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IN

MICROBIOLOGY

BACTERIAL DEGRADATION OF AZO DYES AND ITS DERIVATIVES

BY

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CERTIFICATE

I take pleasure in forwarding the thesis entitled "Bacterial Degradation of azo dyes and its derivatives" of Mr. Himanshu D. Bhimani for the acceptance of the degree of Doctor of Philosophy in Microbiology. Thesis presented here embodies a record of the results of original investigations carried out by him.

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DECLARATION

I, Mr. Himanshu D. Bhimani, the undersigned hereby solemnly declare that the work presented in this thesis entitled "***Bacterial degradation of azo dyes and its derivatives***" is original and independent. I declare further that this work has not been submitted for any degree or diploma to any other Universities or institutions.

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Key words

Anoxic condition
Aromatic amines
Bacterial isolate
Biodegradation
Bioremediation
Co-metabolism
Consortium
Cytotoxicity
Decolorization
Dye mixture
Ecotoxicity
Germination
HPLC
Lysinibacillus fusiformis JTP-23
Phytotoxicity
Recalcitrant
Sequencial static shaking condition
Textile dyes
Textile effluent

Abbreviation

Activated sludge	AS
Black BT	BBT
Black E	BE
Benzidine	BZ
Chemical Oxygen Demand	COD
Common effluent treatment plant	CETP
Complete Medium Broth	CMB
Distilled Water	DW
Fetal Calf Serum	FSC
FF Sky Blue	FFSB
Green B	GB
Minimal Essential Media	MEM
Mineral Salt Medium	MSM
Optical Density	OD
Reactive Black 5	RB 5
Reactive Orange M_2R	ROM_2R
Tripsyn Phosphate Versene Glucose	TPVG

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Its great fortune that in vast universe, we worked on the omnipotent creature of the almighty God.

August, 2011

RAJKOT *Himanshu Bhimani*

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General Introduction

Since last few decades, there is an explosive development in the dye and dying industries. Within the manufacturing of various synthetic dyes many potentially hazardous organic compounds have been introduced into various components of the environment. This process is going on till to date with exponential increase.

The continuous dumping of such hazardous compound in the form of waste has lead to contaminate soil, ground water, sediments, surface water etc. at alarming level. The roots of such waste products found to be an industrial, agriculture, energy production industries, textile industries, and printing houses that produce huge amount of wastes, which ultimately find its way in the environment.

The studies of biodegradation of dyes and its derivates products are of environmental interest because of its recalcitrant nature, carcinogenicity, mutagenicity and toxic effects. As we know that dyes never die, but some of the dyes when degraded biotically or abiotically, produce the end products that are more toxic than native dyes as a result of incomplete degradation. Some azo dyes are effectively reduced to their intermediate structure, thus destroying the apparent color of dye. Many of these intermediates are aromatic amines with constituent side groups. By reducing the dye compounds to their intermediates, the problem of visual pollution is solved, but a larger and more deleterious problem may be created. Higher percentage of these intermediates has been identified as more mutagenic, carcinogenic, and toxic (Brown and DeVito, 1993). Because of the toxic potential of many aromatic amines, further degradation of

the dye compound is necessary if scientific community is seriously thinking and trying to find out some effective way to treat the various sites which are contaminated by dues and other hazardous components.

A dye may be defined as an organic compound containing both chromophore and auxochrome groups linked to benzene ring. Chromophore is responsible for imparting color to the compound and auxochrome imparts the property of electrolytic dissociation. The chromogen-chromophore structure is often not sufficient to impart solubility and cause adherence of dye to fiber. The auxochrome or bonding affinity groups are amine, hydroxyl, carboxyl, and sulfonic radicals, or their derivatives.

Azo dyes are characterized by presence of nitrogen-nitrogen (N=N) in its chemical structure. Many different structures are possible in azo dyes as Monoazo dyes have only one N=N double bond, while diazo and triazo dyes contain two and three N=N double bonds, respectively (Zollinger, 1991). The azo groups are generally connected to benzene and naphthalene rings, but can also be attached to aromatic heterocycles or enolizable aliphatic groups. These side groups are necessary for imparting the color of the dye, with many different shades and intensities being possible (McCurdy, 1992).

Because of the diversity of dye components available for the synthesis, a large number of structurally different azo dyes exist and are used in industry. Approximately 80-95 % of all reactive dyes are based on the azo chromogen (Zollinger, 1991; Edwards, 2000). Reactive dyes are colored compounds that contain one or two functional groups capable of forming covalent bonds with the active sites in fibers. A carbon or phosphorous atom of the dye molecule will bind to hydroxyl groups in cellulose, amino, thiol, and hydroxyl groups in wool, or amino groups in polyamides. Most fiber-reactive azo dyes are used for dyeing cellulosic materials, such as cotton, and are a major source of dye waste in textile effluents.

Bioremediation is the use of biological systems (mainly microorganisms and plants) for the treatment of polluted air, aquatic or terrestrial component of environment. The textile industry is major user of water. Reduced water resources due to rapid population growth and industrial development has triggered need to reuse of municipal and industrial waste water after proper treatment and elimination of potential pollutants. Conventional treatment processes have long been established in removing many hazardous chemicals of public health and environmental concern. These conventional methods have their own disadvantages at large scale (Keharia and Madamwar, 2003).

Microorganisms can breakdown most compounds for their growth and/or energy need. In some cases, metabolic pathways which organisms follow for its own normal growth and development may also be used to beak down pollutant molecules. In this process microorganisms do not benefit directly, but researchers have taken advantages of this phenomenon and use it for the process of bioremediation. Complete degradation of any compound ultimately yields water and either carbon dioxide or methane. Incomplete degradation will yield breakdown products which may or may not be less toxic than the native pollutant (Alleman and Lesson, 1999; Alexander and Lustigman, 1996).

In last few years, several microorganisms have been reported to decolorize and transform to completely mineralize azo dyes. The bacterial degradation of azo dye is initiated by a reductive cleavage of azo bond, which result in the formation of amines. The aromatic amines that are formed in the course of these reactions may be degraded aerobically (Stolz A., 2001).

2

Review of Literatures

History of Dyestuff and Dying

Ever since the beginning of mankind, they have been endeavoring to add color to the world around them. They used natural matter to stain hides, decorate shells and feathers, and paint their story on the walls of ancient caves. Scientists have been able to date the black, white, yellow and reddish pigments made from ochre used by primitive man in cave paintings to over 15,000 B.C. With the development of fixed settlements and agriculture around 7,000-2,000 B.C., man began to produce and use textiles, and would therefore add color to them as well (Grierson, 1989). Organic natural colorants have a timeless history of application, especially as textile dyes.

The first synthetic dye was discovered by William Henry Perkin, a student at the Royal College of Chemistry. He tried to make the drug quinine from aniline (a chemical found in coal). The experiment produced a thick dark sludge. Instead of throwing it away, Perkin tried diluting it with alcohol and found that the solution was purple. He discovered that it would dye silk and that it was a 'fast' dye, resistant to washing and to the fading effects of light.

The concept of research and development in variety of dye manufacturing was soon followed by others and new dyes began to appear on the market. This process was dramatically stimulated by Kekule's discovery of the molecular structure of benzene in 1865. In the beginning of the 20^{th} century, synthetic dyestuffs had almost completely supplanted natural dyes (Welham, 2000).

Textile Dyes

Synthetic dyes are extensively used in textile dyeing, paper printing, color photography, pharmaceutical, food, cosmetics and other industries (Rafi, Franklin and Cerniglia, 1990). Approximately, 10,000 different dyes and pigments are used industrially, and over $7x10^5$ tons of synthetic dyes are produced annually worldwide. In 1991, the world production of dyes was estimated 6,68,000 tons of which azo dyes contributed 70% (ETAD, 1997). During dying process, a substantial amount of azo dye is lost in wastewater. Zollinger, (1987) reported that about 10-15% of dyes were lost in effluent during dyeing process. A common example of an azo dye, Reactive Black 5 also known as Remazol Black B (RBB) that has been taken in this study, is as below.

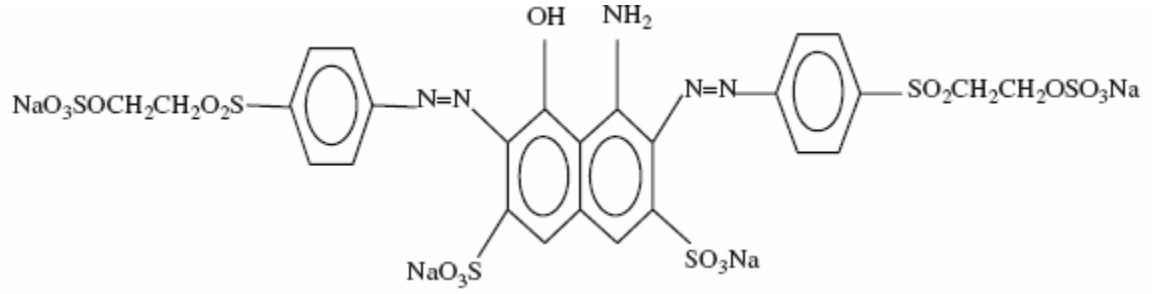

Figure 1.1: Chemical structure of Reactive Black 5

Major classes of synthetic dyes include azo, anthraquinon and triaryl-methane dyes, and many of them are toxic or even carcinogenic compounds with long turnover times. With the increased use of a wide variety of dyes, pollution by dye's wastewater is becoming increasingly alarming.

Classification of Dyes

All aromatic compounds absorb electromagnetic energy but only those that absorb light with wavelengths in the visible range (~400-800 nm) are colored. Dyes contain chromophores (delocalized electron systems with conjugated double bonds) and auxochromes (electron-withdrawing or electron-donating substituents that intensify the color of the chromophore by altering the overall energy of the electron system). Usual chromophores are -C=C-, -C=N-, -C=O, -N=N-, $-NO_2$ and quinoid rings, usual auxochromes are $-NH_3$, -COOH, $-SO_3H$ and –OH (Zee, 2002).

Based on chemical structure of chromophores, 20-30 different groups of dyes can be discerned. Azo (monoazo, diazo, triazo, polyazo), anthraquinone, phthalocyanine, and triarylmethane dyes are quantitatively the most important groups. Most of the commercial available azo dyes are in fact formulations of several components in order to improve the technical properties of the dyeing process. The majority of industrial important azo dyes belong to the following classes: Acid dyes, Basic dyes, Direct dyes, Disperse dyes, Mordant dyes, Reactive dyes and Solvent dyes. The Acid, Basic, Direct and Reactive azo dyes are ionic dyes (Anliker, Clarke and Moser, 1981). They can also be classified according to chromophore they contain. (Wesenberg, 2003) (Table 1.1).

Table 1.1: Classification of synthetic dyes according to Colour index (C.I.)

Code	Chemical Class	Code	Chemical Class	Code	Chemical Class
10000	Nitroso	42000	Triarylmethane	53000	Sulfur
10300	Nitro	45000	Xanthene	55000	Lactone
11000	Monoazo	46000	Acridine	56000	Aminoketone
20000	Disazo	47000	Quinoline	57000	Hydroxyketone
30000	Trisazo	48000	Methine	58000	Anthraquinone
35000	Polyazo	49000	Thiazole	73000	Indigoid
37000	Azoic	49400	Indamine/Indophenol	74000	Phthalocyanine
40000	Stilbene	50000	Azine	75000	Natural
40800	Carotenoid	51000	Oxazine	76000	Oxidation Base
41000	Diphenylmethane	52000	Thaizine	77000	Inorganic

Dye Stuff Industries

Consumption of dyestuff is governed predominantly by several factors. The primary long term factor is demand for textiles, leather and colored paper. Consumption of textiles, the largest end-use market for dyestuff, in turn depends directly on population growth and private consumer spending levels. The most important short-term factor is fashion, which dictates the types of colors used. The quantity of dyestuffs consumed per volume of textile is considerably higher

when bright or dark colors are desired in textile than when only light colors are in demand.

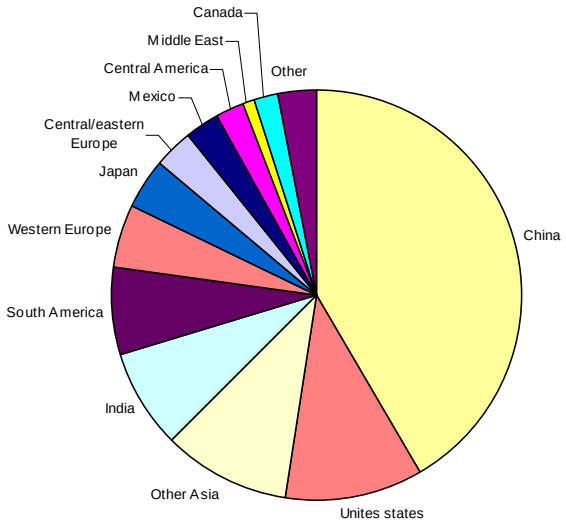

Figure 1.2: World consumption of dyes – 2009.

The world market for dyes, pigments and dye intermediates is estimated at about US $ 23 billion consisting of dyes and pigment market of 1.3 million tones valued at US $ 16 billion and dye intermediates market of US $ 7 billion. Though the overall growth of dyestuff industry during the last 5 years has slowed down, the industry is still expected to maintain a growth of about 2% per annum in the next decade. Globally, reactive dyes account for around 25 percent while disperse dyes account for 20 percent of total dyes production. China, Korea and Taiwan are strong players in disperse dyes while India leads in production of reactive dyes on account of easy availability of intermediates like vinyl suphone in the country

The Indian dyestuff industry, which was primarily started to cater to the needs of domestic textile industry, now not only meets more than 95% requirement of the domestic market, but has gradually also made a dent in the global market. Today, India exports dyes and dye intermediate to the very same countries, on which it was dependent for imports till a decade ago. All range of dyes such as disperse, reactive, vats, pigments and leather dyes are now being manufactured

in India. The dyestuffs industry in India is concentrated in the states of Maharashtra and Gujarat, especially around the city of Ahmedabad, which hosts about 1200 plant and accounts for one-third of India's exports.

Textile Effluent

Textile effluents are highly colored effluent released by textile and dye manufacturing industries that contain large amount of mixtures of dyes. Two major sources of release of dyes into the environment are the textile and dyestuff manufacturing industries (Nigam *et al*., 1995). These dyes are difficult to degraded and pose hazardous effect on aquatic life. In addition to dyes, textile effluent also contains high ionic strength, salt and high pH values as well.

Color Removal Techniques: Physico-chemical Treatments

Literature reveals conflicting findings concerning the ability of textile waste water treatment processes, such as physical, physico-chemcial, chemical and biological. Textile waste water treatment by physical processes is found to be negligible. Various physicochemical processes such as anion-exchange resin, flotation, electro floatation, electro-chemical destruction, irradiation, ozonation, activated carbon, chemical coagulation, chemical oxidation and adsorption have been found to be high cost for treating textile dye wastes (Banat and Nigam, 1996). Most physicochemical dye removal methods has drawback because they are expensive, limited versatility, greatly interfered by other waste water constituents, and/or generate waste product that pose additional serious disposal problem. Different physico-chemical techniques have been discussed with its advantages and disadvantages (Table 1.2). Alternatively, biological treatments by means of microorganisms may present a relatively inexpensive way to remove dyes from waste water.

Table 1.2: Different physical and chemical methods for dye removal from textile effluent with its advantages and disadvantages. (Robinson *et al*, 2002b)

Physical/chemical method	Advantages	Disadvantages
Fentons reagent	Effective decolaration of soluble and insoluble dyes	Sludge generation\
Ozonation	Applied in gaseous state, no alteration of volume	Short half life (20 min)
Photochemical	No sludge production	Formation of by-products
Sodium hypochlorite	Initiates and accelerate azo bond cleavage	Release of aromatic amines
Electrochemical destruction	Break-down compounds are non hazardous	High electricity consumption
Activated carbon	Good removal of wide variety of dyes	Very expensive
Peat	Good adsorbent due to cellular structure	Specific surface area for adsorption are lower than activated carbon
Wood chips	Good sorption capacity for acidic dyes	Require long retention time
Silica gel	Effective for basic dye removal	Side reactions prevent commercial application
Membrane filtration	Removal all types of dyes	Concentrated sludge production
Ion-exchange	No adsorbent loss due to regeneration	Not effective for all dyes
Irradiation	Effective oxidation at laboratory scale	Requires high concentrations of dissolved oxygen
Electrokinetic coagulation	Economically feasible	High sludge production

Bioremediation

The process of bioremediation can be measured by monitoring any of the two factors, (1) by measuring the redox potential, together with pH and temperature, oxygen content and concentration of electron acceptors/donors as well as breakdown products such as carbon dioxide etc. (2) Measurement of chemical oxygen demand (COD) and biological oxygen demand (BOD). Biological oxygen demand represent only the organism matter which is being capable of degraded/oxidized by microbes where as COD represents all the oxidizable matters including organic matter in any particular effluent. In case of colored effluents, bioremediation is measured by estimating the decrease in color intensity. (Marmagne and Coste, 1996)

Bacterial Decolorization

Although it is thought that azo dyes are nearly non-biodegradable or untransformable by bacteria under aerobic condition, efforts to isolate bacteria capable of degrading dye have continued (Zimmermann et al, 1984). Hu (1998) isolated *Pseudomonas luteola* from waste water treatment plant that decolorize reactive azo dyes. Wong and Yuen (1996) isolated a bacterium *Klebsiella pneumoniae* from dye contaminated sludge that could degrade the methyl red upto 100 mg l^{-1} more efficiently than other isolated bacteria.

The ability of two bacterial strains, the Gram –negative *Alcaligenes faecalis* and the Gram-positive *Rhodococcus erythropolis* to decolorize the monoazo dye Acid orange were studied with different initial dye concentrations by Mutafov *et al.* (2007). The diazo dye Reactive yellow 84A was efficiently degraded by a novel bacterial strain *Exiguobacterium* sp. Analytical techniques like HPLC, GCMS, and FTIR demonstrated that degradation of dye resulted with significant reduction of phytotoxicity, confirming the environmentally safe nature of the degradation metabolites (Dhanve *et al.*, 2009)

Anaerobic Decolorization Process

Anaerobic reduction of azo dyes using microbial sludges can be an effective and economic treatment process for removing color from dye house effluents. Previous studies have demonstrated the ability of anaerobic bacteria to reductively cleave the azo linkages in reactive dyes (Razo-Flores *et al.*, 1997; Lloyd, 1992, Brown and Laboureur, 1983, Chung *et al.*, 1978). Although this effectively alters the chromogen and destroys the observed color of the dye, many aromatic groups are not susceptible to anaerobic reduction. However, there is evidence that some azo dye metabolites may be fully stabilized in anaerobic environments (Razo-Flores, 1997).

