco et al., 2000). So a number of oxido reductase enzymes are involved in this process.

This part of present research work involves exploitation of role of redox mediators on dye decolorization by newly isolated bacterial and fungal strains. For this
purpose five compounds were selected including Hydroquinone Uric acid, Sodium benzoate, Mannitol and EDTA. The results obtained are shown in Figure 4.7.1-4.7.15.

**Reactive Black B**

Hydroquinone was added in reaction media from 1-2 mmol$^{-1}$. Control experiments were run as well with no addition at optimal conditions. There was an increase in %decolorization by addition of hydroquinone. An increase of 5% with 1mmol$^{-1}$ addition of hydroquinone with bacterial strain B$_2$. With 5mmol$^{-1}$ of hydroquinone 94.5% of decolorization of Reactive Black occurred. With further increase in hydroquinone a decrease in decolorization occurred and at 20 mmol l$^{-1}$ addition only 58.7% of color removal occurred with a thirty percent decrease. With bacterial strain B$_1$, a slight increase was observed in start. Uptill 5 mmol l$^{-1}$ addition only 2% increase occurred i.e. 80.5% of dye decolorization was noted (78% at 0 mmol$^{-1}$ addition). With fungal strain F$_1$ 7% increase occurred reaching up to 97.5% at 5 mmol l$^{-1}$. However at 20 mmol l$^{-1}$ 50% reduction in color removal occurred and only 40.5% dye decolorization was recorded.

Uric acid showed a very slight non-significant increase at starts i.e. 1 mmol l$^{-1}$ addition with all the three strains. However, with further increase in addition there was decrease in decolorization ability but extent of decrease was different in each case. With Mannitol there was no increase in color removal. There was a continuous decrease in decolorization with all three selected strains at all concentrations. With bacterial strain B$_1$ and B$_2$ 45% and 50% decrease was observed at 20 mmol l$^{-1}$ addition of Mannitol. With fungal strain F$_1$ 50% decrease occurred with 50 mmol$^{-1}$ addition. Similarly Sodium benzoate and EDTA showed a decreasing trend with increasing their concentration on decolorization ability of B1, B2 and F1 at all concentrations.
Fig 4.7.1: Effect of Hydroquinone on % decolorization of Reactive Black-B.
Fig 4.7.2: Effect of Sodium Benzoate on % decolorization of Reactive Black-B
Fig 4.7.3: Effect of uric acid on % decolorization of Reactive Black-B

4.7.4: Effect of mannitol on % decolorization of Reactive Black-B
Reactive Orange 16

At start Hydroquinone showed a positive effect on decolorization rate of Reactive Orange 16. With bacterial strain B₂ 7% increase occurred in decolorization rate at 5 mmol l⁻¹ addition of hydroquinone. With bacterial strain B₁ only 5% occurred, and fungal strain F₁ showed a 4% increase in decolorization of RO16. However, at higher concentration of added hydroquinone as Redox mediator, decolorization rate decreased and only 29.3% (B₂), 50% (B₁) and 41.9% (F₁) of Reactive Orange 16 was decolorized at 20 mmol l⁻¹ of addition. Adding uric acid as redox initiator, there was a continuous decrease in color removal rate of all selected newly isolated microbial strains. Overall there was a 30% decrease in % decolorization for B₂, 25% for B₁ and 70% for fungal strain at 20 mmol l⁻¹ of uric acid. This shows that fungal strain was more sensitive to the addition of Uric Acid as extra redox mediator. Similarly Mannitol has negative correlation with rate of decolorization of reactive Orange 16 with all bacterial as well as fungal strain. Similar to uric Acid addition, fungal strain showed less tolerance to addition of Mannitol as compared to that of bacterial strains. However its sensitivity to Mannitol was lesser when compared with that of Uric Acid. With 20mmol l⁻¹ of Mannitol
40.2% of dye was decolorized with bacterial strain B2, 37% with B1 and 27.5% with fungal strain.

Fig 4.7.6: Effect of Hydroquinone on % decolorization of Reactive Orange 16.
Fig 4.7.7: Effect of Sodium Benzoate on \% decolorization of Reactive Orange 16.

Fig 4.7.8: Effect of Uric Acid on \% decolorization of Reactive Orange 16.
**Fig 4.7.9:** Effect of Mannintol on % decolorization of Reactive Orange 16.

**Fig 4.7.10:** Effect of EDTA on % decolorization of Reactive Orange 16.
**Reactive Brilliant Red K2BP**

Only a slight increase in decolorization rate of Reactive Brilliant Red K-2BP was observed at lower concentrations of hydroquinone. Strain B2 8% increase with 1 mmol l\(^{-1}\) addition while B1 and F1 showed only 2% increase. However at higher concentration a decreasing trend in decolorization was observed. Remaining four redox mediators could not increase color removal of Reactive Brilliant Red K2BP by all three strains. There was a 20% decrease in color removal of Reactive Brilliant Red K2BP for B\(_2\) in case of uric acid, for B\(_1\) 50% decrease and for fungal strain F1 also 50% decrease occurred. However with mannitol tolerance rate was higher and thus lesser decrease in decolorization ability was recorded as compared to Uric Acid and Hydroquinone. With 20mmol l\(^{-1}\) addition of Mannitol 40.3% dye decolorization was achieved with B2, 42% with B1 and 54.5% with fungal strain F1. Sodium Benzoate was also failed to increase color removal rate of Reactive Brilliant red K2Bp at any concentration for all three selected microbial strains. There was a slow but continuous decrease in decolorization rate with increase in amount of Sodium benzoate. With 20 mmol l\(^{-1}\) addition, F1 showed gave only 35.7% decolorization while B2 and b1 gave 42% and 23.2% percent color removal. Depressive effect of sodium benzoate was lesser as compared to mannitol for all three microbial strains for Reactive Brilliant Red K2BP decolorization.
Fig 4.7.11: Effect of hydroquinone on % decolorization of Reactive Brilliant Red 2 KBP