Chung *et al.* (1978) conducted a study measuring the degradability of seven azo dyes using intestinal and other major anaerobes. The studies were carried out using isolated strains of bacterium in suspended cell mediums containing different azo dyes. Although the dyes studied were not fiber-reactive dyes, their

findings showed that the reduction of azo compounds could be accomplished by intestinal and other major anaerobes. Furthermore, the presence of aromatic intermediates was also detected in measurable amounts for each dye. Toxicity tests were not conducted, but some of the intermediates had been previously determined to be mutagenic.

Brown *et al.* (1987) studied the degradability of various azo dyes in both anaerobic and aerobic systems. They investigated the anaerobic degradability of 22 commercial azo dyes. Later in 1987, Brown and Hamburger conducted a study on 14 azo dyes subjected to anaerobic sludge digestion followed by aerobic treatment. The study focused on both the reduction of the dye molecules as well as the production and subsequent degradation of dye metabolites. Brown and Hamburger's results confirmed cleavage of azo linkages, the production of metabolites was also observed, but at less than theoretical concentrations.

The anaerobic reduction of textile mill effluents and the azo dyes Reactive Black 5 and Navy 106 were investigated by Lloyd (1992) and Ganesh (1992) respectively. In both cases, laboratory scale anaerobic reactors were used for dye degradation. The results of Loyd and Ganesh were similar; both observed good decolorization with minimal nutrient removal. These findings concur with many studies found in the literature. While high decolorization of textile effluents is often achieved in anaerobic environments, poor TOC and nitrogen removals are usually observed.

Kothari R. K (2002) and Kothari C. R (2006) reported the effect of static and shaking conditions on decolorization of various textile dyes. Their results suggested that more decolorization is achieved under static culture condition compared to shake flask condition.

Aerobic Decolorization Process

Activated sludge treatment of wastes is effective and highly economic system for reducing organic pollutants in wastewater. However, aerobic treatment of azo dye wastes has proven ineffective in most cases, but it is often the typical

method of treatment used today (Edwards, 2000; Yang *et al.*, 1998). Because aerobic microbes cannot reduce azo linkages, their ability to destroy dye chromogens is less than anaerobic bacterium. However, aerobic sludges have been successfully used to stabilize dye metabolites (Brown and Laboureur, 1983).

The aerobic biodegradability of aniline, o-toludine, p-anisidine, p-phenetidine, o-dianisidine, and 3, 3'- dichlorobenzidine, was investigated by Brown and Laboureur (1983b). These compounds were all aromatic amines and possible by-products of azo dyes degradation. Because many aromatic structures are non-biodegradable in anaerobic environments and are not hydrophilic, they can accumulate in the adipose tissues of organisms. Many aromatics have been identified as possible carcinogens, release of which into the environment is a matter of concern. Previous work by Brown and Laboureur (1983a) indicated that azo dyes may be broken down to their intermediate structures in a reductive environment, but were not amenable to further degradation by anaerobes. Recently the aerobic decolorization of azo dyes has been reported by a number of investigators (Adedayo *et al.*, 2004; Mona *et al.*, 2008).

The degradation of *p*-aminoazobenzene by *Bacillus subtilis* was studied through batch experiment in order to investigate azo dye degradation by Zissi (1997). The results proved that *B. subtilis* cometabolize *p*-aminoazobenzene in the presence of glucose as carbon source, producing aniline and p-phenylenediamine, as the nitrogen-nitrogen double bond is broken. In this study azo dye was found to act as inhibitor to microbial growth. *Bacillus subtilis* HM was able to decolorize aerobically eight different sulfonated azo dyes. Decolorization of Fast Red was achieved through microbial degradation rather than biosorption or adsorption as indicated by the uncolored biomass or its methanol extracts (Mona *et al.*, 2008)

Sequential Anaerobic-Aerobic Process

Generally, bacterial azo dye biodegradation proceeds in two stages. The first stage involves reductive cleavage of the dye's azo linkage, resulting in the formation of generally colorless but potentially hazardous aromatic amine. The

second stage involves degradation of the aromatic amines. Azo dye reduction usually requires anaerobic conditions, whereas bacterial biodegradation of aromatic amines is an almost exclusively aerobic process (Ghatnekar *et al*, 1996). A waste water treatment process in which anaerobic and aerobic conditions are combined is therefore the most logical concept for removing azo dyes from wastewater.

According to the concept of combined anaerobic-aerobic treatment, azo dyes should be removed from the water phase by anaerobic reduction followed by aerobic oxidation of the dyes' constituent aromatic amines. The anaerobic-aerobic reactor studies show that a generally high extent of color removal can be obtained, and several studies furthermore provide evidence for removal of aromatic amines. Combined anaerobic-aerobic treatment therefore holds promise as a method to completely remove azo dyes from wastewater (Naimabadi *et al.*, 2009).

Anaerobic bacteria are often able to reduce the azo linkages, but are generally unable to further stabilize the dye metabolites; it would seem useful to follow anaerobic treatment processes with an aerobic treatment step. As mentioned previously, aerobic organisms can oxidize aromatic ring compounds to simpler molecules. A considerable amount of research has been conducted on ANA/AER sequential step-treatment systems used for degrading textile wastewaters (O'Neill *et al.,* 2000; Loyd, 1992; Kothari and Kothari 2002, 2006).

Biodegradation of two azo dyes, 4-phenyl azophenol (4-PAP) and mordant yellow 10 resulted in accumulation of aromatic amines. Further, 4-aminophenol (a-AP) and aniline are detected from the reduction of 4-PAP. Aniline is degraded further in the presence of oxygen by the facultative aerobic bacteria present in the anaerobic granular sludge. Aerobic enrichment cultures developed on aromatic amines combined with oxygen-tolerant anaerobic granular sludge can be used to completely biodegrade azo dyes under integrated anaerobic-aerobic conditions (Loidl *et al.*, 1990).

Molecular Characterization of Azoreductase Enzyme

Suzuki et al. was the first to describe the sequence and subsequent characterization of a gene encoding an aerobic azoreductase from the soil isolates, Bacillus sp. OY1-2. The protein consists of 178 amino acids and presumably it is a FMN dependent azoreductase. A similar gene encoding azoreductase from *Rhodobacter sphaeroies* AS1.1737 was expressed and partially characterized (Suzuki *et. al*, 2001).

Chen *et al.* was able to identify and express a functional azoreductase gene from *Staphylococcus aureus* in *Escherichia coli*. The *Staphylococcus aureus* azoreductase was found to be a homotetramer with a native molecular mass of 85 kDa containing four noncovalently bound FMN molecules. The enzyme requires NADPH as an electron donor. It was resolve to dimeric apoprotein by removing the flavin prosthetic groups using hydrophobic-interaction chromatography. The dimreic apoprotein was reconstituted on column and in free stage with FMN resulting in formation of a fully functional native-like tetrameric enzyme (Figure 1.3). The Staphylococcus aureus azoreductase was able to metabolize methyl red, orange II, Amarnath, and Ponceau S azo dyes.

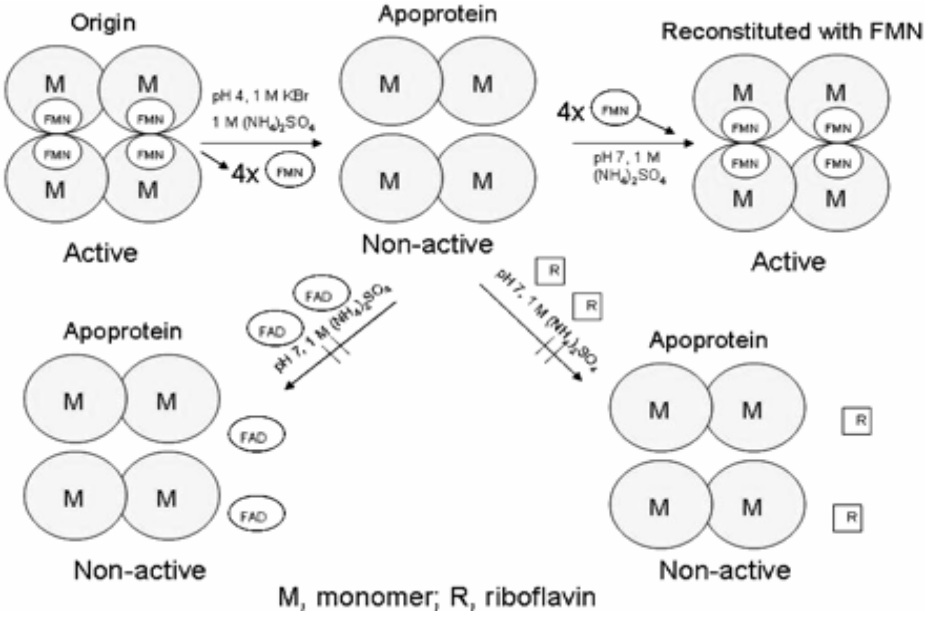

Figure 1.3: Possible mechanism of the dissociation and reassociateion of Azo 1 of S. aureus expressed in *E. coli.* (Chen and Hopper, 2005)

Application of Genetic Engineering

Cloning, overexpression and characterization has become a basic tool in studying genetic engineering for the improvement of any biological process. For the overexpression and characterization of azoreductase, different genetic engineered strains (usually *Escherichia coli*) strains were constructed. Russ *et al*., (2000), transferred flavin reductase (fre) to *Sphingomonas* sp. Strain BN6 and found 30 fold increase in azo reductase activity in the wild-type strain. On the other hand, whole cells of the recombinant *Sphingomoas* sp. BN6 showed only approximately threefold increase in the reduction rate for azo dyes Amarnath and Mordant Yellow three compared to wild type strain.

It has been reported by Blumel *et al.* 2002 that *E. coli* strain carrying the azoreductase gene (azo B) from *X. azovorans* KF46F, expressed almost 50 times higher azoreductase activity in its cell extracts than that observed in wild type strain. However, the resting recombinant cell suspension showed no detectable azoreductase activity.

Dyes, Environmental Concern

Wastewater from the textile industry is a complex mixture of many polluting substances ranging from organochlorine-based pesticides to heavy metals associated with dyes or the dyeing process (Correia *et al*.,1994). Many dyes are visible in water at concentrations as low as 1 mg l^{-1}. Textile-processing wastewaters, typically with dye content in the range 10-200 mg l^{-1} (O'Neill *et al*., 1999) are therefore usually highly colored and discharge in open waters presents an aesthetic problem. As dyes designed to be chemically and photolytically stable, they are highly persistent in natural environments.

The majority of dyes pose a potential health hazard to all forms of life. These dyes may cause allergic responses, skin dermatoses, eczema (Su and Horton, 1998), and may affect the liver, the lungs, the vasco-circulatory system, the immune system and the reproductive system (Nikulina and Deveikis, 1995) of experimental animals as well as humans.

Textile dyes have found to be toxic, genotoxic and mutagenic in various test systems. Dyes with azo bonds nitro-or amino-groups are carcinogenic, causing tumors of liver and urinary bladder in experimental animals (Puvaneshwari *et al.*, 2006). However, reduction of azo dyes, i.e. cleavage of the dye's azo linkage(s), leads to formation of aromatic amines and several aromatic amines are known mutagens and carcinogens. In mammals, metabolic activation (reduction) of azo dyes is mainly due to bacterial activity in the anaerobic parts of the lower gastrointestinal tract. Various other organs, especially the liver and the kidneys, can, however, also reduce azo dyes (Zee, 2002).

The toxicity of aromatic amines depends on the nature and location of other substituents. As an example the substitution with nitro, methyl or methoxy groups or halogen atoms may increase the toxicity; whereas substitution with carboxyl or sulphonate groups generally lower the toxicity (Chung and Cerniglia, 1992). As most soluble commercial azo dyestuffs contain one or more sulphonate groups, insight in the potentials danger of sulphonated aromatic amines is particularly important. Sulphonated aromatic amines, in contrast to some of their unsulphonated analogues, have generally no or very low genotoxic and tumorigenic potential (Jung and Steinle, 1992).

Environmental Concerns of Small Scale Industries in India

Small-scale industries (SSIs), having capital investment up to Rs.10 millions, have a very important role in overall industrial development in India and growth of SSI units has been actively promoted by Government of India. It is estimated that more than 300,000 SSI units are spread all over India, mainly in about 867 clusters/industrial estates of the country.

It is difficult for each industrial unit to operate individual wastewater treatment plant because of the scale of operations or lack of space or technical manpower. However, the amount of pollutants emitted by SSIs clusters may be more than an equivalent large-scale industry. One possible reason for this may be the inefficient production technologies adopted by SSIs.

Common Effluent Treatment Plant (CETP)`

Keeping in view the key role played by SSIs and the constraints in complying with pollution control norms individually by these units, The Ministry of Environment and Forests (MoEF) initiated an innovative technical and financial support scheme of common facilities for treatment of effluents generated from SSIs located in clusters. The financial assistance provided under this Common Effluent Treatment Plant (CETP) scheme was as: Central Government - 25%, State Government - 25%, Loans from financial institutions- 30%, and Entrepreneurs' contribution- 20% of the project capital cost. The CETP scheme was instituted initially for a period of 10 years with effect from the year 1991 but MoEF has decided to continue financial assistance under the scheme beyond this period. Table 1.3 shows number of CEPT with their treatment capacity of selected sates having larger industrial zones.

Table 1.3: State wise distribution of CETPs and their capacity in MLD.

State	No. of CETP	Combined Capacity of CETPs, MLD	Combined Capacity as %of total capacity
Delhi	11	133.2	24.1
Gujarat	16	156.3	28.2
Maharashtra	11	63.25	11.43
Tamilnadu	29	71.15	12.85
Total (India)	**88**	**559.77**	**100**

Total of 88 CETPS have been constructed throughout the country under this scheme. The Central Pollution Control Board had checked performance of 78 CETPs operating throughout the country and submitted report in October 2005. The performance of all the CETPs in terms of general parameters like pH, Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Total Suspended Solids (TSS) and Total Dissolved Solids (TDS) was assessed.

It was observed that out of the total 78 CETPs studied, only 20 (i.e. 25.6%) complied with the prescribed limits for general parameters pH, BOD, COD and TSS but 15 of these were not able to comply with the prescribed limit for TDS. Thus, only 5 (i.e. 6.4%) CETPs were complying all general parameters including

TDS. In the state of Gujarat, only 2 CETP falls in this category. These two CETP are Ankleshwar and Sachin CETP which has small capacity up to 0.5 MLD where as state's cumulative is 156.3 MLD. Out of 16 CETPs of Gujarat, none was found to meet the prescribed limits of general parameters pH, BOD, COD, TSS and TDS (Figure 1.4). From the data it is observed that Gujarat being the state with highest effluent treatment capacity (28.2% of total capacity throughout country), we can conclude it is highly industrialized and highly effluent generating state. Still, there is no single CETP that meets the criteria to release effluent in nature after treatment.

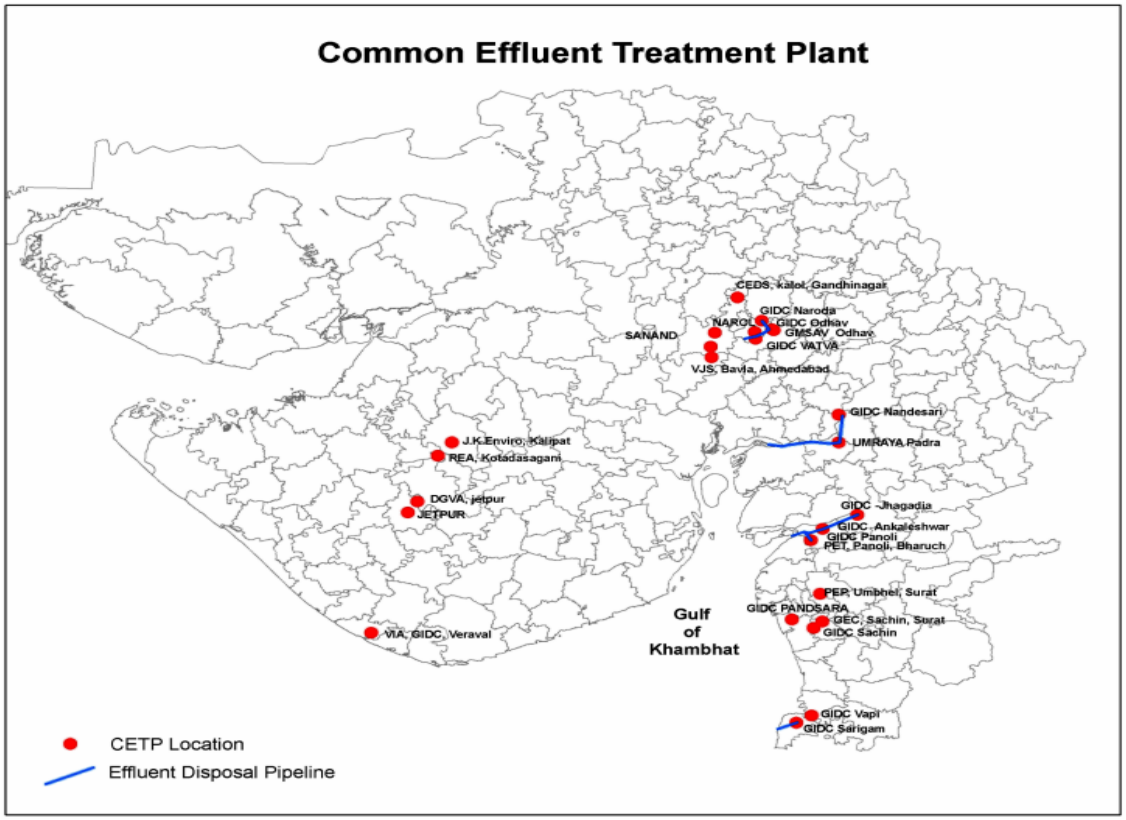

Figure 1.4: The Sites of Common Effluent Treatment Plants (CETP) in Gujarat.

DESCRIPTION OF SAMPLE COLLECTION SITE

JETPUR (CETP in Gujarat)

Geographically Jetpur lies in the heart of Saurashtra. Arabian see covers Saurashtra from west and south side; where as Gulf of Kutch is on north and Gulf of Khambhat to the southeast. This rise in population in last decade has indicated rapid industrialization of this town of Rajkot district. The industrialization is due to one major industry - textiles. There are around 200 dyeing and printing units in the town having an average annual turnover of more than Rs 150 crore. In addition, there are around 500 ancillary units all of which combine to produce, on an average, two million meters of printed cloth per day which is enough to make 40,000 cotton saris.

Water pollution is the major ecological threat that Jetpur faces. The river Bhadar which turned seasonal with the construction of a dam upstream has now turned red due to the untreated effluents from dyeing units which are emptied into the river (Figure 1.4 to 1.7). The chemicals used for printing saris have sunk deep into the soil and has even killed the grass," says a leader of town Dhoraji, downstream of Jetpur, who has been leading an agitation for clean water. Water in Jetpur and nearby villages/towns is highly contaminated.

After various court cases and agitations, the high court ordered closure of certain units that were polluting the water and the Gujarat Pollution Control Board (GPCB) ordered construction of a common effluent treatment plant. The plant, however, had only a limited capacity against a huge daily discharge. Few units are willing to spend on effluent treatment processes and state bodies are reluctant to order units to shut down fearing large-scale unemployment.

Short falls in design and construction part: Influent characteristics and hydraulic load are not representative of actual design criteria. For example, in Vapi, Gujarat, the design concentration of influent COD is 1000mg/l but actual

influent concentration of COD is more than 3000mg/l. Many important parameters like NH3 – N, Phenol and other toxicants including heavy metals were not included into the design aspects of CETP, resulting in improper treatment and noncompliance of prescribed norms. Toxic effluents some time destroy whole biomass of CETP.

Short falls in operation and maintenance part: Jetpur CETP plant receives 70% industrial effluent from member units and 30% city sewage. At the time of inspection of the plant by Gujarat Pollution Control Board in January 2005, the treated effluent was not meeting the standards in terms of BOD, COD, TSS, TDS and NH3-N. CETP does not have laboratory facility. The effluent collection system is not fool proof. [Performance status of common effluent treatment plant, CPCB Report October 2005,]

Figure 1.4: The untreated effluent is taken to CETP located 2 km far from industrial area in Jetpur. The bridge is constructed in Bhadar River.

Figure 1.5: Blockage of canal with waste and leakage/overflow of untreated effluent directly into the Bhadar River.

Figure 1.6: Untreated, highly colored and polluted effluent finding its way in river.

Figure 1.7: Effluent collected and deposited in Bhadar River.

3

Isolation, Screening and Identification of Dye Decolorizing Bacteria

INTRODUCTION

The treatment of textile effluent containing dye has been carried out by various physical and chemical methods over the last two decades for the removal of color from waste water. These methods have limited applicability as they are expensive and lead to the production of solid waste. The treatment processes are based on the microorganisms capable of decolorizing or degrading these recalcitrant compounds. These biological processes are environmental friendly and can lead to complete mineralization of xenobiotic compounds.

Over the past decade, many organisms capable of dye decolorization at lab scale have been reported, but there are few reports available on their exploitation in treatment processes. The most widely studied white-rot fungus, in this regard is *Phanerochaete chrysosporium* (Reddy, 1995). Efforts to isolate bacterial culture capable of degrading azo dyes started in the 1970s with reports of a *Bacillus subtilis* (Horitsu *et al.*, 1977). Bacterial isolates from soil and sludge sample belonging to *Bacillus* sp. *Alcaligenes* sp. and *Aeromonas* sp. were found to have high dye decolorization ability (Sharma and Saini, 2004). Cynobacteria like *Gloeocapsa pleurocapsoides* and *Phormidium ceylanicum* decolorized Acid Red 97 and FF sky Blue dye more than 80% after 26 days (Parikh and Madamwar, 2005). Decolorization of Direct yellow and Erio red dyes by bacterial and actinomycetes were studied by Waffa and Moawad, 2003. Other reports

suggested that *Pseudomonas* sp. (Kothari, 2002) *Escherichia coli*, sulfate reducing bacteria (Yoo, 2002) are efficient dye decolorizer. The effectiveness of these treatment systems depends upon the survival and adaptability of microorganisms during the treatment processes.

This part of the study was undertaken to isolate microorganisms capable of decolorization/degradation various textile azo dyes used in industry situated in central Saurashtra region. The use of isolated bacteria either individually or as consortium was envisaged to develop efficient biological process for the treatment of effluents containing different dyes.

MATERIALS AND METHOD

Sample Collection

Samples were collected from in and around Jetpur Common Effluent Treatment Plant. Samples were collected from different places, such as drainage canal that carry textile effluent to CETP located about 2 km far from dying industries, various stages of CETP, and soil samples of agriculture field where treated effluent is used for irrigation. Samples were in the form of liquid untreated effluent, treated effluent, sludge, and soil. All the samples were collected in sterile glass-screw cap tubes and preserved at 4^{o}C in refrigerator and samples were tested within 24 hrs of collection.

Physico-chemical characterization of samples

The effluent samples, mainly before treatment and after treatment were tested for its physico-chemical characteristics like, color, pH, COD, BOD, TSS, TDS, etc.

Dyes

All azo dyes including Remazol black B, sky blue FF, Green B, Black E, Black BT, Reactive Orange H_2R, and Reactive Orange M_2R were procured from local dye manufacturing unit GIDC, Ahmedabad. The main dye in this study Reactive Black 5 also known as Remazol Black B has H-acid core molecule with two vinylsulphone groups in its structure (Figure 1.1). All other chemical for preparing various media were purchased from Hi-media Pvt. Ltd. Mumbai, SRL India.

Chemicals

The Mineral Salt Medium (MSM) consisted of (g l^{-1}) $Na_2HPO_4 \cdot 7H_2O$ 3.6; $(NH_4)_2SO_4$ 1.0; KH_2PO_4 1.0; $MgSO_4$ 1.0; $Fe(NH)_4$ citrate 0.01; $CaCl_2 \cdot 2H_2O$ 0.1; and 10 ml of trace element solution per liter. The trace element solution contained (mg l^{-1}): $ZnSO_4 \cdot 7H_2O$ 10; $MnCl_2 \cdot 4H_2O$ 3; $CoCl_2 \cdot 6H_2O$ 1; $NiCl_2 \cdot 6H_2O$ 2; $Na_2MoO_4 \cdot 2H_2O$ 3; H_3BO_3 30; $CuCl_2 \cdot 2H_2O$ 1. The pH of the medium was adjusted to 7.

Complete Medium Broth used for decolorization study contained:(g l^{-1}); Peptone 5; Yeast extract 3; glucose 2; NaCl 5; K_2HPO_4 5; KH_2PO_4 1; $MgSO_4$. $7H_2O$ 0.1. The pH of the medium was adjusted to 7.0.

Enrichment and isolation of dye decolorizing bacteria

All samples were used for isolation of dye decolorizing bacterial cultures by enrichment culture techniques using MSM supplemented with glucose (0.2 % w/v) and yeast extract (0.2 % w/v) containing mixture of two azo dyes (RB5 and Reactive Orange M2R) with the final dye concentration of 100 mg l^{-1}. The enrichment was carried out in 200 ml MSM medium in 500 ml Erlen-meyer flask by adding 10 ml, 1g, and 10 ml of effluents, sludge, and soil suspension, respectively. The culture flasks were incubated on orbital shaker with 120 rpm, at 30^oC. After every 3rd days of incubation (i) a loop-full of medium was streaked onto sterile nutrient agar plates and incubated at 37^oC for 24 to 48 h, and (ii) 1 ml of the enriched culture was transferred to fresh medium. Such serial transfers were performed till 7 days. At the end of incubation 1ml of sample was serially diluted from each flask and plated on the agar medium with same concentrations of ingredients. The pure cultures of individual bacterial strains were maintained by streaking on nutrient agar slant and stored at 4^oC, as well as in 40% glycerol stored at -20^oC.

Screening of dye decolorizing bacteria

The thirty seven morphologically distinct bacterial isolate were tested for their ability to decolorize textile azo dyes. Overnight grown culture of these 37 isolates were use to inoculate with 1ml in 250 ml Erlenmeyer flask containing 100 ml CMB supplemented with Reactive Black 5 and Green B (100 mg l^{-1}) dye. The inoculated flasks were incubated under static conditions. 2 ml sample was taken out aseptically and centrifuged at 6000 rpm for 15 min. The cell free supernatant was used to determine the percentage decolorization of the added dye. Six morphologically distinct bacterial isolates showing more than 60% decolorization of the added dye were selected for further studies.

Growth and colony characteristics

Growth curve pattern for all the 37 bacterial isolates were carried out by inoculating loop full culture into Nutrient broth medium. Cultures were grown overnight in CMB medium and next day a loop-full young culture was transferred to CM agar plates and slants. They were incubated at 37^{o}C for 24 h.

Gram reaction and cell morphology

Gram's staining of the 24 h old cultures of all the isolates was performed to study Gram reaction and the cell morphology.

Biochemical tests

All required media for biochemical tests were prepared in respective, test tubes, flasks, and petri dishes. Reagents required for different biochemical tests were prepared and stored at 4^{o}C in refrigerator. Overnight grown cultures of all 37 isolates were inoculated 10 µl in media and incubated at 37^{o}C for 24 hrs.

DNA extraction PCR amplification of 16S r RNA gene

The PCR primers used to amplify 16S rDNA fragments were the bacteria-specific primers (Lane, 1991) a forward primer F27 (5'-AGAGTTTGATCMTGGCTCAG-3'); and a reverse primer R1492 (5'-ATAGGYTACCTTGTTACGACT-3'). A total of 25 µl of reaction mixture consisted of 10 pmol of each primer, 5 ul from colony suspension as template DNA, 12.5 µl of Master mix (Fermentas, UK). The PCR amplification was performed by Thermal Cycler (ABI, USA) using the following program: Denaturing at 95°C for 5 minutes, followed by 30 cycles of 30 seconds of denaturing at 95°C, 30 seconds of annealing at 50°C and 2 minutes of elongation at 72°C with a final extension at 72°C for 10 minutes for first set. The PCR product (1400 bp) was cleaned by using a Qiagen DNA Gel Extraction Kits (QIAGEN, CA) in accordance with the directions of the manufacturer.

Sequencing

Sequencing was performed (purified PCR product) by using above primer (above pcr conditions) with an ABI Prism 310 Genetic analyzer (Applied

Biosystems Inc., CA) using BigDye Terminator (version 3.1) at Animal Biotechnology laboratory, AAU, Anand, Gujarat, India.

Phylogenetic analysis and Sequence Analysis

The 16S rDNA sequences were initially analyzed at NCBI server (http://www.ncbi.nlm.nih.org) using BLAST tool and corresponding sequences were down loaded. Evolutionary history was inferred using the Neighbor-joining method (Patil *et al.*, 2008). The tree was drawn to the scale, with branch lengths in the same units as those of the evolutionary distance used to infer the phylogenetic tree (Dhanve *et al.*, 2009).

Assay of decolorization

Decolorization activity was expressed in terms of percentage decolorization and was determined by monitoring the decrease in absorbance at absorption maxima (λ_{max}) of respective dyes (i.e. 598 for Reactive Black 5 and 634 for Green B). The uninoculated CMB supplemented with respective dye was used as reference. The culture suspension was centrifuged at 6,000 rpm for 15 min for removal of the biomass. The degree of decolorization of the tested dye was measured at its respective maximum absorbance wavelength using supernatant by UV-visible spectrophotometer (1800, Shimadzu, Japan). The biomass was determined by resuspending the biomass palate in 2 ml sterile distilled water and at λ_{660nm}. The decolorization assay was calculated according to the following formula.

Decolorization activity (%) = (A-B)/A x 100

Where A = initial absorbance

B = Observed absorbance

RESULTS AND DISCUSSION

Physico-chemical characterization of collected samples

The samples were collected in sterilized container from respective sites (Figure 2.1 to 2.4). The color, temperature and pH of the sample were recorded on the site and samples were transported to the laboratory by storage at 4^{o}C. Other physico-chemical characteristics like BOD, COD, TSS, TDS etc. were measured on the same day of collection of sample as per table 2.1. The raw sewage was dark green in color because of the types of dyes generally used.

As the stages of treatment progressed, the color of effluents changed from dark green blue-light green- light yellow and finally light brown. The green and bluish color of the incoming effluent is due to wide use of green and blue color dye in dyeing and printing industries, thus, it contributes more to the effluent's color compared to other dyes. The light brown color of the finally released effluent after treatment may be due to the dirty water condition. The pH of the untreated effluent was 9.8, which reduced during treatment to near neutral 7.8.

Table 2.1: Characteristics of samples collected from different stages of CETP, Jetpur.

Sl	**Sample**	**Nature of Sample**	**Color**	**pH**	**BOD** (mg/l)	**COD** (mg/l)	**TSS** (mg/l)	**TDS** (mg/l)
1	Inffluent - drainage line	Liquid	Dark Green to Blue	9.8	398	1692	223	2930
2	Oil and Grease separated	Liquid	Light green	9.5	331	1734	119	2201
3	After PAC & PE treatment (after chemical mixing)	Liquid	Light Yellowish	8.2	290	1324	109	2016
4	After aeration (Effluent)	Liquid	Light Brown	7.8	80	421	78	1817

Figure 2.1: Aeration tank for treatment of effluent (CETP, Jetpur, Gujarat)

Figure 2.2: Sample collection from sludge bed (CETP, Jetpur, Gujarat)

Figure 2.3: Treated effluent storage - used for agricultural irrigation (CETP, Jetpur, Gujarat)

Figure 2.4: Samples collected before and after treatment of textile effluent (CETP, Jetpur, Gujarat)

Isolation and screening of bacterial strains

The selective enrichment of liquid effluent, sludge, and soil sample collected from the CETP and waste disposal sites, led to the isolation of 37 morphologically different bacterial isolates. Gram strain of all isolates indicated the presence of 20 Gram positive and 17 Gram negative organisms (Figure 2.5). The pure cultures were preserved on N-agar medium at 4^{o}C. All 37 isolates were tested individually for their ability to decolorize RBB and Green B separately at the

concentration of 100 mg l^{-1} each (Table 2.2). All isolates decolorize both the dyes with different capacity ranging from lowest 3% to highest 97% in case of Reactive Black 5 and 3% to 92% in case of Green B.

Six potential isolates namely; JTP-5, JTP-13, JTP-23, JTP-30, JTP-32 and JTP-37 showed good decolorization efficiency in RB5 and Green B, were tested for their ability to degrade other dyes namely FF sky blue, Black BT, Black E, and Reactive orange M_2R. The dye concentration in effluent from textile printing house is approximately in the range of 50 to 200 mg l^{-1}. This value is typical of those used in studies on treatment for azo dye containing effluent (Zhao and Hardin, 2007). However, change in operating processes may lead to still high concentration of dye in effluent. Keeping in mind the above fact, we used 100 mg l^{-1} dye concentrations to check their ability to decolorize different dyes.

Decolorization of FF sky Blue was around 99% by JTP-13 and JTP-30. JTP-23, JTP-37, JTP-32 and JTP-5 decolorized this dye at, 77%, 54%, 35% and 24%, respectively. The dye that has been mainly studied, Reactive Black 5, was decolorize more than 70% by all the isolates whereas this dye was decolorize up to 98% by JTP-23, the most studied organism in this study. The lowest and highest decolorization of different dyes by selected organisms were in the range of 59% to 92% for Green B, 25% to 86% for Black E, 6% to 69% for Black BT, and 23% to 94% for Reactive Orange M_2R (Figure 2.6). The difference in decolorization pattern is due to the dissimilarity in specificities, structure and complexity, particularly on the nature and position of substituent in the aromatic rings and the interaction with azo bond with different dyes as reported by many authors (Sani and Benerjee, 1999; Radha and Raghupati, 2005; Vijaykumar and Vaishampayan, 2007).

The isolation of different microorganisms from the sample indicates the natural adaptation of microorganisms to survive in the presence of toxic dyes. The difference in their rate of decolorization may be due to the loss of ecological interaction, which they might be sharing with each other under natural conditions. (Sharma and Saini, 2004).

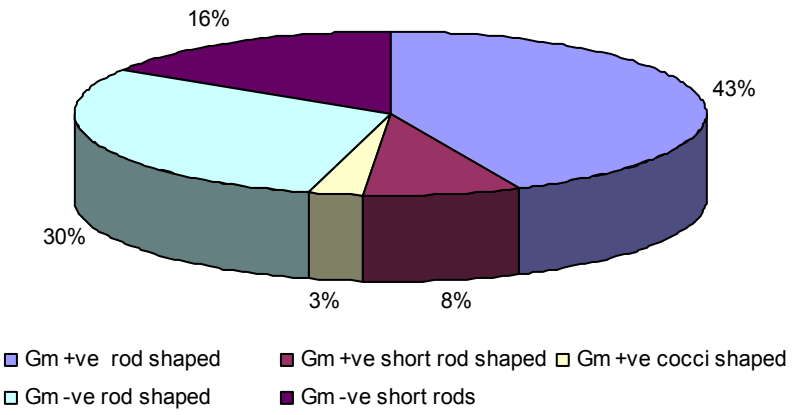

Figure 2.5: Diversity of bacterial isolates at CETP, Jetpur, Gujarat

Table 2.2: Decolorization of RB5 and GB by bacterial isolates JTP-1 to JTP-37 (Dye 100 mg l^{-1})

Bacterial isolates	RB5	Green B
	Decolorization (%)	Decolorization (%)
JTP-1	64	53
JTP-2	24	17
JTP-3	12	9
JTP-4	11	20
JTP-5	85	90
JTP-6	56	62
JTP-7	23	14
JTP-8	3	9
JTP-9	23	30
JTP-10	16	7
JTP-11	7	9
JTP-12	7	10
JTP-13	73	82
JTP-14	51	62
JTP-15	9	5
JTP-16	35	69
JTP-17	28	38
JTP-18	12	7
JTP-19	17	10
JTP-20	19	9

Bacterial isolates	RB5	Green B
	Decolorization (%)	Decolorization (%)
JTP-21	14	17
JTP-22	33	21
JTP-23	98	92
JTP-24	43	38
JTP-25	5	3
JTP-26	47	45
JTP-27	3	5
JTP-28	5	3
JTP-29	4	3
JTP-30	88	73
JTP-31	2	2
JTP-32	83	59
JTP-33	2	5
JTP-34	13	21
JTP-35	9	17
JTP-36	34	12
JTP-37	74	80

Growth and morphological characteristics

Growth curve was obtained for all the 37 bacterial isolates. Wide variation in curve patterns was found indicating diversified bacterial species in textile effluent. JTP-5 and JTP-7 showed rapid biomass synthesis, JTP -13 and JTP-32 had moderate biomass synthesis, while JTP-30 and JTP-37 had comparatively long lag phase as compare to other potential organisms (Figure 2.7).

The Gram's staining indicated that out of 37, Gm +ve rods - 16, Gm +ve short rods - 3, Gm +ve cocci - 1, Gm -ve rod - 11 and Gm -ve short rod - 6 (Figure 2.6). The additional information from Gram staining was in the form of cell morphology and arrangement. The growth pattern of these isolates on nutrient agar slant was filiform, echinulate and arboescent with moderate or large growth abundance (*Table 2.3*). It was found that most of the organisms were of rod shaped including short and big rods (Figure 2.8). The potential dye decolorizers were found in, Gm +ve and Gm -ve group.

When organisms were grown on N-agar plate, there was characteristics pigmentation of colonies like white, dirty white, gray, light yellow and light brown. Two isolates JTP-16 and JTP-17 were found to produce dark pigmentation of yellow and red, respectively (Figure 2.9). Size of colonies varied from small to moderate to large having smooth or rough texture with even, uneven, wavy filamentous margins and circular, rhizoid and irregular forms (Table 2.4).