Fig 4.7.12: Effect of Sodium Benzoate on % decolorization of Reactive Brilliant Red 2 KBP
FIG 4.7.13: Effect of Uric Acid on %
decolorization of Reactive Brilliant Red 2 KBP

% Decolorization

conc (mmol⁻¹)

B2
B1
F1
**Fig 4.7.14:** Effect of Mannitol on % decolorization of Reactive Brilliant Red 2

**Fig 4.7.15:** Effect of EDTA on % decolorization of Reactive Brilliant Red 2KBP
DISCUSSION

Reactive sulphonated azo dyes are considered highly persistent to the environment (Riger et al., 2002). The main reason for this recalcitrant nature is presence of very polar sulphonic group due to which intact of dye with microbial cell becomes very difficult and thus cleavage of azo bond by reduction becomes lower (Mechsner and Wuhrmann, 1982). However it has been well documented that addition of redox mediators (low molecular weight reductants) like hydroquinone could enhance this reaction by accelerating the shuttling of electrons between microbial cells and azo dye. Due to this reason it can be predicted that extra cellular azo bond reduction occurs in high rate by these redox mediators (Keck et al., 1997; Keck et al., 2002).

The first step in bacterial degradation of azo dyes occurring in anaerobic environment or aerobic conditions is generally considered to be reduction of azo bond; the chromophoric group of dye (Pandey et al., 2007). The mechanism of this reduction is different depending upon conditions, Redox mediator and presence of enzymes. It might be through extra cellular or intracellular enzymes. The presence of azo reductase in obligatory aerobic microorganism (as those under investigation) has been now well established fact (Zimmermann et al., 1982, 1984; Suzuki et al., 2001; Ghosh et al., 1992; Chen et al., 2003).

Rau et al. (2002) has reported that anthraquinone-2-sulfonate and lawsone which is 2-hydroxy-1, 4 naphthoquinone acting as redox mediators could successfully enhance anaerobic dye decolorization of azo by different bacterial cultures. Keck et al. (1997) have shown that some bacterial cultures causing aerobic degradation of aromatic compounds can generate redox intermediates which could enhance rate of dye removal (decolorization under anaerobic conditions. Quinone and 1 hydroxy benzenetriazole mediated accelerated anaerobic decolorization of dyes has been reported earlier by Vander Zee et al. 2003, Su et al. 2009 and Sadhasivam et al. 2009. However the effect depended on type of quinine applies and concentration. Low molecular weight, redox mediators which are diffusible generally provide high redox potentials and thus can attack very rapidly to aromatic ring of recalibration azo dye (Hussain, 2006).

This discussion shows that a number of research reports on the role of redox mediators in dye decolorization in anaerobic cultures both by fungi and bacteria are
available but a very little has been performed about effect of redox mediators in aerobic condition on rate of sulphonated reactive azo dye degradation.

Due to highly polar and charged nature of reactive sulphonated azo dyes, extra cellular reducing activity is involved. Redox mediators compounds such as flavins can help to achieve it. They will facilitate the extra cellular non-enzymatic reduction of dyes (Plumb et al., 2001). Redox mediators are required in a very small amount to accelerate this reaction. Synthetic electron donors can also have a positive role in these mechanisms. Quinones and hydroquinone are proven to be helpful in azo reduction (Keck et al., 1997). Quinones can undergo one electron or the electron reductions. Our results also confirm the findings of previous studies for involvement of Quinones in azo dye reduction. At higher concentrations effectiveness of hydroquinone as redox mediators decreased because by losing its proton the pH of the decolorization culture media would tend to decrease shifting to more acidic side which could lead to a decrease in decolorization potential.

Among the applied mediators other than quinine none was proven to enhance rate of degradation. Rate determining factors for azo dye reduction with reference to redox mediators may involve redox potential of mediator in relation to azo dye under investigation and more specifically correlation of reducing enzyme specificity with respect to compound being used as mediator (Pearce et al., 2003). This might be one of reason for failure of other redox mediator being checked in present study.

EDTA (ethylene diamine tetra acetic acid) is a very good chelating agent. Thus sequestering a number of metal ions like Ca$^{+2}$, Fe$^{+2}$, Mn$^{+2}$ etc. This chelating effect may lead to inhibition of various enzymes which might involve in dye decolorization as dye degradation by microorganisms is a biochemical reaction in which enzymes play a key role. Many of these (like Laccasse, peroxidases) have metals as cofactors. So a strong chelating agent may lead to a depression in dye degradation. Similar results have been reported by Ghodake et al. (2009) who observed a 30% decrease in decolorization of DBMR in presence of EDTA mediated by lignin peroxidases. Harazono et al. (2003) also reported inhibitory effect of EDTA on dye decolorization by manganese peroxidase and reported that this depressive effect might be due to metal chelating activity of EDTA.
Uric acid is a potent antioxidant so is a strong reducing agent (electron donor) it should apparently enhance the rate of dye removal but in present study it reduced rate of dye degradation significantly at high level of addition. This might be due to reason that it was being used itself by microorganism as source of nitrogen due to its simpler structure as compared to dye. This competitive inhibition for inorganic nutrient would result in decrease rate of dye degradation by isolated microbial strains.