Table 2.4: Colony characteristics of dye decolorizing bacterial isolates on Nutrient agar

Isolates	Colony characteristics							
	Size	Shape	Margin	Elevation	Surface texture	Consistency	Opacity	Pigmentation
JTP-1	M	Round	Irregular	F	Rough	B	TL	LC
JTP-2	M	Round	Even	Low Convex	Smooth	B	TL	DW
JTP-3	L	Irregular	Uneven	Flat	Smooth	B	OP	White
JTP-4	L	Round	Entire	Flat	Smooth	B	OP	DW
JTP-5	L	Round	Entire	Flat	Rough	B	OP	LY
JTP-6	S	Round	Irregular	Convex	Rough	Solid	OP	LY
JTP-7	S	Round	Even	Convex	Smooth	B	OP	DW
JTP-8	L	Uneven	Radiating	Flat	Rough	B	TL	DW
JTP-9	L	Round	Uneven	Flat	Smooth	B	OP	DW
JTP-10	S	Round	Entire	Flat	Smooth	B	OP	DW
JTP-11	L	Round	Even	Flat	Smooth	B	TL	LC
JTP-12	M	Round	Entire	Low Convex	Smooth	B	OP	DW
JTP-13	L	Round	Radiating	Flat	Rough	B	OP	DW
JTP-14	M	Round	Entire	Low Convex	Smooth	B	TL	DW
JTP-15	S	Round	Even	Low Convex	Smooth	B	TL	DW
JTP-16	B	Round	Lobed	Convex	Rough	B	OP	Red
JTP-17	S	Round	Entire	Convex	Smooth	B	OP	Yello
JTP-18	M	Round	Entire	Convex	Smooth	Gummy	OP	DW
JTP-19	S	Round	Entire	Flat	Smooth	B	OP	White
JTP-20	L	Irregular	Uneven	Flat	Rough	B	OP	White
JTP-21	L	Irregular	Uneven	Flat	Rough	B	OP	LC
JTP-22	M	Irregular	Uneven	Flat	Smooth	B	OP	DW
JTP-23	S	Round	Even	Low Convex	Smooth	Watery	TP	NP
JTP-24	M	Irregular	Lobed	Flat	Rough	B	OP	LY
JTP-25	S	Irregular	Uneven	Flat	Rough	B	OP	NP

Table 2.4: Colony characteristics of dye decolorizing (Continued)

Isolates	Colony characteristics							
	Size	Shape	Margin	Elevati-on	Surface texture	Consist ancy	Opacity	Pigment-ation
JTP-26	M	Round	Entire	Flat	Smooth	B	OP	NP
JTP-27	M	Round	Raised	Flat	Rough	B	OP	NP
JTP-28	M	Round	Uneven	Flat	Rough	B	OP	NP
JTP-29	L	Round	Radiating	Flat	Rough	B	OP	DW
JTP-30	L	Round	Uneven	Flat	Smooth	B	OP	DW
JTP-31	S	Round	Entire	Convex	Smooth	B	TL	NP
JTP-32	M	Round	Even	Flat	Smooth	B	OP	L.Brown
JTP-33	M	Round	Uneven	Convex	Smooth	B	OP	DW
JTP-34	L	Irregular	Uneven	Flat	Rough	B	OP	LC
JTP-35	M	Regular	Even	Flat	Smooth	B	OP	NP
JTP-36	S	Round	Even	Flat	Smooth	B	OP	LC
JTP-37	S	Round	Even	Convex	Smooth	Gummy	TL	DW

S, small; M, Moderate; L, Large; B, Buterious; G, Gray; DW, Dirty White; LY, Light Yellow; LC, Light Cream; NP, No Pigment; TL, Translucent; TP, Transparent; OP, Opaque

Biochemical Tests

Thirty seven bacterial isolates were considered for their characterization, based on Gram's reaction, cell morphology, colony characteristics, growth patterns in nutrient broth and biochemical tests. Table 2.5 and Table 2.6 show results of various characters studied. JTP-8, JTP-11 and JTP-23 produce gas and H_2S while growing on TSI. JTP-2, JTP-4, JTP-9, JTP-10, JTP-16, JTP-27, JTP-28, and JTP-29 produced acid present in TSI which is evidenced b conversion of slants from red to yellow. From the analyzed characters, the isolates were tentatively identified up to generic levels (Table 2.7). The result showed that many of them occurred commonly in such environment.

16S rDNA sequencing Phylogenetic analysis

16S rDNA sequences were determined for three different bacterial isolates namely, JTP-07, JTP-23 and JTP-37. These sequences were submitted to NCBI. To analyze the phylogenetic position, the 16S rDNA sequence of the strain JTP-07 (530 bp, GU137541), JTP-23 (530 bp, GU172172) and JTP-37 (516 bp, GU137542) were determined. Figure 2.10 showed the phylogenetic relationship between the isolated bacterial strains and other related bacteria found in the GenBank database. The homology indicated that the strain JTP-07, JTP-23 and JTP-37 were in the phylogenetic branch of the genus *Alcaligenes, Lysinibacillus*, and *Pseudomonas*, respectively. These isolates were identified as *Alcaligenes faecalis* JTP-07, *Lysinibacillus fusiformis* JTP-23, and *Pseudomonas aeruginosa* JTP-37.

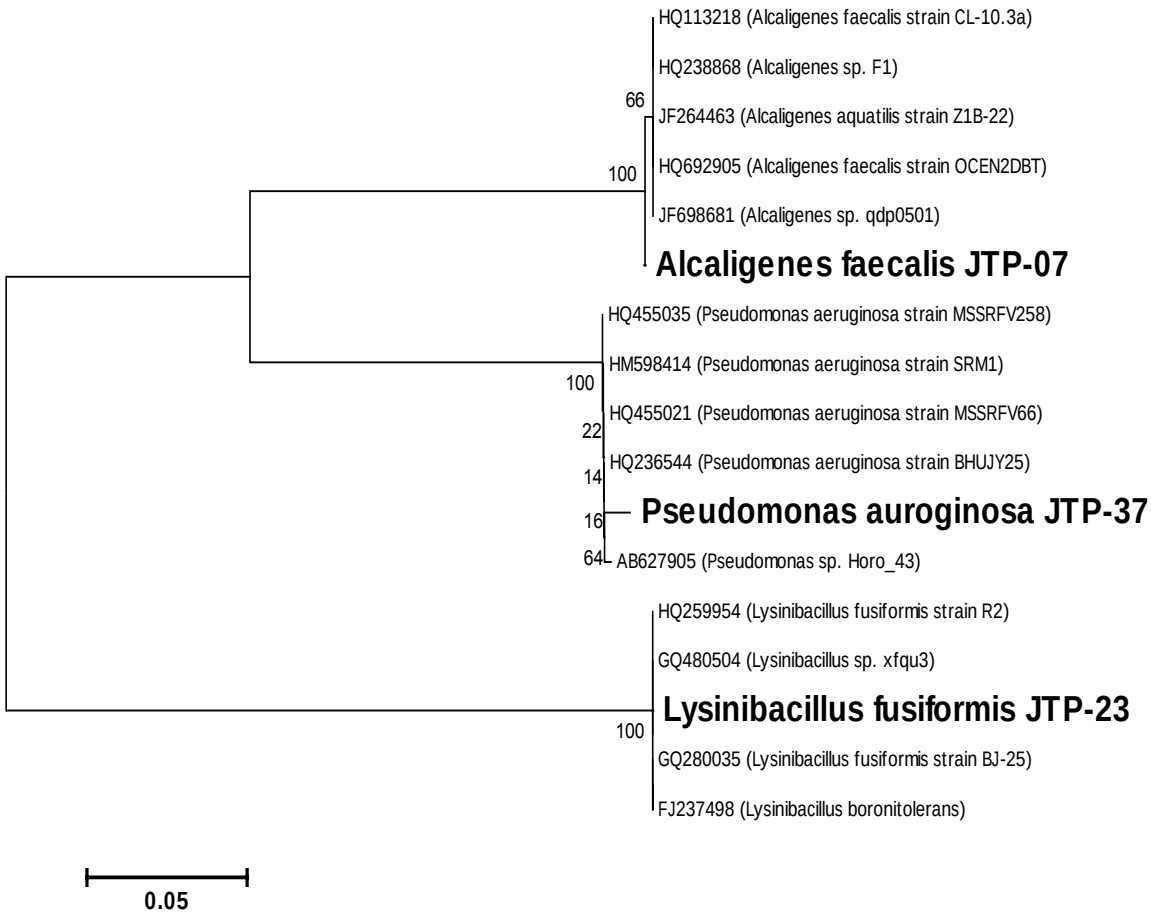

Figure 2.10: The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4. The scale bar represents 5% sequence divergence

Table: 2.7 Tentative identification of all 37 isolated bacteria on the basis of morphological, cultural, and biochemical test.

Isolate	Identification	Isolate	Identification
JTP-1	Pseudomonas sp.	JTP-20	Bacillus sp.
JTP-2	Acetobacter	JTP-21	Pseudomonas sp.
JTP-3	Bacillus sp.	JTP-22	Bacillus sp.
JTP-4	Bacillus sp.	JTP-23	Lysinibacillus sp.
JTP-5	Lysinibacillus sp.	JTP-24	Bacillus sp.
JTP-6	Bacillus sp.	JTP-25	Bacillus sp.
JTP-7	Alcaligenes sp.	JTP-26	Xenthomonas sp.
JTP-8	Escherichia sp.	JTP-27	Bacillus sp.
JTP-9	Bacillus sp.	JTP-28	Agrobacter sp.
JTP-10	Xenthomonas sp.	JTP-29	Lactobacillus sp.
JTP-11	Staphylococcus sp.	JTP-30	Lysinibacillus sp.
JTP-12	Actinomycetes sp.	JTP-31	Pseudomonassp.
JTP-13	Bacillus sp.	JTP-32	Lysinibacillus sp.
JTP-14	Enterobacter sp.	JTP-33	Azatobacter sp.
JTP-15	Azatobacter sp.	JTP-34	Bacillus sp.
JTP-16	Pseudomonas sp.	JTP-35	Escherichia sp.
JTP-17	Staphylococcus sp.	JTP-36	Pseudomonas sp.
JTP-18	Lysinibacillus sp.	JTP-37	Pseudomonas
JTP-19	Bacillus sp.		

CONCLUSION

Textile effluent and sludge produced by effluent treatment plant is rich source of dye decolorizing bacterial population. Total 37 isolates were selected on the basis of their Gram reaction, colony characteristics, growth curve pattern, and cell morphology. Samples contain both the types of organisms, Gm +ve and Gm –ve with dominated Gm +ve species. Initially, all 37 isolates were tested for their ability to decolorize two azo dyes, Reactive Black 5 and Green B. Then, six potential isolates were selected on the basis of their dye decolorizing ability and further tested with FF sky Blue, Black E, Black BT and Reactive Orange M_2R. Differences in structures and complexity of the dye led to variable percentage of decolorization of different dyes by the same organism.

4

Influence of environmental parameters on the process of dye decolorization by *Lysinibacillus fusiformis* JTP-23

INTRODUCTION

Dyes released from the textile processing and dyestuff industries result in the increase of organic load of the natural reservoirs (Dhanve *et al.*, 2009). The effluents from these industries are complex; contain a wide variety of dyes and other products such as dispersants, acids, bases, salts, detergents, humectants, oxidants etc. A very small amount of dye in water (10 to 50 mg l^{-1}) affects the aesthetic value, transparency, and gas solubility of water bodies (Banat and Nigam, 1996).

A lot of information is available on the removal and degradation of Remazol black B using pure strains of bacteria, fungi, algae and yeast including *Shewanella strain* J18143, *Rhizopus arrhizus*, *P. chrysosporium*, *Chlorella vulgaris*, *Candida tropicalis* and *Saccharomyces cerevisiae* (Akshu and Tezer, 2000; Swamy and Ramsay, 1999; Akshu and Donmez, 2005). Under aerobic conditions, most azo dyes are not degradable by bacteria. However, under anaerobic conditions, the azo linkage in the dye molecule can be reduced to form colorless aromatic amines which are occasionally toxic and carcinogenic (Zimmerman and Kulla, 1982). The diverse group of anaerobic bacteria contributes to the anaerobic decolorization of azo dyes via reduction of azo bonds to produce colorless

metabolites (kim *et al.*, 2007). Under anaerobic conditions, azo dyes act as terminal electron acceptors during microbial respirations (Ramalho *et al.*, 2004).

The pH has major effect on the efficiency of dye decolorization, and the optimal pH for color removal is often between 6 to 10 for most of the dyes. However, the decolorization rate decreases under extremely alkaline pH (>pH 12) and under acidic pH (<pH 6). Azo dyes inhibit nucleic acid biosynthesis and cell growth, so dye tolerance is also an important consideration for industrial applications (Chen et al., 1999). Kapil kumar *et al.*(2009) tested various initial concentrations of dye ranging from 25 to 300 ppm and found that percentage removal of dye decreased with an increase in dye concentration.

Various physical and chemical methods have been studied for degradation of Remazol Black B (Vinodgopal *et al.*, 1998). In biological process, to maintain purity of culture in large scale as well as their inability to degrade all different dyes present in the actual effluent are the major drawbacks for it application.

In the present study, attempt has been made for the degradation of an azo dye (Reactive Black 5) using a potential dye decolorizer *Lysinibacillus fusiformis* JTP-23, isolated from effluent contaminated site. Effect of various parameters, such as pH, temperature, initial dye concentration, static and shaking culture condition, size of reaction mixture, etc. have bee studied

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MATERIALS AND METHODS

Dyes and Chemicals

Reactive Black 5, an azo dye was procured from local manufacturer located at GIDC, Ahmedabad. All chemical used were of highest purity or analytical grade, obtained from recognized chemical suppliers. All other chemical for preparing various media were purchased from Hi-media Pvt. Ltd. Mumbai, and SRL India. Complete Medium Broth used for decolorization study consisting of (g l^{-1}); Peptone 5; Yeast extract 3; glucose 2; NaCl 5; K_2HPO_4 5; KH_2PO_4 1; $MgSO_4$. $7H_2O$ 0.1. The pH of the medium was adjusted to 7.0.

Preparation of dye solution

Reactive black 5 is acidic and soluble in water. A stock solution of 1000 ppm was prepared and autoclaved. Solution of the desired concentrations was obtained by successive dilution.

Determination of bacterial growth

(i) Biomass (dry weight, g/l): 2 ml of culture was taken in pre-weighed Eppendroff tubes and centrifuged at 10,000 rpm for 15 min to separate biomass. It was washed thrice, supernatant discarded and tube was allowed to dry at 60^{o}C.

(ii) Turbidometric analysis: Biomass was separated by centrifugation at 6000 rpm for 15 min. pallet was washed (3x), resuspended in equal volume DW and measured for OD at 660nm.

Effect of physico-chemical parameters on decolorization of azo dyes

Decolorization of azo dye by *Lysinibacillus fusiformis* strain JTP-23 was studied. The effect of initial dye concentration (100 – 1000 mg l^{-1}), Inoculums size (2, 5, 8, 10 and 15 % v/v), pH (4 – 11) and temperatures ^{o}C (10, 20, 30, 35, 40, 50) on decolorization process was studied.

A working volume of 100 ml CMB medium in 250 ml Erlen-meyer flask was employed through out the study unless otherwise stated. The culture was grown overnight in CMB to obtain optical density of 0.5 at λ_{660nm}. The reaction medium

was inoculated with 5% v/v in 100 ml of CMB containing 100 mg l^{-1} RB5 unless otherwise stated. The flasks were kept in incubator and orbital shaker respectively, for static and shaking conditions at 37^{o}C. In case of anoxic reactions, 33 ml CMB medium was filled in 35 ml glass serum tubes with air tight cap containing 100 mg l^{-1}. The pH of the medium was adjusted to 4, 5,6,7,8,9,10, and 11 with the help of sterile 1N HCl and 1N NaOH. In case of temperature effect on decolorization, the flasks were incubated at (^{o}C) 10, 20, 30, 35, 40 and 50 temperatures. For studying effect of initial dye concentration, dye aliquots were added in the medium so as to get final concentration of 100, 200, 300, 400, 500, 750, and 1000 mg l^{-1}. Overnight grown culture of *Lysinibacillus fusiformis* JTP-23 was used as an inoculum. Different flasks were inoculated with fixed volume in the range of 2, 5, 8, 10 and 15% v/v. Uninoculated controls were also included to check abiotic decolorization of azo dye. The effect of static, shaking, and anoxic condition on decolorization were examined by incubating flasks.

COD analysis

The culture was observed for the change in Chemical Oxygen Demand (COD) at different time interval during decolorization. COD was determined by standard procedures (APHA, 1992). To study the effect of aeration on COD reduction, the culture medium was subjected to sequential Static and Shaking culture condition. The experiment was carried out in triplicate.

Analytical method

At different time intervals, the samples were collected from reaction mixture and centrifuged at 6000 rpm for 15 min to separate biomass. The concentration of dye in the supernatant was determined by absorbance at 598nm. The absorbance was compared with standard curve plotted using different concentrations of the dye. The measurement of absorbance was made by Simadzu UV-1800 spectrophotometer.

RESULTS AND DISCUSSION

Effect of incubation conditions

As per Xu *et al.* 2006, 2007, the highest biomass was obtained in aerobic condition, while the fastest color removal was observed in anoxic condition. After 4 hrs incubation, 49 % dye was decolorized under anoxic condition while 3% and 23% decolorization was observed under aerobic and microaerophilic (static) cultural conditions respectively (Figure 4.1). In both microaerophilic and aerobic conditions, the presence of oxygen would normally inhibit the activity of decolorization, resulting in less efficiency of color removal capacity by the same organisms (Meiying and Guoping, 2007). Complete removal of dye was observed by the organism at 12 hrs and 16 hrs incubation under anoxic and static condition respectively, while in aerobic condition only 31% decolorization was observed at 24 hrs incubation. High dye removal capacity was observed even with less biomass that occurred in anoxic condition indicating the condition most favorable for dye decolorization (Figure 4.2).

Change in COD

Figure 4.3 illustrated decolorization of dye and reduction in COD during sequential static-shaking condition. The results showed that majority of the decolorization was obtained during static culture conditions, while the COD was reduced under shaking culture conditions. The initial COD load of the reaction flasks consisting of 100 mg l^{-1} dye was 2000 mg l^{-1}. After 24 hrs incubation under static culture condition 98% dye was decolorized without significant reduction in COD. After 24 hrs, the decolorized sample was subjected to shaking culture conditions and COD reduced to 1493, 1054, 428, 349 mg l^{-1} at 30, 36, 42, and 48 hrs, respectively. The COD of medium control was 337 mg l^{-1}. Thus, the COD load exhibited due to RB5 was completely removed during shaking flask culture.

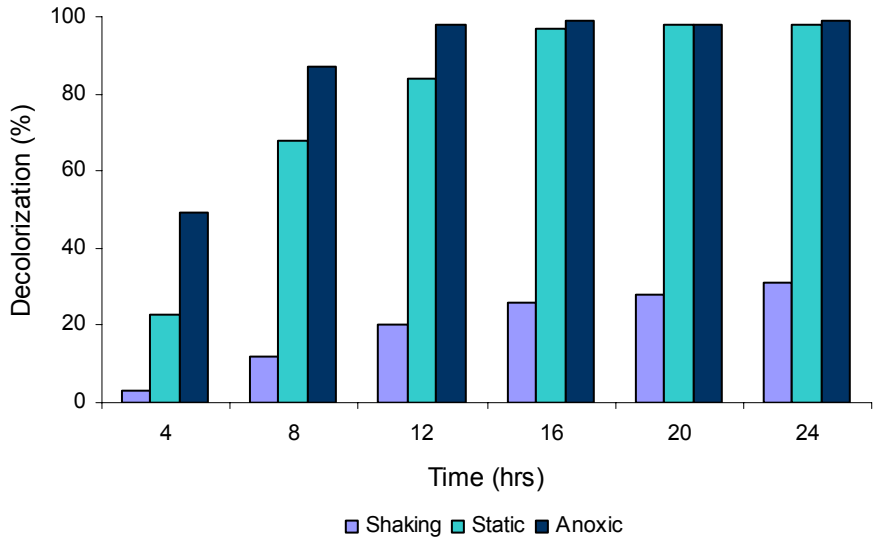


Figure 4.1: Effect of cultural conditions on the decolorization of Reactive Black 5 by *Lysinibacillus fusiformis* strain JTP-23

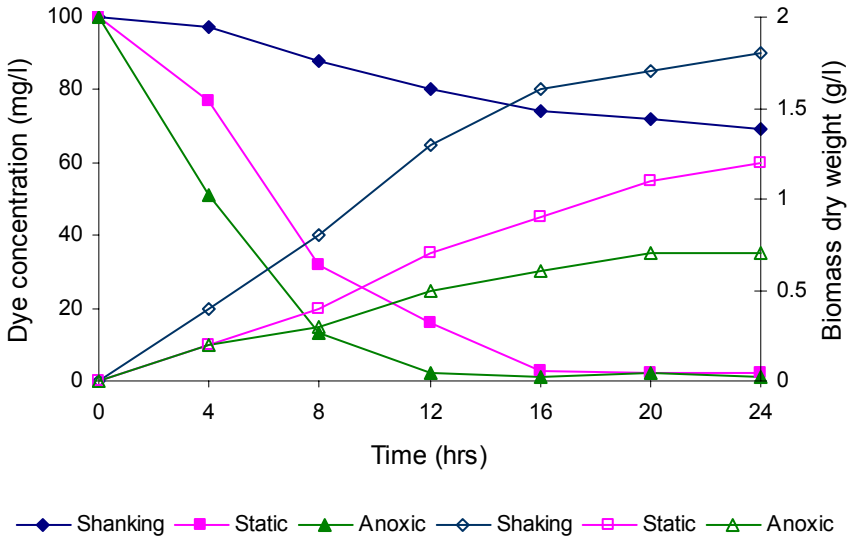


Figure 4.2: Comparison of shaking, static and anoxic conditions on the cell growth and dye removal capacity of *Lysinibacillus fusiformis* strain JTP-23. Open symbols indicates biomass and closed ones dye concentration.

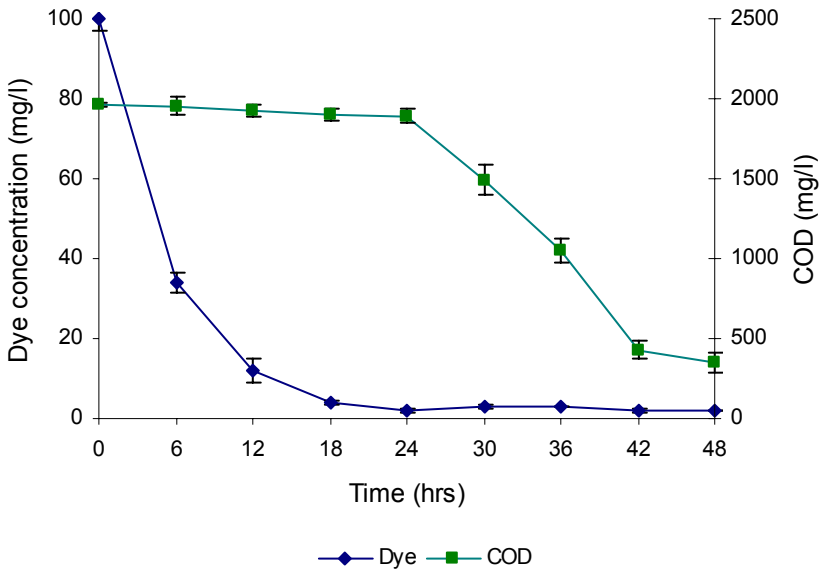


Figure 4.3: Dye decolorization and corresponding COD removal in static (upto 24 hrs) followed by shaking condition by *Lysinibacillus fusiformis* strain JTP-23

Effect of dye concentration

In order to study the effect of initial concentration of Reactive black 5, the experiments were carried out at a fixed biomass (5% inoculums v/v) at different dye concentrations (100, 200, 300, 400, 500, 750 and 1000 mg l^{-1}) for different time intervals (12 , 24, 36 , and 48 hrs) at 35^{o}C under static culture condition. Figure 4.4 demonstrate the effects of initial dye concentration on decolorization. The decolorization at the lowest (100 mg l^{-1}) and highest dye concentration (1000 mg l^{-1}) was 99% and 57%, respectively.

The higher concentration of azo dye inhibits nucleic acid biosynthesis and cell growth (Chen *et al.*, 2003), so the effect of dye concentration on growth of organisms is an important consideration for its field application. The dye concentration in effluent from textile printing house is approximately 200 mg l^{-1}. This value is typical of those used in studies on treatment for azo dye containing effluent (Zhao and Hardin, 2007). At concentrations up to 200 mg l^{-1}, up to 85% decolorization of azo dye within 12 hrs of incubation was observed. On extended incubation, up to 48 hrs, 97% decolorization was observed. In case of 300, 400,

500, 750 and 1000 mg $l^{-}1$ dye concentrations, the decolorization achieved was 87, 85, 82, 78 and 57%, respectively. Khehra *et al.* (2005) suggested that the decrease in decolorization efficiency might be due to the toxic effect of dyes. Initial concentration provides an important driving force to overcome all mass transfer resistance of the dye between the aqueous and solid phases (Parshetti *et al.*, 2006)

Effect of pH

The pH tolerance is an important consideration for industrial applications because processes using reactive azo dyes are usually performed under alkaline conditions (Aksu and Tezer, 2005). The experiment was performed in 150 ml Erlen-meyer flasks containing 50 ml CMB medium. It was observed that the percentage of dye decolorization varied with change in pH of the medium (Figure 4.5). Although decolorization rate peaked around pH 9 at 6 hrs, the organism decolorized more than 85% of the dye by incubation up to 24 hrs on wide range of pH (6-10). However, organism showed very poor decolorization at the pH 4 and 5 with 1% and 22% level, respectively (Figure 4.6). No growth was observed at pH 4 and only limited growth was observed at pH 5. These observations indicated that the organism can treat basic dyeing waste water at normal operational pH and decrease the cost of acidification (Ayed *et al.,* 2009). Figure 4.7 depicted the effect of various pH on decolorization process under anoxic condition. Under static condition *Lysinibacillus fusiformis* JTP-23 showed reduced decolorization of 87 and 48% at pH 10 and pH 11, respectively; while under anoxic condition the organism showed increased decolorization of 100% and 93% at pH 10 and pH11. At lower pH values, the H^{+} ions compete effectively with dye cations, causing a decrease in color removal efficiency. Furthermore, at high pH, the surface of biomass gets negatively charged, which enhance the positively charged dye cations through electrostatic force of attraction (Lamia *et al*, 2009).

Effect of temperatures

The mesophilic range is traditionally used since it is generally thought that maintaining high temperature would be uneconomical, while dye degradation within the psychrophilic range is too slow (Varel *et al.*, 1980). In order to determine the optimum temperature, decolorization assay were performed over 10 - 50^{o}C temperature range. Figure 4.8 shows that the decolorization rate increases with increase in temperature from 10^{o}C to 35^{o}C. At 40^{o}C there was not much adverse effect on the decolorization. At 50^{o}C, the decolorization ability was sharply reduced to 24% (Figure 4.9). Biomass also increased with increase in temperature from 10^{o}C to 35^{o}C. At 10^{o}C and 50^{o}C the percentage decolorization was almost similar but biomass was nearly double at 50^{o}C as compared to 10^{o}C. The biomass at 20^{o}C and 50^{o}C were 0.5 and 0.6 g l^{-1}, while decolorization was 66% and 24%. This might have occurred due to adverse effect of high temperature on the enzymatic activities (Cetin and Donmez, 2006).

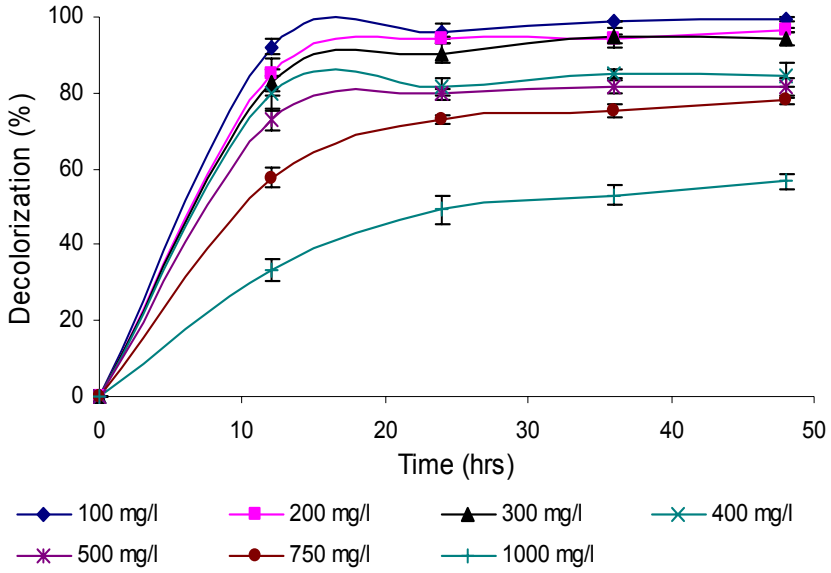


Figure 4.4: Effect of different concentration of RB 5 on process of decolorization by *Lysinibacillus fusiformis* JTP-23

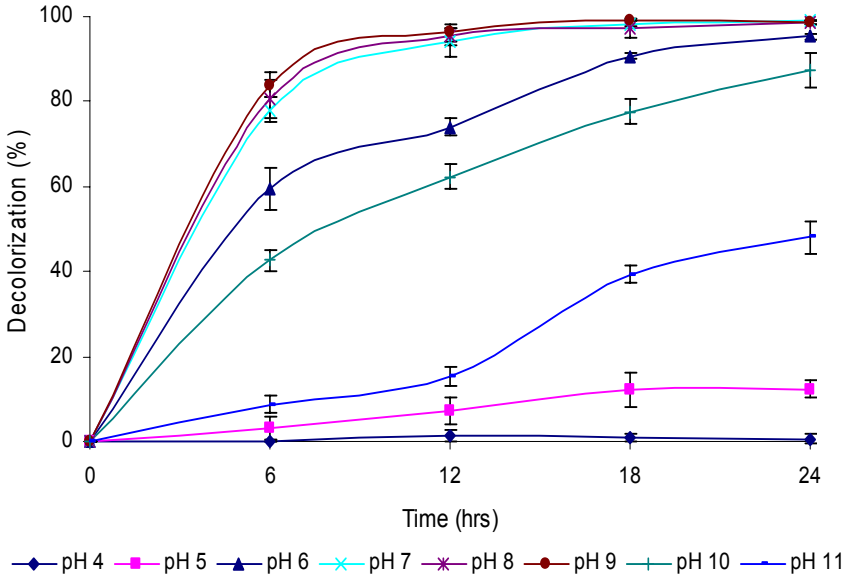


Figure 4.5: Effect of various pH on decolorization of RB 5 by *Lysinibacillus fusiformis* JTP-23 under static culture condition.

Figure 4.6: Effect of different pH on decolorization of RB 5 by *Lysinibacillus fusiformis* JTP-23 under static culture condition

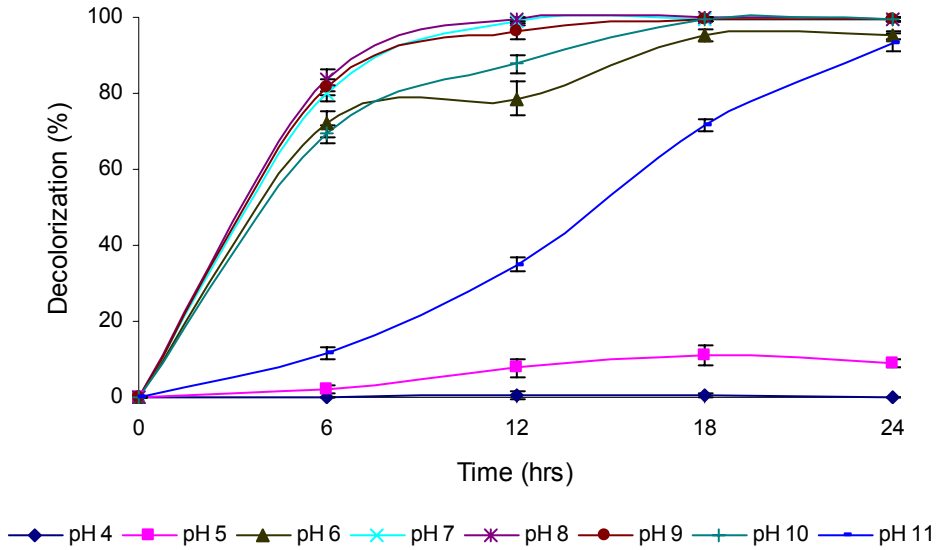


Figure 4.7: Effect of different pH on decolorization of RB 5 by *Lysinibacillus fusiformis* JTP-23 under Anoxic culture condition.

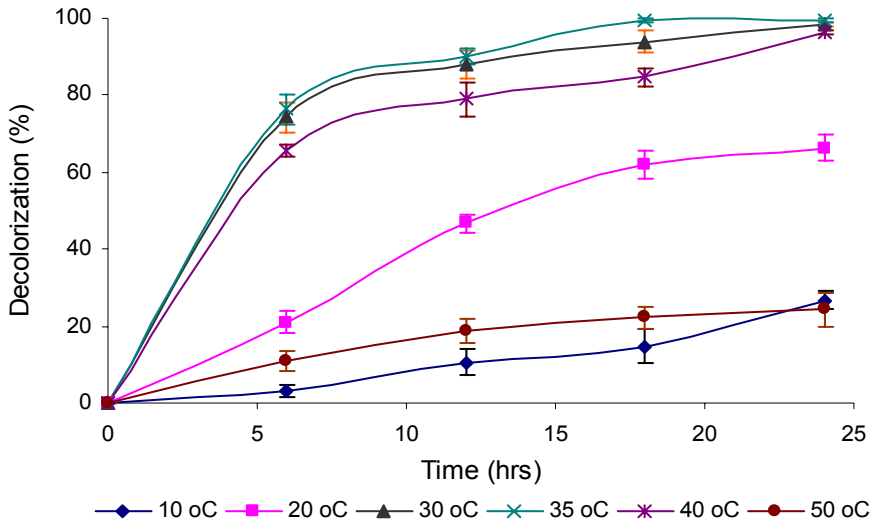


Figure 4.8: Effect of temperatures on decolorization of RB 5 by *Lysinibacillus fusiformis* JTP-23 under static culture condition.

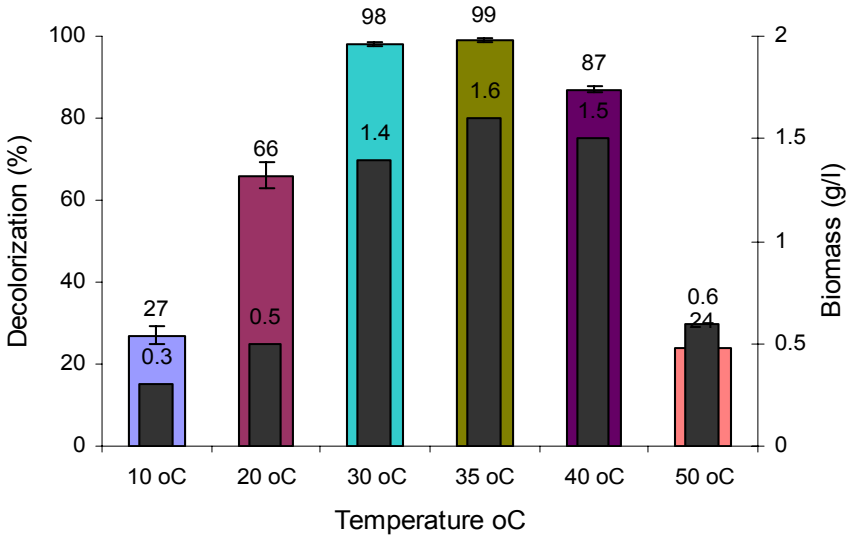


Figure 4.9: Effect of temperatures on biomass and decolorization by *Lysinibacillus fusiformis* JTP-23. Multicolor bars - Decolorization, Black bars – Biomass.

Effect of initial bacterial biomass

To estimate the optimum initial biomass of *Lysinibacillus fusiformis* strain JTP-23, for 100 mg l^{-1} dye concentration, different inoculum sizes were applied. The results indicated that, under static cultural condition, the dye removal capacity remained unaffected by any of the inoculum size used. The rate of decolorization was almost similar with any size of the inoculum used. Similar pattern was observed by Sani and Banerjee (1999) who reported that there was no proportionate increase in decolorization with increase in inoculum size of *Kurthia* sp. for the treatment of the textile effluent. Under anoxic conditions different inoculum gives different rate of decolorization of RB 5 (Figure 4.10 and 4.11). When inoculum size was 2%, decolorization was 81% and 99% at 12 hrs and 24 hrs, respectively. In case of 15% inoculum, decolorization was 44% and 70% at 12 hrs and 24hrs, respectively. In anoxic condition it was observed that as the inoculum was increased, there was decrease in decolorization rate. So, highest decolorization was achieved with the lowest inoculum size. This observation was contradictory to a report where dye removal capacity increased significantly with the increase in initial biomass (Lamia *et al.*, 2009, Kothari, 2006).