Mannitol (Hexane 1,2,3,4,5,6 hexol) has tendency to lose proton in aqueous solution, thus it could trigger dye degradation. But at same time it reduces pH of media transferring it to acidic side. The microorganism understudy worked better around neutral pH. Moreover it has a negative heat of solution which leads to cooling effect which could also disturb optimal condition for decolorization. As is a sugar with very simple structure so it can act as co-substrate proving to be good source of carbon thus leading to inhibition of dye removal having a complex structure.

The fifth chemical compound under investigation was sodium benzoate. It is bacteriostatic, fungi static. Thus it could inhibit the growth of microorganism responsible for dye degradation.

An extra cellular oxido-reductase enzyme activity is responsible for the degradation of high molecular weight and highly polar sulphonated azo dyes. (Reiger et al., 2002 and Pearce et al., 2003). Redox mediators facilitate this reaction particularly in absence of oxygen. However in present project aerobic microorganisms in presence of oxygen are involved in dye decolorization which resulted in different findings confirming the fact that redox mediators are now busy with oxygen rather than with reduction of dye under study. So it could be suggested that azo dye reduction is not initial step in dye decolorization in shaking condition by aerobic micro flora.
4.8. STUDY EIGHT

ECOLORISATION BY ACTIVE VERSUS INACTIVE CELLS

This part of the study was conducted to study the fact that either the decolorization is due to adsorption of dye to cell or it involved a chemical reaction leading to degradation of dye. For this purpose three types of decolorizing media were prepared; one labeled as Biotic Control (having living cells, dye and MSM), Second one: Autoclaved Control having autoclaved cells of strains, dye and MSM. While the third one named as Biotic Control having living cells, dye and MSM media.

Reactive Black B

Similarly in the case of Reactive Black B (Fig4.8.1-4.8.6) autoclaved cells bacterial strain B₁ gave 30.9% adsorption (dye recovery 90.8%) with no color removal till 240 hrs. B₂ (autoclaved cells) showed 46% removal in first 96 hrs while no further change in dye concentration for next 144 hrs. Extractable dye by methanol was 92%. Autoclaved cells of fungal strain responded in similar fashion, 33% of dye disappeared in first 44 hrs and no appreciable loses thereafter. Methanol extraction recovered 96% of dye. However when similar experiments were run with living cells of B₁, B₂ and F₁, 75%, 87% and 90.5% of dye was decolorized. Furthermore recovery of dye from living cells through extraction with methanol was also negligible which confirmed that there was no adsorption in the case of living cells and dye decolorization involved some enzymatic degradation of dye.
**Fig 4.8.1:** % decolorization of Reactive Black -B by autoclaved cells of B1

**Fig 4.8.2:** % decolorization of Reactive Black B by autoclaved cells of B2
Figure 4.8.3. % decolorization of Reactive Black B by autoclaved cells of F1

Figure 4.8.4. UV-Vis spectra of % Decolorization of Reactive Black-B by B-1 (Autoclaved Cells)
Figure 4.8.5. UV-VIS Spectra of % Decolorization of Reactive Black-B by B-2 (Autoclaved Cells)

Figure 4.8.6. UV-VIS Spectra of % Decolorization of Reactive Black-B by F-1 (Autoclaved Cells)
Reactive Orange 16

From Fig 4.8.7-4.8.12 it is clear that autoclaved cells of B2 showed 51.4% dye removal in first 96 hrs however in the next 144 hrs there was no change in dye removal. When supernatant of culture was extracted with methanol 80% of dye was recovered which showed that dye removal was due to adsorption by non living cells. UV-VIS spectral scanning also confirmed it as there was no change in $\lambda_{\text{max}}$ of dye showing no chemical reaction by autoclaved cells. With B1, 40.9% of dye was removed in first 48 hrs but no further change occurred. Extraction with methanol recovered 90.2% of dye whose UV-Vis scanning also showed no structural change during whole course of removal confirming involvement of adsorption in whole process. With F1 37.4% of dye was removed in first 48 hrs and 89.7% of which was recovered by methanol. There was no shift in $\lambda_{\text{max}}$ which again confirmed absence of any dye degradation.

However living cells showed same pattern as obtained previously during optimization of reaction conditions. In all the three cases i-e B1,B2 and F1 methanol fraction gave extractable dye extent than 5%. This clearly indicates that overall decolorization of Reactive Orange 16 by bacterial and fungal strains (i-e living cells)is a biological process involving no dye adsorption.

**Fig 4.8.7:**% decolorization of Reactive orange 16 by autoclaved cells of B1
Fig 4.8.8: % decolorization of Reactive Orange 16 by autoclaved cells of B2

Fig 4.8.9: % decolorization of Reactive Orange 16 by autoclaved cells of F1
Figure 4.8.10. UV-VIS Spectra of % Decolorization of Reactive Orange 16 by B-1 (autoclaved Cells)

Figure 4.8.11. UV-VIS Spectra of % Decolorization of Reactive Orange 16 B by B-2 (Autoclaved Cells)
Reactive Brilliant Red K 2BP

The dye decolorization with living and nonliving (autoclaved) cells for Reactive Brilliant Red K 2BP is shown in fig4.8.13-4.8.18

With living cells of $B_2$, 77% of dye was decolorized and only 1.5% of which could be recovered by methanol extraction. However with non living cells 50.3% color removal was observed in first 48 hrs of incubation and no more color was removed further uptill 240 hrs. 90.4% of dye (unchanged as clear from UV-Vis spectra) was recovered with the help of methanol.