Effect of aeration and volume of reaction mixture

The decolorization of Reactive Black 5 in static and shaking culture condition in combination with different volume of media (25ml, 50ml, 75 ml, 100ml) was studied. Data shown in Table 4.1 proves that lower decolourization was exhibited in shacked culture compared to static culture conditions. In all volumes studied, it was observed that highest decolorization was achieved with static condition. In case of 50, 75, and 100 ml, 95% or more decolorization was achieved in 12h incubation in static condition. For 50 rpm in shake flask culture, 20%, 24%, 25%, and 25% decolorization was observed for 25, 50, 75, and 100 ml volumes, respectively. Similar results were observed for 100 rpm shake flask culture. Figure 4.12, suggests that decolorization performance of *Lysinibacillus fusiformis* JTP-23 was better in low oxygen content. This could be due to competition of abundant oxygen and the azo compounds for the reduced electron carriers under aerobic conditions (Mona and Honda, 2008). It is reported by other authors that to achieve an effective color removal agitation and vigorous aeration should be avoided (Chen and Huang, 1999; Khehra and Saini, 2005).

Table 4.1: Effect of different reaction volume and cultural condition of RB5 decolorization by *Lysinibacillus fusiformis* JTP-23. (dye concentration 100 mg l^{-1})

Volume (ml)	Cultivation condition		Decolorization (%)					
			4h	8h	12h	16h	20h	24h
25	ST		42	68	85	90	96	98
	SH	50 rpm	6	9	12	15	19	20
	SH	100 rpm	6	8	10	14	18	22
50	ST		46	79	95	95	97	99
	SH	50 rpm	5	8	11	14	22	24
	SH	100 rpm	5	9	11	16	19	25
75	ST		49	78	95	98	98	100
	SH	50 rpm	8	8	13	16	24	25
	SH	100 rpm	8	10	13	17	22	27
100	ST		52	83	97	99	100	99
	SH	50 rpm	7	10	12	14	26	25
	SH	100 rpm	7	11	13	18	25	35

ST, Static condition; SH, Shaking condition

Figure 4.12: Effect of reaction volume and cultural condition on decolorization of RB5 by *Lysinibacillus fusiformis* JTP-23. **a**. static condition, **b**. shaking condition.

Decolorization of Black E

Lysinibacillus fusiformis JTP-23 was tested for its ability to decolorize different dyes as mentioned ahead. The organism was added in the flask containing 100 ml of CMB medium and screw cap tube containing 33 ml CMB medium with final concentration of 100 mg l^{-1} Black E dye. After 5 hrs incubation, the floccules of dye were observed, and the color of the supernatant turned lighter. As the incubation time extended, more floccules were formed and precipitated at the bottom of the container (Figure 4.13).

Figure 4.13: Decolorization of Black E by *Lysinibacillus fusiformis* JTP-23. Dye solution (flocculation) after 5 hrs incubation.

CONCLUSION

Various aspects of environmental and cultural parameter were studied for decolorization of Reactive Black 5 by *Lysinibacillus fusiformis* JTP-23. The results indicated that the new species had high capacity for rapid decolorization of azo dyes. *Lysinibacillus fusiformis* JTP-23 being aerobic organisms produces abundant biomass under shaking condition, but decolorization capacity was sharply reduced under this condition. Anoxic condition produced less biomass with fastest decolorization process. Maximum decolorization was observed in static condition, while COD was decreased in subsequent shaking condition. Further, it observed that with increasing dye concentration, efficiency of decolorization decreased.

The suitable initial pH for decolorization of RB 5 was in the range of 6 to 9 under static condition and 6 to 10 under anoxic condition. Optimum temperature for decolorization process was 30-35^{o}C. Results on the effect of size of inoculum indicated that under anoxic condition smallest of size 2% v/v was ideal for rapid decolorization of RB 5 dye.

Textile industries use wide variety of structurally different dyes, so effluents are extremely variable in dye composition. Therefore, treatment of such effluent and decolorization of dye is difficult (Kothari, 2006). Wide range of pH, salt concentrations and heterogeneous hazardous chemical substances often add to the difficulties in its treatment.

The newly isolated organism *Lysinibacillus fusiformis* JTP-23 decolorize dye very efficiently in different cultural and environmental condition. Therefore, this culture can be used on large scale application for the treatment of textile effluent.

5

Influence of Nutritional Parameters on Dye Decolorization by *Lysinibacillus fusiformis* JTP-23

INTRODUCTION

The textile industry is major user or water, starting from washing raw wool or manmade fiber production up to garment manufacturing. Waste water may be defined as utilized water that is loaded with many dissolved and suspended waste from residential, commercial, agricultural and industrial establishments. Sometimes wastewater contains numerous pathogenic organisms including organisms from human origin. Numerous organic materials with nutritive value can stimulate growth of undesired aquatic organisms. Therefore, effective and economical waste water treatment facility is required to overcome health and environmental hazardous problems (Kothari *et al.*, 2006; Banat et al., 1996; Brown and Laboureur, 1983; Brown and Vito, 1993).

Treatment of effluents containing dye has been conventionally carried out by physical or chemical methods, but these methods are of limited applicability. The treatment system based on microorganisms capable of decolorizing/degrading these recalcitrant compounds are environmental friendly and can lead to mineralization of target compound (Sharma and Saini, 2004).

Bacterial decolorization of azo dyes under aerobic conditions usually results in adsorption of dyestuffs on bacteria, rather than oxidation (Pagga and Brown, 1986). White rot fungi can decolorize and degrade wide variety of azo dyes with the help of extracellular degradative enzymes (Glenn and Gold, 1983). It is

difficult to keep bacteria in functional form in activated sludge system, because of their special nutritional requirement and environment conditions (Kapdan and Kargi, 2000).

In last few years, several organisms have been found to decolorize and degrade azo dyes up to mineralization level. The bacterial metabolism of azo dyes is initiated by reductive cleavage of azo bond, resulting in the formation of amines. These reductive processes have been studied in some aerobic bacteria, which grow on azo compounds. It is effective method to add some specific substrate to induce bacterial decolorization of azo dyes. Dong *et al.,* (2003) found that decolorization of Reactive brilliant blue by *Rhodocyclus gelatinosus* XL-1 was greatly enhanced with increase in peptone concentration in reaction medium. It has been observe that increasing yeast extract concentration resulted in higher decolorization rates (Chen *et al.*, 2003). Glucose has been added to enhance the decolorization of various dyes in some studies (Haug *et al.*, 1991; Kapdan *et al.*)

Reactive dyes are highly soluble in water due to high degree of sulfonation and non-degradable under typical aerobic condition found in conventional biological treatment system. Lot of information about the transformation of azo compound is available, while relatively limited is known about the transformation of complex reactive textile azo dyes, despite their applications being increased due to the worldwide increase in cotton use.

In the present study, the decolorization of textiles azo dyes was studied in presence of various nutrients, such as carbon sources and nitrogen sources. Various concentrations of salt and glucose were examined for their effect on decolorization. Different organic and inorganic nutritional factors have remarkable effect on decolorization of dye. Plackett-Burman experimental design was applied as a tool for optimizing bacterial decolorization process and to test the relative importance of various organic and inorganic variables.

MATERIALS AND METHODS

Dyes and Chemicals

Reactive Black 5, an azo dye was procured from local manufacturer located at GIDC, Ahmedabad. All chemical used were of highest purity or analytical grade, obtained from recognized chemical suppliers. All other chemical for preparing various media were purchased from Hi-media Pvt. Ltd. Mumbai, and SRL India. Complete Medium Broth used for cultivation of selected bacteria and decolorization study consisting of (g l^{-1}); Peptone, 5; Yeast extract, 3; glucose, 2; NaCl, 5; K_2HPO_4, 5; KH_2PO_4, 1; $MgSO_4$. $7H_2O$, 0.1. The pH of the medium was adjusted to 7.0.

The composition of Mineral Salt Medium was as per chapter 2 and preparation of dye solution as described in chapter 4.

Determination of bacterial growth

(i) Viable count: Standard method of viable count was performed to quantify number of cell to be used as inoculum of the reaction mixture.

(ii) Turbidometric analysis: Biomass was separated by centrifugation at 6000 rpm for 15 min and the pallet was washed with sterile normal saline (3x), resuspended in equal volume of DW followed by the measurement of absorbance at 660nm.

Effect of Carbon and Nitrogen sources on Decolorization

Mineral Salt Medium was used along with 200 mg l^{-1} yeast extract and 100 mg l^{-1} RB5 for studying effect of various carbon and nitrogen sources of the process of dye decolorization. Carbon sources like lactose, tryptone, starch, glucose, dextrin, sucrose and maltose were sterilized (10 psi 10 min) separately and added in the reaction mixture with final concentration of 1%. The medium was inoculated with 2% v/v 18 hrs old culture containing approximately 1.3×10^{10} cells ml^{-1}. In case of nitrogen sources, $(NH_4)_2SO_4$ was replaced by peptone, yeast extract, meat extract, ammonium nitrate, sodium nitrate, pep. nitrate, ammonium sulfate with 1% concentration in final reaction mixture. Experiments

were carried out in flask containing 100 ml medium and incubated in static conditions at 35^{o}C.

Effect of Salt and Glucose concentrations on decolorization

Effect of different salt concentrations on the growth of the organisms and decolorization was studied by using different sodium chloride concentrations: 0.2, 1, 2, 3, 4, 5, 7.5 and 10% in complete medium broth. The change in color of the mediumand growth of bacterial biomass was observed at 598 nm and 620 nm, respectively at different time interval. Study of such effect has been also reported earlier (Salah *et al.,* 2007; Dhanve and Kalyani, 2009). Anoxic cultural condition was also tested by using 35 ml screw cap glass serum tubes.

Most of the organisms prefer glucose as a source of carbon. Various concentrations of glucose: 0.2, 0.5, 1, 2 and 3% were tested for its effect on the process of RB5 decolorization by *Lysinibacillus fusiformis* JTP-23 under both static and anoxic conditions.

Influence of $HgCl_2$ and preadaptation on decolorization

250-ml EM flasks containing 100 ml CMB devoid of $MgSO_4$ were autoclaved (121^{o}C, 20 min). Appropriate amount of sterile stock solution of $HgCl_2$ was added to get 0 to 10 mg l^{-1} concentration in the reaction mixture with Reactive Black 5 (100 mg l^{-1}). These flasks were inoculated with 2% inoculum (*ca*. 1.3 ×10^{10} cells.ml^{-1}) and incubated at 37^{o}C. Inoculated and un-inoculated controls were also included. Samples were harvested at specified time intervals and analyzed.

Reaction mixture flasks were inoculated with equal volume of cultures grown in presence and absence of dye in two different flasks to check the effect of preadaptation of the organisms to dye for its decolorization.

(i) Pre-adapted cells preparation: Cells were harvested after 12 h incubation, in CMB medium supplemented with RB 5 (100 mg l^{-1}), by centrifugation and resuspending them in sterile normal saline.

(ii) Non-adapted cells preparation: Cells were harvested after 12 h incubation, in CMB medium without any dye, by centrifugation and resuspending in sterile normal saline.

Media optimization

In order to approach a near optimal response region of the medium ingredients, a fractional factorial Plackett - Burman design was applied (Mona *et al.*, 2008). In this experiment, seven independent variables were screened in eight combinations organized as per Plackett – Burman matrix. All variables were tested in two concentrations, high level (+) and low level (-). Trials were performed in duplicate and results were treated as the responses. The main effect of each variable on decolorization was determined using the equation:

$$E_{xi} = (\Sigma M_{+1} - \Sigma M_{-1}) / N$$

Where, E_{xi} = Variable main effect
M_{+1} = Calculated decolorization percentage recorded by trail which contains high level (+).
M_{-1} = Calculated decolorization percentage recorded by trail which contains low level (-).
N = Number of trails divided by 2.

A main effect figure with a positive sign indicted that the high concentration of this variable is close to its optimum concentration in media, while a negative sign indicated that the low concentration of this variable is near to the optimum concentration.

Analytical method

At different time intervals, the samples were collected from reaction mixture and centrifuged at 6000 rpm for 15 min to separate biomass. The concentration of dye in the supernatant was determined by reading absorbance at 598 nm by Simadzu UV-1800 UV-visible spectrophotometer. To monitor adsorption of dyes, the cell pellets after centrifugation were resuspended in an equal volume of methanol to extract the dye. The suspension was mixed thoroughly and centrifuged at for 15 min at 10000 rpm. The supernatant was again examined at λ_{max} of respective dye (Khera *et al*., 2005). Uninoculated control flasks as control were kept to record dye removal if any.

RESULT AND DISCUSSION

Effect of Carbon and Nitrogen sources on decolorization of RB5

An attempt was made to test decolorization of RB5 by *Lysinibacillus fusiformis* JTP-23 under influence of various carbon and nitrogen sources. The importance of carbon source to achieve successful decolorization could be inferred from Figure 5.1a and 5.1b. The organism was able to grow on all tested carbon sources but its decolorization activity was observed to be influenced by the type of Carbon sources. Decolorization activity at 12 h under static condition was in the range of 19-24 % for starch, sucrose and maltose, while in the range of 52-72% for lactose, tryptone, glucose and dextrin. At the end of 36 h incubation, more than 90% decolorization was achieved in all tested carbon sources except starch and sucrose that could decolorize 55% and 63%, respectively. Extended incubation up to 48 h resulted in complete removal of dye in lactose, tryptone, glucose, dextrin and maltose.

The potential of *Lysinibacillus fusiformis* JTP-23 to decolorize RB5 dye with glucose as carbon source and various nitrogen sources is depicted in Figure 5.2a and 5.2b. Organism growing on these media decolorized 38-86% dye in the first 12h in the presence of all nitrogen sources. Peptone was the best nitrogen source that yielded highest, 86%, decolorization at 12 h, while Yeast extract and meat extract were better nitrogen sources with around 75% decolorization. Complete removal of dye was observed in all tested nitrogen source at 48 h except ammonium nitrate.

Effect of salt and glucose concentrations on decolorization of RB5

Satisfactory growth and decolorization was observed upto maximum of 5% salt concentration in CMB medium under static condition (Table 5.1). At salt concentrations higher than 5%, there was sharp reduction in biomass and decolorization capacity of *Lysinibacillus fusiformis* JTP-23 (Figure 5.3a, 5.3b). Halophilic bacteria have also been reported to decolorize azo dye under high salt conditions (Guo *et al.*, 2007). Anoxic cultural condition results were quite similar

to static condition (data not shown). Exposure of microorganism to high salt concentration may cause plasmolysis and/or loss of activity of cells.

Earlier reports on decolorization on dye decolorization indicated the necessity of glucose to enhance the decolorization process (Swamy and Ramsay, 1999; Kapdan and Kargi, 2000). Figure 5.4 illustrates the results of glucose concentration on decolorization under static condition. The reaction mixture without glucose achieved only 45% decolorization as compared to 0.2 and 0.5% glucose concentrations, with nearly 100% decolorization under static condition, indicating glucose being unavoidable ingredient for the effective treatment of dye effluent. Similar results were reported by Mona et al., 2008, where removal of glucose from the basal medium inhibited the decolorization performance of *Bacillus subtilis* HM by almost 55% compared to control. As glucose increased to 1% and above, the decolorization efficiency was reduced to less than 20%.

Decolorization process was enhanced at all glucose concentrations under anoxic conditions. Under this condition, reaction without glucose gave increased decolorization upto 90% as compare to 45% under static condition. 2% and 3% glucose also give good decolorization of 75% and 87%. Methanol extract of centrifuged biomass was colorless indicating no adsorption or absorption occurred and color removal was due to decolorization of dye.

Influence of $HgCl_2$ and Pre-adaptation of cells on decolorization

Microbial enzymes can be permanently inactivated by $HgCl_2$ (Chen *et al.*, 1999). The purpose for this experiment was to study the effect of various concentrations of $HgCl_2$ on the process of Reactive Black 5 decolorization by *Lysinibacillus fusiformis* JTP-23. Decolorization activity was decreased with increased in $HgCl_2$ concentration (Figure 5.5). The dye was completely decolorized in $HgCl_2$ concentration 0, 1, 2 and 3 mg l^{-1} in 18, 36, 75 and 125 hrs, respectively. Concentration of $HgCl_2$ higher than 4 mg l^{-1} did not give considerable decolorization even on extended incubation up to 125 hrs (Figure 5.6).

The properties of enzyme that mediates dye decolorization are diverse (Zimmermann *et al.*, 1982; Ramalho *et al.*, 2002). This study aimed at evidences regarding the constitutive or inducible nature of the enzyme involved in decolorization. The decolorization rates by cells pre-adapted to the dye were compared with those cells grown in medium without a dye, but otherwise of identical composition. The results in figure 5.7 clarify indicated that decolorization of RB 5 was mediated by the constitutive enzymes and there was no significant difference in decolorization pattern with pre-adapted and non-adapted cells of *Lysinibacillus fusiformis* JTP-23.

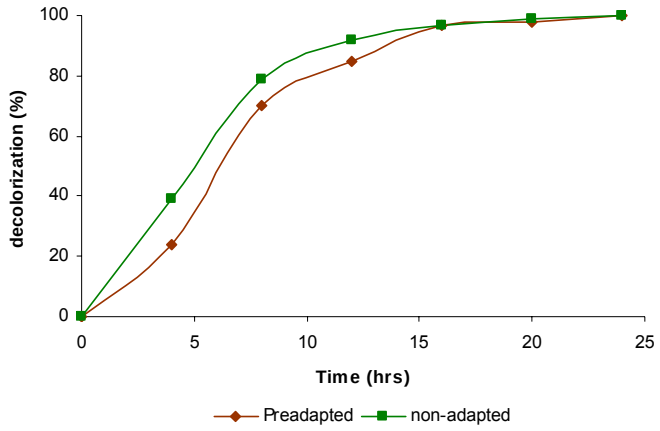


Figure 5.7: Effect of pre-adapted cells on the process of RB5 decolorization by *Lysinibacillus fusiformis* JTP-23 under static culture condition

Optimization of media for RB5 decolorization by Plackett – Burman design

In order to determine the effect of nutritional factors on dye decolorization and to overcome their negative interactions, a statistical design was carried out. Plackett – Burman design (Plackett and Burman, 1946) was constructed to determine the nutritional requirements for color removal (Table 5.2). Reactive black 5 concentration was treated as a constant factor (100 mg l^{-1}), that is in the range of dye concentration usually found in dye house effluent (Nigam *et al.*, 2000).

The main effect of variable was calculated according to RB5 decolorization by *Lysinibacillus fusiformis* JTP-23 under anoxic and static cultural conditions (Figure 5.3). Increasing concentrations of peptone, KH_2PO_4, $MgSO_4$ and NaCl had positive effect on RB5 decolorization under anoxic condition, while, glucose, K_2HPO_4 and yeast extract had negative effect. Glucose and K_2HPO_4 had positive effect on decolorization under static culture condition. This trend was in agreement with other researchers (Mona *et al.*, 2008; Radha *et al.*, 2005).

Table 5.2: Plackett - Burman design for high level and low level of variable in different trials

Trial	G	P	K2	K	Mg	Na	YE
1	+	+	+	-	+	-	-
2	+	+	-	+	-	-	+
3	+	-	+	-	-	+	+
4	-	+	-	-	+	+	+
5	+	-	-	+	+	+	-
6	-	-	+	+	+	-	+
7	-	+	+	+	-	+	-
8	-	-	-	-	-	-	-

According to data obtained, it can be predicted that the near optimal medium for RB5 decolorization by *Lysinibacillus fusiformis* JTP-23 can be formulated by taking higher concentrations of the variables that show positive effect and lower concentrations of variable that shows negative effects on dye decolorization process (Figure 5.8). Thus, optimized media for anoxic culture condition could be as follow, (g l^{-1}): glucose, ; peptone, ; K_2HPO_4, ; $MgSO_4$, ; KH_2PO_4, ; NaCl, ; and

yeast extract, . Optimized media for static culture condition could be formulated as follow, (g l^{-1}): glucose, ; peptone, ; K_2HPO_4, ; $MgSO_4$, ; KH_2PO_4, ; NaCl, ; and yeast extract.

Table 5.3: Assigned concentrations of variable at different levels of the Plackett-Burman design for seven factors.

Variable	Symbol	Level (g l^{-1})			Main Effect	
		Low (-)	(regular)	High (+)	Anoxic culture	Static culture
Glucose	G	1	2	3	- 4.75	9.00
Peptone	P	2	5	8	18.75	12.5
K_2HPO_4	K2	1	3	5	- 5.25	3.00
KH_2PO_4	K	0.5	1.0	1.5	5.25	- 3.50
$MgSO_4.7H_2O$	Mg	0.05	0.1	0.15	1.25	3.50
NaCl	Na	2	5	8	5.25	8.25
Yeast Extract	YE	1	3	5	- 3.25	- 4.00

A verification experiments was performed to evaluate the unoptimized (CMB) versus the optimized medium. The data obtained revealed that the decolorization process was fast and almost 100 % decolorization achieved in 8 hrs incubation for optimized media, instead of 12 hrs incubation in unoptimized media under anoxic condition. Similar results were observed in static culture condition with 4 hrs reduced time required to achieve complete decolorization as compared to unoptimized media.

CONCLUSION

Various nutritional parameters were examined for their effect on decolorization of RB5 by *Lysinibacillus fusiformis* JTP-23. Analysis of carbon sources showed that lactose, tryptone and glucose were most suitable. Organic nitrogen sources in the form of peptone, yeast extract and meat extracts were most appropriate as compared to other inorganic nitrogen sources.

Decolorization of dye was almost unaffected under different salt concentrations. Salt at higher than 5% (w/v) had negative effect on the biomass synthesis and there was sharp reduction in the decolorization. It was observed that glucose was unavoidable in small amount for better decolorization. However, the increased glucose concentrations retarded the decolorization process.

Metals are commonly found in waste water from dying industry. At high concentrations of $HgCl_2$, microbial enzymes are inactivated (Chen *et al.*, 1999). It was observed that increased concentration of metal reduced decolorization efficiency. *Lysinibacillus fusiformis* JTP-23 did not display any advantage of previous exposure to the dye for decolorization, indicating the constitutive nature of the enzymes involved in the process.

Analysis of Plackett-Burman statistical design for the determination of optimum nutritional requirement for the decolorization revealed that peptone favored decolorization. Glucose and K_2HPO_4 exhibited negative effect on the decolorization under anoxic condition, but both showed positive main effect under the static culture condition.

There are few reports on the biological decolorization and degradation of textile and dyestuff industrial wastes containing azo dyes. The reports so far available are on a small scale and no large scale data on biological decolorization and degradation is available (Kothari, 2006). Biological treatment is the only way for ultimately controlling pollution generate by textile and dyes stuff industries. However, further research would be required to develop a viable alternative process for the treatment of dye wastewaters.

Our isolate *Lysinibacillus fusiformis* JTP-23 decolorized Reactive Black 5 and other textile dyes optimally under employed nutritional parameters: sources of carbon and nitrogen, glucose and salt concentrations and presence of heavy metals. Thus, the isolate displayed potential ability to decolorize various textile dyes under varied cultivation conditions.

6

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

INTRODUCTION

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

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

Decolorization of azo dyes normally begins with initial reduction cleavage of azo bond anaerobically, which results in colorless but toxic aromatic amines. This is

followed by complete degradation of aromatic amines strictly under aerobic conditions (Kothari, 2006). Therefore, anaerobic/aerobic processes are crucial for complete mineralization of azo dyes. However, not all bacteria have both anaerobic and aerobic properties. Usually consortia are routinely used for the degradation of azo dyes.

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

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

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

MATERIALS AND METHOD

Dyes

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

Decolorization of mixed azo dyes

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

Development of bacterial consortium

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

Dye decolorization experiments

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

Decolorization of industrial effluents

Industrial effluent containing a mixture of various textile dyes was obtained from a drainage line taking effluents to Common Effluent Treatment Plant in Jetpur (Figure1.5). The effluent was centrifuged at 12,000 x g for 10 min to remove insoluble materials (Joe *et al.,* 2008). This supernatant was used in place of water for preparation of CMB. After adjusting to pH 8 with 1 N HCl, the medium was sterilized by passing through 0.45 um membrane filter. 250 ml EM flask containing 100 ml medium was inoculated with 5% overnight grown culture of *Lysinibacillus fusiformis* JTP-23 and incubated at 30^{o}C under static – shaking sequential culture condition.

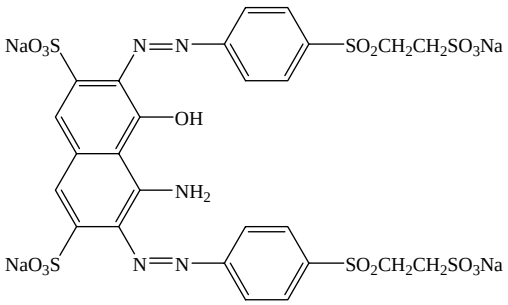
Analysis of decolorization

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

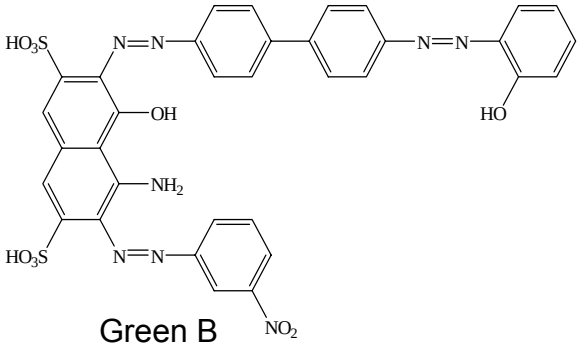
Phytotoxicity study

The effect of original dye and its degradative metabolite on germination and early seedling growth of two plants; *Triticum aestivum* and *Phaseolus mungo* was evaluated. The degradation metabolites of Remazol Black 5, Green B, and Black BT were extracted in ethyl acetate were dried and dissolved in water to form the final concentration of 1000 ppm. The dye solutions were also prepared with concentration of 1000 ppm for phytotoxicity studies.

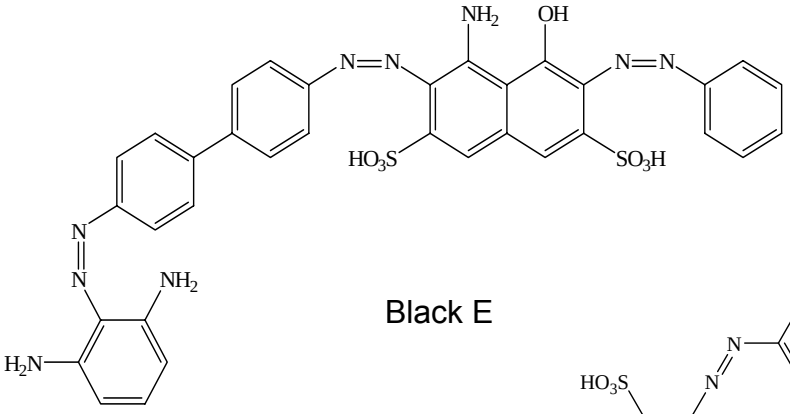
The seeds were germinated in sterile 10 cm petri dishes, layered with sterile filter paper. Seeds were sterilized as described earlier (Somasegaran and Hoben, 1985) before transferring to the surface of the paper in petri dish. The phytotoxicity study was carried out at room temperature (32 ± 2^{o}C) in relation to *Triticum aestivum* and *Phaseolus mungo* seeds (10 seeds per plate) by watering separately 5 ml samples of dye and its degradation product per day. Seeds germinated in water irrigated petri dish were used as a control. Length of plumule (shoot), radicle (root) and germination (%) were recorded after every alternate day.



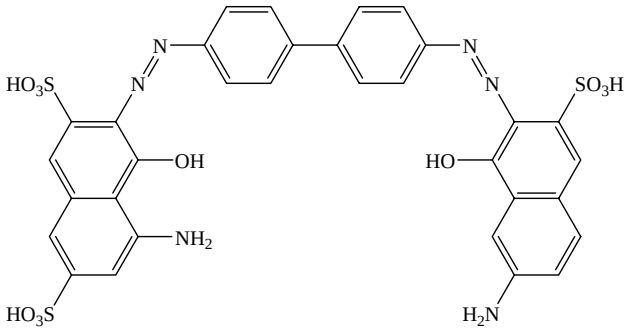
Reactive Black 5



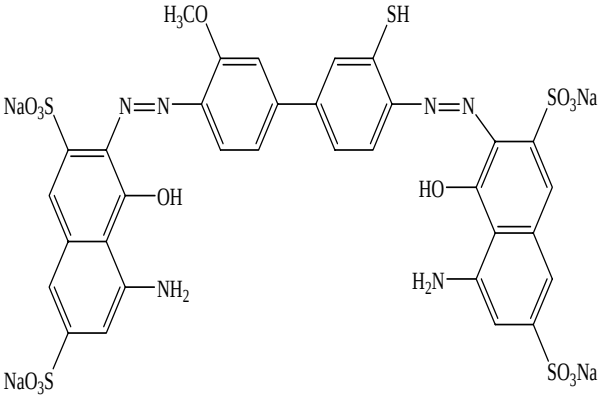
Green B



Black E



Black BT



Sky Blue FF

Figure 6.1: Chemical structures of azo dyes used in this study.

RESULT AND DISCUSSION

Decolorization of mixed azo dyes

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

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

Decolorization by bacterial consortium

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

When combination of two bacterial isolates (JTP-23 and JTP-5, JTP-23 and JTP-7, JTP-23 and JTP-13, JTP-23 and JTP-30) was employed, decolorization achieved was more than 99% with no remarkable difference in decolorization

pattern under static condition (Figure 6.4), while less decolorization was achieved 65, 11, 84, and 63 % respectively under shaking culture condition (Figure 6.5).

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

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

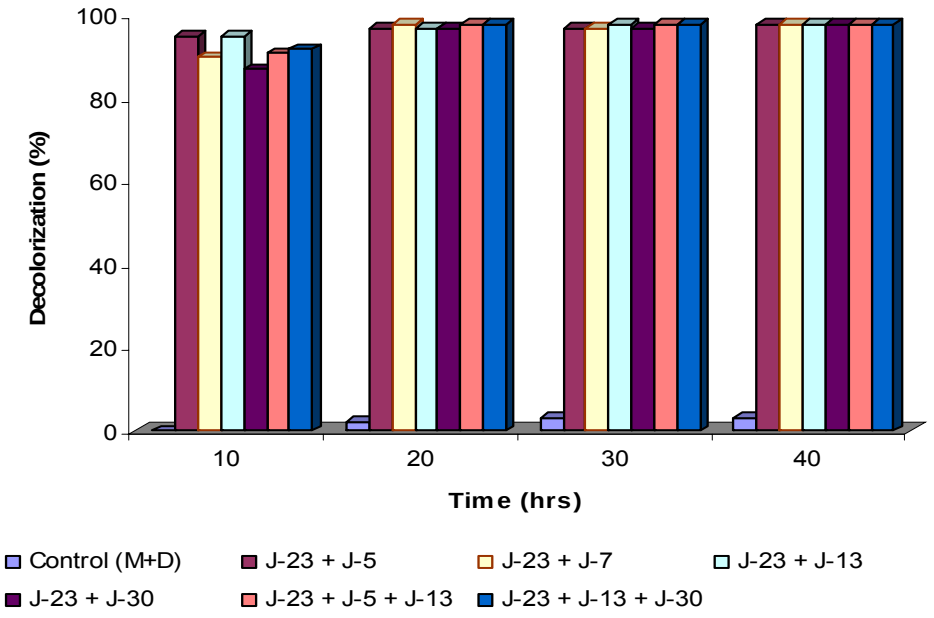


Figure 6.4: Decolorization of Reactive Black 5 by different consortiums under static culture condition.

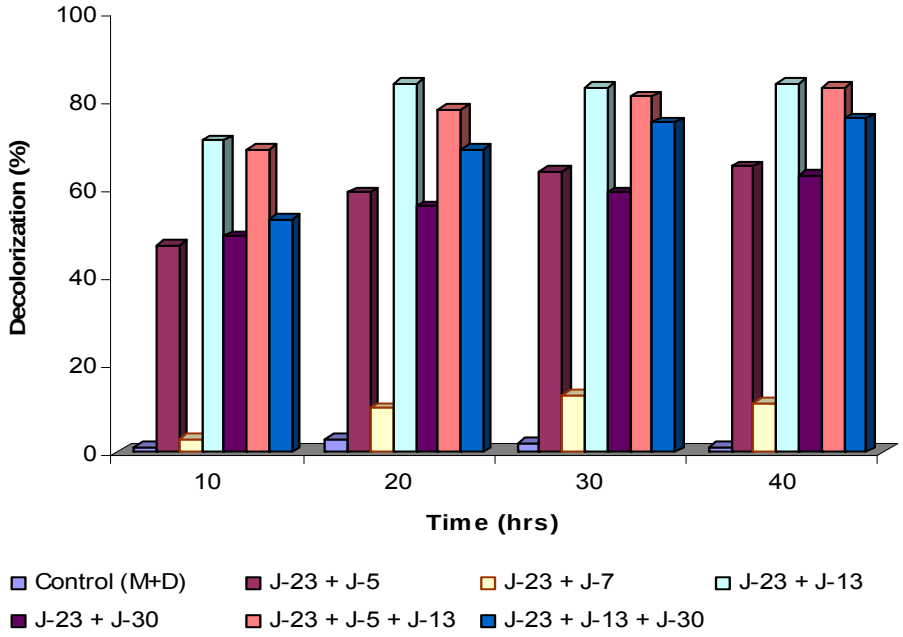


Figure 6.5.: Decolorization of Reactive Black 5 by different consortiums under shaking culture condition.

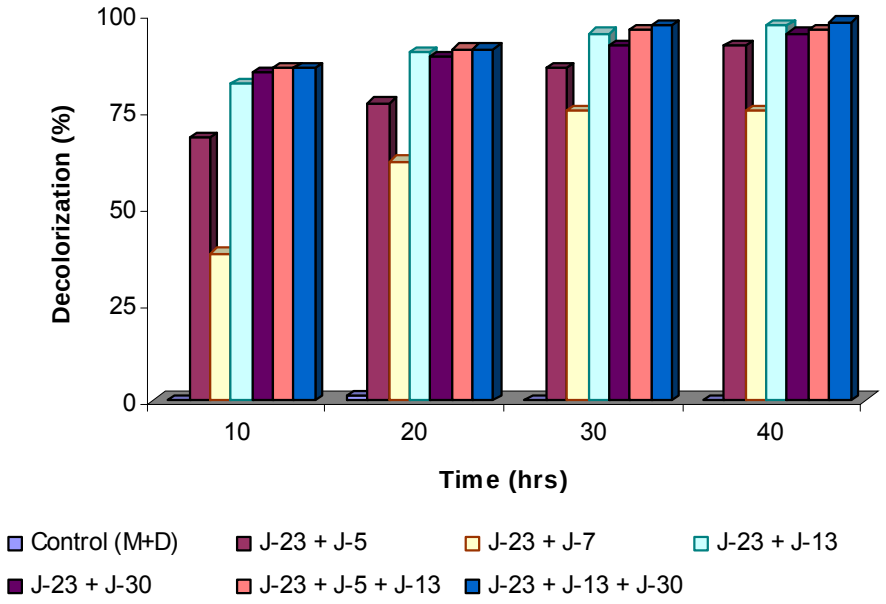


Figure 6.6: Decolorization of Black BT by different consortiums under static culture condition.

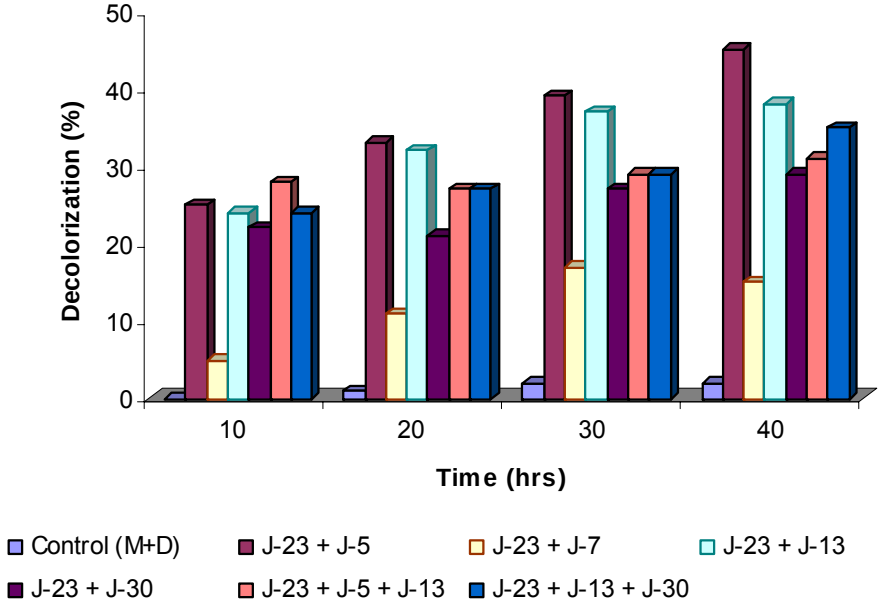


Figure 6.7: Decolorization of Black BT by different consortiums under shaking culture condition.