With $B_1$, 49.8% dye was removed by absorption on non living cells (evidenced by dye removal 95% by methanol) in first 48 hrs. On the other hand 84% dye was decolorized by living cells methanol extraction of which was negligible. With fungal strain $F_1$, 92.6% of dye was removed by living cells. Only 3% of dye could be extracted by methanol extraction showing it to be enzymatic decolorization. However non living cells showed a rapid removal of 40% dye in first 48 hrs but no more color removal in upcoming 172 hrs 96% of dye was recovered back by methanol extraction. UV-VIS scan
of this extract showed no extra peak other than \( \lambda_{\text{max}} \) of dye which confirmed the involvement of adsorption as principle mechanism of dye removal with autoclaved cells. The comparison of dye decolorization ability of newly isolated unidentified strains \((B_1, B_2, F_1)\) with biotic control and non living cells (autoclaved culture) has confirmed that dye decolorization potential of these microorganisms was due to their biotic activity and their was no adsorption of dyes. Moreover data also shows that no dye removal occurred with abiotic control. This indicates that none of the chemical added in composition of media was involved in color removal. Almost 90% fraction of dyes removed by nonliving cells of bacterial and fungal strains could be recovered with methanol extraction. However there was negligible recovery of dye with methanol extracts obtained after dye decolorization by living cells. Thus it could be concluded that dye removal by autoclaved cells mainly involved sorption of dyes. Similar results have been reported by Khehra et al., (2005) who report decolorization of Azo dye AR-88 by living cells of microbial consortium HM-4 and moreover autoclaved cells showed 70% of dye decolorization by adsorption. The results presented here are in accordance

With those reported by Wang et al., (2005) who provided an evidence of biodegradation of reactive Red 18 by bacterial strain. The results of present project support the earlier conclusion that decolorization by bacteria strain conclusion that decolorization by bacteria involves biodegradation rather adsorption on inactive surface (Asad et al., 2005, Fan et al 2008, Joshi et al, 2008).

Dye adsorption can also be inspected by observing the cell mats. Cell mats become deeply colored because of adsorption of dyes however if biodegradation is involved in dye removal they will retain to their original color. (Yu and Wen, 2005). During the course of our study i.e. after 240 hrs of incubation at first there was appearance of color on fungal and bacterial strains but it changed to white or almost colorless till the maximum decolorization was achieved which made it clear that dye decolorization was happening because of some metabolic activity of microorganisms involving chemical changes.

In adsorption of dyes, examination of UV-VIS spectra will show that all peaks decrease in proportion to each other. (Knapp and Newby, 1995, Chen et al., 2003). If the dye removal is due to biodegradation, major visible peak will be disappeared or some new peaks of metabolites will be formed. In present study spectrophotometric monitoring of color removal by autoclaved cells showed no disappearance of major peak or no appearance of new peak which confirmed the idea of biodegradation is principal phenomenon by living cells of strains under study for the removal of selected reactive azo dyes.
Fig 4.8.13: % decolorization of Reactive brilliant Red 2KBP by autoclaved cells of B1

Fig 4.8.14: % decolorization of Reactive Brilliant Red 2KBP by autoclaved cells of B2
Figure 4.8.15: % decolorization of Reactive Brilliant Red K2BP by autoclaved cells of F1

Figure 4.8.16. UV-VIS Spectra of % Decolorization of Reactive Brilliant Red K2BP by B-1(Autoclaved Cells)
Figure 4.8.17. UV-VIS Spectra of % Decolorization of Reactive Brilliant Red K2BP by B-2 (Autoclaved Cells)

Figure 4.8.18. UV-VIS Spectra of % Decolorization of Reactive Red K2BP by F-1 (Autoclaved Cells)
4.9. STUDY NINE

DEVELOPMENT OF CONSORTIUM TO STUDY SYNERGISTIC EFFECT

Keeping all experimental conditions at optimum level for decolorization of selected three azo dyes a comparison was made between efficiencies of three strain individually (Figure 4.9.1-4.9.3). For Reactive Black B maximum decolorization was shown by fungal strain (F\textsubscript{1}) i.e. 89.5% while B\textsubscript{1} was least active with a value of 73%. While in the case of reactive Orange 16 95% decolorization was shown by F\textsubscript{1} 73% by B\textsubscript{1} and 77% by B\textsubscript{2}. So in short selected fungal strain was able to decolorize almost 90% of all the three reactive azo dyes individually understudy. Moreover, it showed 10% more efficiency for decolorization than the other two bacterial strains. This implies that the fungal strain F\textsubscript{1} carries an effective enzymatic system responsible for cleavage leading to high rate of biodecolorization of selected reactive sulphonated of azo bonds dyes under shaking conditions at an optimum level of 100 mg l\textsuperscript{-1} both in liquid and solid media. The findings may also imply that F\textsubscript{1} have great potential to decolorize structurally diverse dyes and could potentially be used on large scale textile wastewater for the treatment of contaminated with reactive azo dyes of complex and diverse structure.

These three laboratory isolates designated as B\textsubscript{1}, B\textsubscript{2} and F\textsubscript{1} having best decolorization efficiency than others were used further for consortium development. To determine the effect of their concerted metabolism on decolorization efficiency, they were mixed in 1:1 ratio by volume so that each isolate contributed equally to optical density. The decolorization efficiency of different consortia thus developed was determined as per procedure (chapter three). The results obtained reveal that consortium designated BF-3 based on all three isolates showed a small increase in decolorization efficiency however no significant concerted metabolic potential of microbial populations to decolorize colored wastewater of these selected dyes separately could be observed. Similarly none of the other consortia consisting of two strains showed better decolorization than the individual isolates. These results are in contrast with those reported by Knapp and Newby (1995) who suggested a synergistic role of bacterial species in decolorization of azo dyes. Biodegradation of azo dyes by mixed bacterial
cultures with a higher level of mineralization have been reported earlier by Knackmuss, 1996. Joshi, et al., (2008) have also reported decolorization of AO7 in a consortium at a much higher level than with individual strains (TJ-11, TJ-12 and TJ-13) and supported the idea of concerted metabolic role of individual isolates.

There are many reports available in literatures which are in harmony with our findings where capabilities of pure microbial cultures have been discussed to decolorize complex azo dyes (Change et al., 2001; and Hu, 2001). Inability of mixed consortium for color removal of selected dye might be due to fact that primary metabolites produced by one microbial strain may act as toxic compound for growth and enzymatic action of other present in consortium. Presence of more than one bacteria or substrate may reduce degradation of target compound due to reduction of oxygen level and competition for inorganic nutrients (Steffensen and Alexdender, 1995). Although mixed cultures of microorganisms are more practical with reference to application however on the other side they can also provide an average view of insight mechanism and thus effective interpretation of results become very difficult. (Pearce et al, 2003). Biodecolorization data can only be reproduced by the use of pure bacterial and fungal strains. Complete and detailed pathway of biodegradation can be provided only with single strains. Moreover kinetics of dye decolorization can be determined only with use of single strains which is very useful information.
4.9.1: Synergistic effect of microbial consortium on %

% decolorization of Reactive Black-B
Fig 4.9.2: **Synergistic effect of microbial consortium on % decolorization of Reactive Orange 16**

![Bar chart showing synergistic effect of microbial consortium on % decolorization of Reactive Orange 16]
Fig 4.9.3: Synergistic effect of microbial consortium on % decolorization of Reactive Brilliant Red K2BP
4.10. STUDY TEN

A) Biodegradation analysis via HPTLC

In order to determine biodegradation products HPTLC analysis was performed. The results obtained clearly support the idea of dye degradation as developed by UV-Vis spectra rather than dye removal by adsorption. Abiotic control containing all growth media along with dye but no living cells. Biotic control (culture growth with microorganism but no dye) was run along with experiments of three dyes and three strains separately. The extracts after fixed time of incubation were withdrawn and treated (procedure given in chapter 3). The final extract obtained was run through three solvent systems.

i. n-propanol methanol: ethyl acetate water. Glacial acetic acid [3:2:1:0.5].


However during this screening the first solvent system proved to be more effective in separating dye metabolites. The plates showed different spots. Each plate was screened under UV-light. The spots with R_f value 0.65, 0.66, 0.67, 0.68, 0.74 were not visible under UV-light which confirmed that they were non aromatic substituents and moreover none of them was amine in nature the spots with R_f value of 0.55, 0.43 were found to be derivatives of Naphthalene sulfonic acid. Various other standards 4-aminophenol, 4 nitrophenol, 4, nitro aniline, benzidine were also naphthalene based dye degradation having azo group as chromophore and sulfonic acid group as substituent. Haug et al., (1991) and Khehra et al (2005) have reported complete degradation of such dyes. TLC analysis was done for best decolorizing strain among the three selected strains for each dye (Asad et al., 2005 and Moosvi et al., 2005). As R_f value of dye and products (from decolorization) media are not the same indicating biodegradation of dye. Mane et al (2008) has also found TLC an important and simple tool to study degradation for navy blue Rx. Kalyani et al. (2008) has also studied biodegradation of reactive dye Red BLI very TLC. The standard of H acid, J acid ortho aniline, para hydroxyl aniline, α Naphthyl amine, β-naphthyl amines. Ortho aniline vinyl suphones were also run on separate plates. However R_f values did not matched to any of the sports observed in chromatograms of dyes under
study. The results are in constraint of those reported by Bhatt et al. (2005) who studies
decolorization of diazo dye (Reactive Blue 172) in unagitated anaerobic media by
*Pseudomonas aeruginosa*. A no of aromatic amines giving fluorescent bands under UV
light were observed. Moreover dye intermediates like H acid, J acid were also formed
during dye decolorization. These at were attributed to the fact that anaerobic condition
bacterial degradation lead to formation of aromatic amines which are not further
degraded due to absence of oxygen (Stolz 2001). In contrast for going results confirmed
complete oxidation f dye degradation products as there is no aromatic amine formed by
selected strains growing under aerobic condition along with continuous supply of O₂ in
form of shaking.
Figure 4.10.1. Identification of Dye (RBB) Metabolites by HPTLC
Figure 4.10.2. Identification of dye (RO 16) Metabolites by HPTLC
Figure 4.10.3. Identification of Dye (RBR 2KBP) Metabolites by HPTLC
B) Biodegradation Analysis Using UV-Vis Spectra

Evidence of dye removal can be obtained by absorbance at $\lambda_{\text{max}}$ which should be virtually zero at maximum decolorization time of incubation (McMullan et al., 2001; Moosvi, et al., 2005; Daneshvar et al., 2007). The results obtained in the form of spectra 4.10.4-4.10.12.

Reactive Black B

UV-visible spectra of the effluent in liquid culture incubated with B$_1$, B$_2$ and F$_1$ separately for 0 hrs and 192 hrs (time at which maximum decolorization of dye was achieved) was scanned from 200 nm to 750 nm (Fig 4.10.4-4.10.6). Maximum optical density was observed at 597 nm which is $\lambda_{\text{max}}$ of the dye: Reactive Black B. After 192 hrs of incubation there was a significant decrease in peak intensity almost equal to base line showing decolorization of dye completely. However a small peak at 250 nm shows the formation of some phenolic derivative as dye metabolites. Decrease in intensity of visible peak at $\lambda_{\text{max}}$ indicates disruption of chromophoric group i.e. azo group here. However no aromatic amines could be recorded by UV-Vis scanning.

The peaks between 220-260 nm (0 hrs of incubation were the absorption of $\pi-\pi^*$ transitions due to amino group bonded to naphthalene ring present in dye molecules (Wu et al, 2000, Xiong et al. 2001, Wang et al, 2005). However at 192 hrs incubation this peak disappeared dramatically showing opening of naphthalene ring during course of decolorization. Appearance of a less intense peak at 250 nm refers to the formation of some phenyl derivative as metabolite but it can’t be amino substituted as they absorb at lower side of UV-spectra. In the case of F, a base line absorbance occurs with no peak between 200-700 nm which suggests breakage of azo nuclei leading to some aromatic intermediates (phenyl or naphthyl) which finally could be cleaved leading to final products, alcohols, or aliphatic hydrocarbons or even it could be mineralized completely leading to CO$_2$ and H$_2$O during entire reaction. N$_2$, NH$_3$ formed from azo linkage might be evolved.
**Reactive Orange 16**

A spectrophotometric scanning (200-700 nm) of Reactive Orange 16 have a maximum absorbance peak at 494 nm and very less intense peak at 260 nm corresponding to phenyl ring in dye. The dye decolorization was apparent by a gradual decrease in absorbance at $\lambda_{\text{max}}$. Absorbance spectral pattern and decrease rate was not similar to each other and to initial one (0hr) for each applied strain showing a change in enzymatic system and change in affinity for dye for these strains.

**Reactive Brilliant Red 2-KBP**

Maximum decolorization of Reactive Brilliant Red 2KBP was achieved after 96 hrs of incubation for B$_2$, 144 hrs for B$_1$ and 96 hrs for F$_1$. So spectrophotometer analysis of extracts from culture was performed at 0 hrs and time where maximum decolorization was attained. $\lambda_{\text{max}}$ of Reactive Brilliant Red K2BP is 531nm. At start there was a single major peak of absorbance at $\lambda_{\text{max}}$ showing no decolorization with B$_1$. After 48 hrs, there was no other peak as well but decrease in absorbance intensity at $\lambda_{\text{max}}$ was recorded which shows that color removal have been occurring with no production of major metabolite. After 144 hrs complete disappearance of absorbance peak is observed. Similar was the case with bacterial strain B$_2$. Disappearance of the peak at 531nm demonstrates that dye structure has been broken significantly especially conjugated molecular structure based on azo group has been decomposed which was mainly responsible for the color of dye. With fungal strain F$_1$ at 48 hrs of incubation there was an absorbance peak at 320 nm corresponding to naphthalene derivatives (NIST library), showing formation of some naphthalene based intermediate during the course of decolorization. However at 96 hrs of incubation (when F$_1$ gave maximum decolorization of dye) this peak as well as peak at $\lambda_{\text{max}}$ was completely disappeared confirming dye degradation. Generally a decrease of this kind (observed with all dyes) is attributed to the dye biodegradation rather than its adsorption on microbial cells (Wesenberg et al., 2002, Vijakumar et al., 2006). A marked difference in intensity of absorbance peak after complete decolorization in visible region and no peak in UV region explains clearly that
Figure 4.10.4. UV-VIS Spectra Showing Degradation of Reactive BLACK-B by B1

$A = 0\text{hr}$
$B = 192\text{hr}$
Figure 4.10.5. UV-VIS Spectra Showing Degradation of Reactive Black-B by B2

A=0 hr
B=192 hr
Figure 4.10.6. UV-VIS Spectra Showing Degradation of Reactive Black-B by F1

A = 0 hr
B = 192 hr
Figure 4.10.7. UV-VIS Spectra Showing Degradation of Reactive Orange 16 by B1

A=0hr
B=48hr
C=192hr
Figure 4.10.8. UV-VIS Spectra Showing Degradation of Reactive Orange 16 by B2

A=0hr
B=48hr
C=192hr
Figure 4.10.9. UV-VIS Spectra Showing Degradation of Reactive Orange 16 by F1

A = 0hr
B = 48hr
B = 192hr
FIG. 4.10.10: UV-VIS Spectra showing degradation of Reactive Brilliant Red K2BP by B1

Figure 4.10.11. UV-VIS Spectra Showing Degradation of Reactive Brilliant Red K2BP by B2
Figure 4.10.12. UV-VIS Spectra Showing Degradation of Reactive Brilliant Red K2BP by F1
mechanism of decolorization for the dyes under study involve biochemical destruction of dye by bacterial as well as fungal strains. These results are in agreement with those of Sponza and Isik (2002) and Li and Xi (2004). However these are in contradictory to the hypothesis that removal of dye under aerobic environment occurred firstly by biosorption onto floc matrix and then bond cleavage of dye occurred (Li and Xi, 2004).

If we compare among three dyes for changes in absorbance maxima each dye has behaved differently with similar bacterial and fungal strains. Similar variable response to dye decolorization has been reported earlier by Joe et al. (2008) using three dyes and three strains. It is very much strongly recommended that the difference in chemical structure of dyes is responsible for variation in dye decolorization rate (Paszczyszynski et al., 1992). Chen et al. (2003) has postulated that dye removal by biodegradation can be monitored through UV-Vis spectra. Dye removal by biodegradation leads a complete reduction of major absorbance peak may or may not be accompanied by appearance of new peaks. Our results are in accordance to above requirements as complete disappearance of visible peaks occurred at time of incubation where maximum decolorization was achieved. Chances of adsorption have already been negated in earlier studies.

If maximum wavelength of dye ($\lambda_{\text{max}}$) shifts from longer wavelength to shorter wavelengths with the course of time of incubation (it mans it will shift to UV region) which corresponds to most of aromatic amines intermediates (Hsuch and Chen, 2007). If azo bond of a dye molecule disintegrates (i.e. chromophoric group of dye) in an anaerobic environment by azo reductase, $\lambda_{\text{max}}$ (maximal wavelengths) will be shifted to a shorter wavelength. (Hsuch and Chen, 2007; O’Neill et al, 2000, Asad et al, 2007) the reason behind this shifting is that conjugated $\pi$ electron system of azo dye refers to lower energy of absorption than respective aromatic amines. This sort of shifting has been earlier reported in the case of methyl orange which was converted to sulfanilic acid and N, N dimethyl aniline (Hsuch and Chen, 2007). However there was no observation of any peak in UV region for any of three dyes under study. So metabolic by products formed by selected microbial strains are different from aromatic amines produced by anaerobic decolorization (Chang et al. 2001). As present project involved aerobic environment so
these bacterial and fungal strains are leading to completely oxidized degradation products having no absorbance in UV-region.

C) Biodegradation Analysis VIA GC/MS

The monitoring of UV-Vis spectra gave information about nature of color removal with emphasis and shifts in maximum absorbance confirming presence of some chemical change in extended conjugated double bond and chromospheres groups of dyes. It did not provide any clue about products formed as a result of that degradation. In order to identify dye metabolites formed after complete dye degradation GC/MS analysis was performed. Probably the most common use of mass spectrometry coupled with gas chromatography is accurate determination and precise information about molecular weight and structure of an organic compound.

As in study presented here fungal strain showed maximum decolorization at optimal conditions with all the three dyes so only its liquid extract was run through GC/MS.

In the case of Reactive Brilliant Red K2BP a strong peak at m/z 113 shows formation of 1,3,5 triazine 2, 4 diol which confirms the degradation of dye involved oxidases or peroxidase enzyme in the primary cleavage which lead to asymmetric cleavage. Similar cleavage has been proposed earlier by Kalyani et al, 2008. No such peak was seen with other two dyes i.e. Reactive Black B and Reactive Orange 16 as they have vinyl sulfone group as reactive group.

A very weak peak at m/z 492 represents a minor hydrolyzed form of Reactive Orange 16. Two strong peaks appeared at 294 and 283 referring to 6-acetamido-3, 4-dioxo-3,4 dihydroanaphthalene-2-sulfonate and 2-(4 acetamidophenyl)-1-carboxyethene sulfonate respectively (NIST Library). Svobodora et al (2007) studied degradation of Reactive Orange 16 with ripe lacteus and observed three metabolites with m/z 294, 284 and 201, similar to present study however in present work no metabolite at m/z = 20 could be observed. This difference is due to the reason that every fungal strain has its own substrate affinity and specificity, various enzyme systems with different activities. Thus metabolic pathway showed by different strain with same substrate could be different.
Presence of –OH and –Amino group ortho to azo group causes a difference in reactivity for cleavage of two azo groups present in dye. Both of these groups are good attractive activating for electrophilic substitutes. Lower molecular weigh aromatic compounds including phenols have been formed during dye degradation as clear for GC-MS spectra.

None of product formed was from list of banned amines Novotny et al. (2004) reported a decrease of 95% in mutagenic character of RO16 after biodegradation by fungus I lacteus.

Some times biodegradation of azo dyes lead to more complex high molecular weight polymeric structure by coupling after breakage of chromoporic groups (Zille et al., 2005). In present study no dimeric or trimeric coupling product could be identified with any dye. These results are in contrast with findings of Field et al. (1995) who reported that oxygen can interact via free radical mechanism with aromatic products and thus a number of colored oligomers and polymers (more toxic than dye)could be formed in reaction media.

Oxidative biodegradation involves mainly laccasses and peroxidase. Sulphonated reactive azo dyes have been successfully degraded by involvement of fungal laccasses and peroxidases (Kandelbauer et al, 2004, Kalme et al, 2007). Pasti et al, (1996) have reported presence of extra cellular azo dye oxidizing peroxidase in bacterial strain. Conclusively from observation of these mass spectra (4.10.13-4.10.15)it can be proposed that degradation of dyes under study through fungal strain in aerobic conditions have involved essentially oxidases, peroxidase enzymes, deamination, desulphonation, and hydroxylation etc. which resulted in no formation of amines as major metabolites. These results support previous theories that fungal degradation essentially did not require azo reductase as primary key factor in dye degradation (Tan et al., 1999). Theory by Kulla et al (1991) shows that despite oxygen is present in environment the initial step of dye degradation could be a reduction of azo linkage by an oxygen insensitive azo reductase adapted in aerobic bacterial and fungal microorganisms. Even if this mechanism was involved as primary step in degradation of dyes under study, amines formed as a result were further metabolized leading to low molecular weight non-toxic simpler organic compounds. In anaerobic conditions aromatic amines with sulfonic acids as substituents
show resistance to any further change. Some researchers have confirmed that presence of sulfonic groups on azo dye acts as a rate limiting factor in anaerobic conditions (Mendaz et al., 2005). In contrast results of foregoing project show that sulphonated reactive azo dyes are successfully oxidized leading to simpler non-toxic products after degradation with selected strains which is in harmony with results reported Tan et al., 1999. Desulphonation of aromatic moiety of dye molecule coupled with reductive cleavage of azo bond would suppers formation of any aromatic amine (reported to be toxic and recalcitrant) thereby detoxification of sulphonated reactive azo dye occurs (Oranuri and Ogugbue, 2005).

In earlier studies about aerobic biodegradation of sulphonated azo dyes an unusual desulphonation of recalcitrant dye has been reported which involved hydroxylation as major chemical reaction being catalyzed by oxygenase (mono or di). As a result of these reactions catechol sulphonates have been identified as intermediates (Fiegl and Knackmuss, 1993; Junker et al, 1994 and Panday et al., 2007).

For the present study it could be supposed that if azoreductase acts as first part and aniline like amino derivatives are formed on one side and naphthalene derivatives on other side with further degradation particularly in aerobic environment C-N bond would be broken and deamination would result in form of oxidized phenyl derivatives or naphthyl derivatives. As phenolic like catechols, resorcinols in way of degradation are easily mineralized to aliphatic and aromatic carboxylic acid through ring opening. These carboxylic acids can easily be changed to CO₂ and H₂O leading to complete mineralization of recalcitrant organic dye molecules.
LIST OF METABOLITS

6-amino-4-hydroxy-2-naphthalene sulfonic acid

1-naphthol

sulfanilic acid

2-naphthol

salicylic acid
Figure 4.10.13. GC/MS Analysis of Degradation of Reactive Black-B by F1
Figure 4.10.14. GC/MS Analysis of Degradation of Reactive Orange 16 by F1
Figure 4.10.15. GC/MS Analysis of Degradation of Reactive Brilliant Red K2BP by F1
Chapter # V

SUMMARY

The textile industry, especially dye manufacturers, is facing regulations and standards for their discharge effluents. To comply with the discharge permits, waste generators have to consider alternatives before disposal of their effluents. Owing to the diversity in structure of dyes, the composition of dye wastewater may be very complex and thus difficult to be decolorized by simple conventional treatments. Keeping in view this environmental condition, present project was designed which aimed to study feasibility of application of biotechnology for the degradation of selected reactive azo dyes considered to be recalcitrant.

Three microbial strains were isolated from local textile effluent channel. Optimization of environment factors showed that best color removal was achieved at 37°C, neutral pH, and 240 hrs of incubation in presence of oxygen. However, slight variation occurred among strain’s ability for decolorization of three different dyes under study. Among various strains different tolerance rates as well as color removal rates were recorded as different microorganisms have various enzyme systems responsible for dye degradation. Moreover, structural differences among dyes, presence of different substituents as well as reactive groups strongly affected the overall processes. Isolated strains showed better potentials for removal of dyes than developed mixed consortium, omitting any chances of synergistic effect. No dye adsorption could be observed so chemical changes mediated by enzymes are responsible for color removal. Additional C and N sources significantly reduced decolorization potential of dyes. As reactive azo dyes have complex structure and recalcitrant in nature so added simple C and N suppliers were being used as co substrate. Added metals inhibited enzymes activity of microorganisms involved in decolorization thus causing depletion in decolorization rate for all dyes and strains under study. Hydroquinone proved to be a mild enhancing agent for decolorization potential while others like mannitol, EDTA, uric acid and sodium benzoate failed to show any positive response to decolorization of these dyes.
Furthermore the end products formed as results of dye decolorization were identified by UV-VIS, HPTLC, and GC/MS. A number of products were formed as a result of decolorization. 90% of them were successfully identified for their chemical structure. No amine formation was observed which confirmed the idea that aerobic decolorization of azo dyes did not essentially involved azo reduction. Most of the products formed were hydroxylated, and oxidized majorly phenol derivatives and carboxylic acid derivatives. Thus it could be concluded that degradation of Reactive black B, Reactive orange 16 and Reactive Brilliant Red, K 2BP under aerobic conditions did not lead to any toxic, banned and mutagenic product formed with the help of isolated strains.

It can be concluded that indigenous microorganisms have promising potential to degrade azo dyes. There exits a need for collaborative efforts between industrialists and chemists to develop treatment technology and pilot scale plants for treatment of textile wastewater having reactive azo dyes with help of microorganisms leading to biodegradation.


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