Decolorization of industrial effluent

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

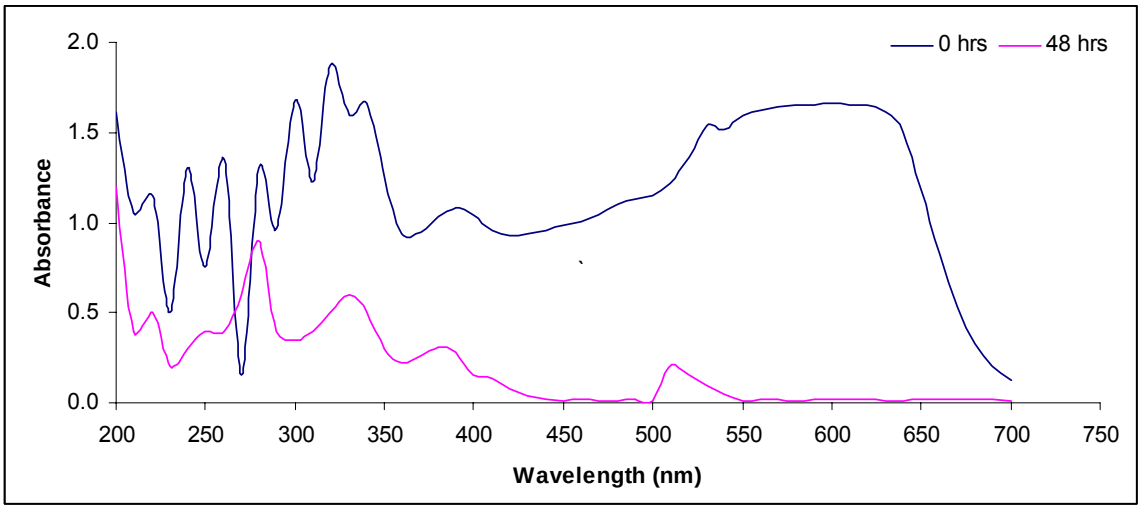


Figure 6.8: UV-Visible spectral analysis of textile effluent before and after treatment with *Lysinibacillus fusiformis* JTP-23

Phytotoxicity study

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

and radicle were significantly affected by Reactive Black 5 than its degradative metabolites (Table 6.1), indicating less toxic nature of degradation metabolites as compared to dye.

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

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

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

Table 6.1: Phytotoxicity study of Reactive Black 5 and its degradation metabolites on seed germination and growth. **a**, *Triticum aestivum*; **b**, *Phaseolus mungo*.

a

Parameter studied	*Triticum aestivum*			
	Water	Reactive Black 5	Extracted metabolites-1	Extracted metabolites-2
Germination (%)	100	36.67	56.67	86.67
Plumule (cm)	5.55	1.55	2.30	4.98
Radicle (cm)	8.77	2.36	5.34	8.16

b

Parameter studied	*Phaseolus mungo*			
	Water	Reactive Black 5	Extracted metabolites - 1	Extracted metabolites – 2
Germination (%)	100	66.67	ND	100
Plumule (cm)	4.47	1.19	ND	3.95
Radicle (cm)	2.26	0.20	ND	2.19

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

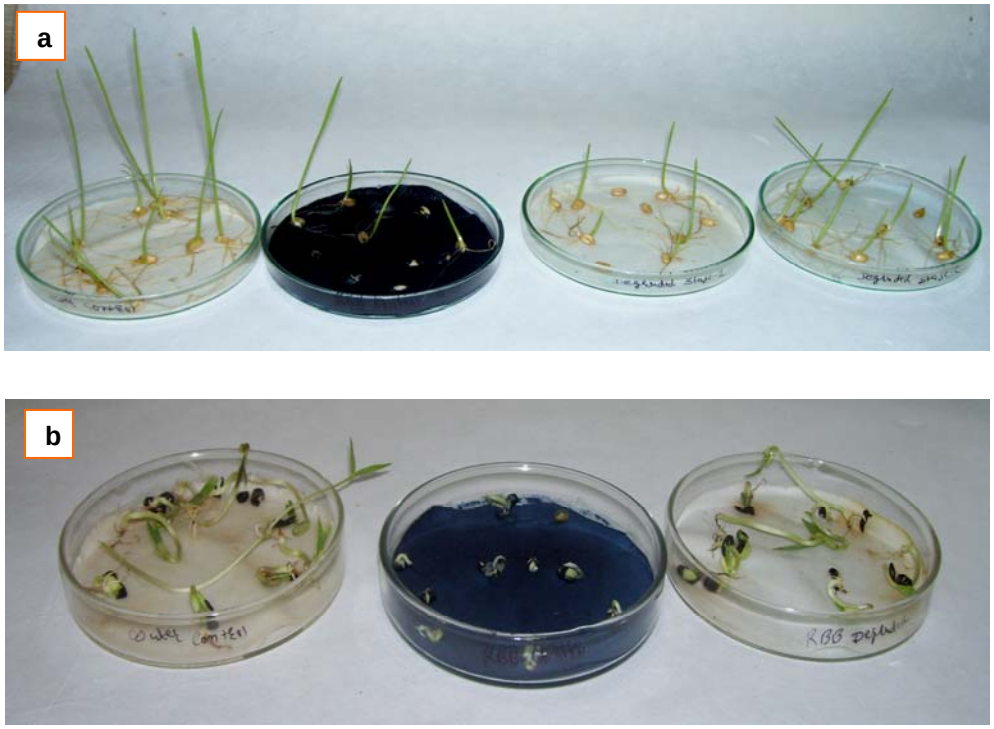


Figure 6.9: Germination of seeds irrigated with water control, RB5 dye control and degraded metabolites respectively from left to right. **a**, *Triticum aestivum*; **b**, *Phaseolus mungo.*

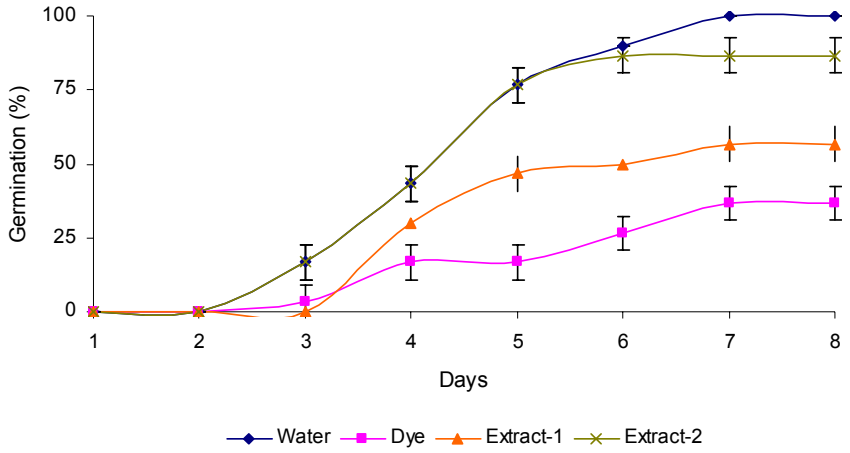


Figure 6.10: Germination percentage of *Triticum aestivum* seeds irrigated with different samples.

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

a

Parameter studied	***Triticum aestivum***			***Phaseolus mungo***		
	Water	Green B	Extracted metabolites	Water	Green B	Extracted metabolites
Germination(%)	100	46.67	96.67	100	70	100
Plumule (cm)	5.56	0.24	4.60	4.47	1.01	3.68
Radicle (cm)	8.77	1.74	8.17	2.26	0.24	2.01

b

Parameter studied	***Triticum aestivum***			***Phaseolus mungo***		
	Water	Black BT	Extracted metabolites	Water	Black BT	Extracted metabolites
Germination(%)	100	40	96.67	100	66.67	100
Plumule (cm)	4.81	0.82	4.72	3.51	0.92	3.18
Radicle (cm)	8.17	1.60	8.31	1.75	0.27	1.39

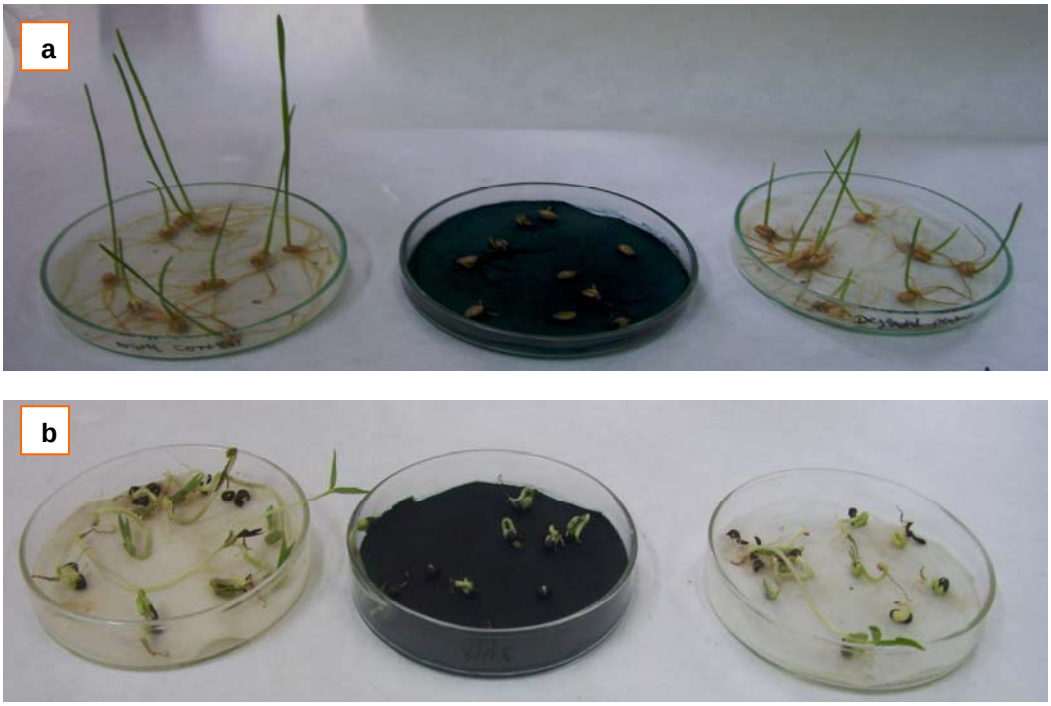


Figure 6.11: Germination of seeds irrigated with water control, Green B dye control and degraded metabolites respectively from left to right. **a**, *Triticum aestivum*; **b**, *Phaseolus mungo.*

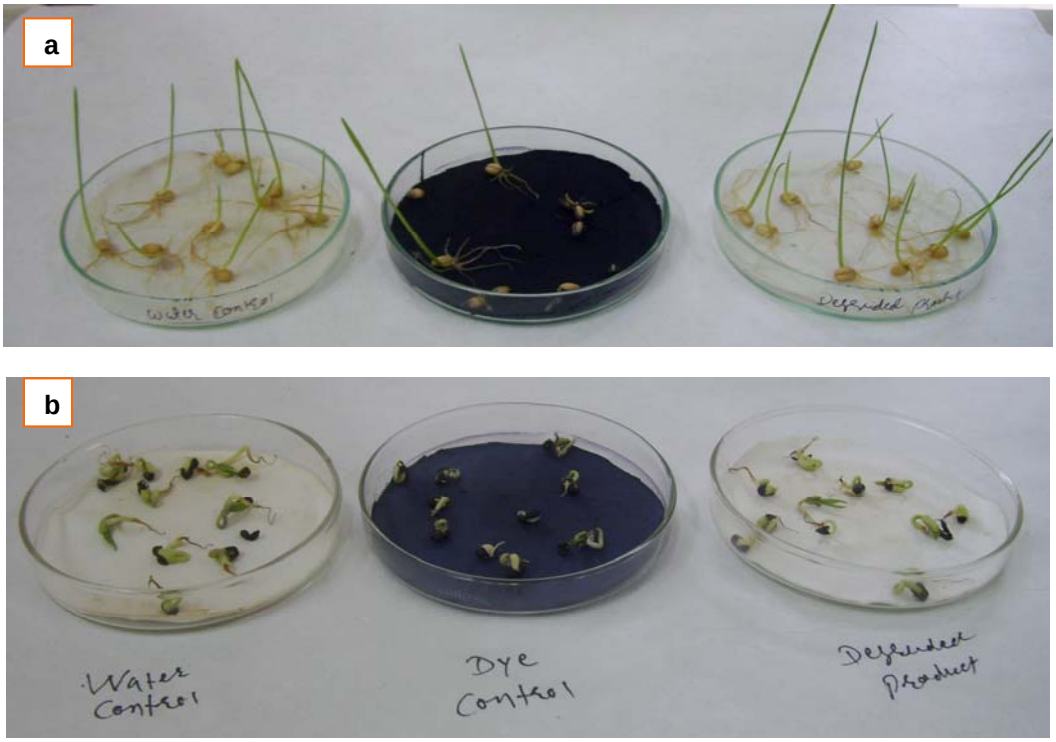


Figure 6.12: Germination of seeds irrigated with water control, Black BT dye control and degraded metabolites respectively from left to right. **a**, *Triticum aestivum*; **b**, *Phaseolus mungo.*

CONCLUSION

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

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

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

7

Degradation of Azo Dyes by *Lysinibacillus fusiformis* JTP-23 Degraded Product Analysis and Evaluation of Cytotoxicity

INTRODUCTION

A wide variety of azo dyes with antraquinone, polycyclic and triphenylmethane groups are being increasingly used in textile dyeing and printing processes. They are important in biodegradation process. They pose toxicity in the form of genotoxicity, mutagenicity and carcinogenicity to aquatic organisms, such as fish, algae and bacteria as well as humans and plant that come in its contact (Puvaneshwari *et al.*, 2006).

Benzidine (BZ) based dye are widely used in dye manufacturing, textile printing, color paper printing and leather industry. BZ has long been recognized as a human urinary bladder carcinogen and tumorogenic in a variety of laboratory animals (Haley, 1975). Since, BZ is used as a reactant in dye synthesis; workers could be directly exposed to the carcinogen. Experimental studies with rats, dogs, and hamsters have shown that animals administered BZ and BZ based dye excrete potentially carcinogenic amines in their urine (Nony and Bowman, 1980). Definite/possible carcinogenic substances are as per table 7.1.

Chemical analysis could be insufficient to provide insight into the potential ecological risk, since they do not allow an evaluation of possible combined effects of the different contaminant mixed together as well as their bioavailablity. Bioassays which can mitigate these constraints are, therefore, recommended for the assessment of ecological risks (Conder *et al.*, 2001).

Table 7.1: Substances and mixtures evaluated by IARC as definite or possible human and animal carcinogens (Siemiatycki *et al.*, 2004)

Substances	Occupation or Industry	Human Evidence	Animal Evidence	Site(s)
Aromatic amine dye (definite)				
4-Aminobiphenyl	Production; dyestuff and pigment	Sufficient	Sufficient	Bladder
Benzidine	Production; dyestuff and pigment	Sufficient	Sufficient	Bladder
2-Naphthylamine	Production; dyestuff and pigment	Sufficient	Sufficient	Bladder
Azo dyes (possible)				
O-aminoazotolune	Production; textiles and leather	NA	Sufficient	-
CI Acid Red 114	Production; textiles and leather	NA	Sufficient	-
CI Direct Blue 15	Production; textiles and leather	NA	Sufficient	-
Oil Orange SS	Production; dyes/pigment, varnish, oils, fats, and waxes	NA	Sufficient	-
Ponceau 3R	Production; textile	NA	Sufficient	-
Trypan Blue	Production; texteils and printing; biological stains	NA	Sufficient	-

NA: Not available.

In the present study, the efficiency of bacteria to degraded azo dyes was analyzed by TLC and HPLC. The toxicity assessment of the dye and its degraded products was examined using the degrading bacterium itself as well as on African green monkey kidney cell line.

MATERIALS AND METHODS

Toxicity on *Lysinibacillus fusiformis* JTP-23

Microbial toxicity of Reactive Black 5 and its degradative metabolites (1000 ppm) were carried out using the diffusion assay according to Parshetti *et al.,* (2006) with slight variation. Melted CMB agar medium was inoculated with overnight grown culture of *Lysinibacillus fusiformis* JTP-23 in order to get loan growth in petri dish. 100 µl sample of dye and its degraded extracts were added in well.

Thin Layer Chromatography

Dye and its degradative products were analyzed on Thin Layer Chromatography (TLC). Commercially available TLC plates coated with silica gel 60F 254 on aluminum foil having dimensions 1-0 cm x 5 cm x 0.25 mm (Merk, Germany) were used. Reactive Black 5 dye was decolorized by *Lysinibacillus fusiformis* JTP-23 and the cell free supernatant obtained at 48 hrs were extracted with equal volume of ethyl acetate. The extracts were dried by evaporating ethyl acetate at ambient temperature. The dried extracts were dissolved in 1 ml methanol and 100 µl was loaded on the TLC plates. Solvent system consisting of n-butanol: acetic acid: water (4: 2: 4). TLC plates were observed in short wavelength UV (254nm) and long UV (365).

Cytotoxicity analysis on VERO cell line

Maintenance of cell line and subculturing

Preservation of African green monkey kidney cell line was done in repository. Subculturing was done by wiping the mouth of the bottle with cotton soaked in spirit to remove the adhering particles. Then, 4-5 ml of Minimal essential media (MEM) without FCS (Fetal calf serum) was added and gently rinsed with tilting. The dead cells and excess of FCS was washed out and the medium was discarded. Tripsyn phosphate versene glucose (TPVG) was added over the cells and incubated at 37oC for 5 min for disaggregation. The cell becomes individual and after addition of 5 ml 10% MEM with FCS by serological pipette it presents

the suspension of cell line. The cells were splited into 1:2, 1:3 etc. for cytotoxicity study through plate method.

Seeding of cells

1 ml of suspension was poured in to 24 well plates. Each well was added with 1 ml of the suspension and kept in desiccators in 5% CO_2 atmosphere. At the end of 2 days incubation cells were observed in inverted microscope.

Sample preparation

The dye (1000 ppm) and extracted metabolites (1000 ppm) were dissolved in media (Minimal essential media) to make up the dilutions. Degradation metabolites of RB5 by *Lysinibacillus fusiformis* JTP-23 were extracted after 24 h incubation in static culture condition. 500µl of MEM without FCS was taken in 9 eppendroff tubes. Then 500µl of the working conc. was added to the first eppendroff tube and mixed well then 500µl of this volume was transferred from first to last tube by serial dilution to obtain the desired concentration of the sample to be tested.

Cytotoxicity assay

Assay was carried out with 48hr monolayer culture VERO cells at a concentration of one lakhs cells / well seeded in 24 well titer plates. Each dilution of the samples was added to the respective wells. The plates were incubated at 37ºC in 5% CO_2 environment and observed for cytotoxicity using inverted microscope.

MTT assay

After the examination of 24 well titre plates, medium was removed carefully for MTT assay. Each well was wash with MEM (w/o) FCS for 2 – 3 times and added with 200µl of MTT (5mg/ml). Plates were incubated for 6-7 h in 5% CO_2 followed by addition of 1ml of DMSO in each well, mixed and left for 45 seconds. The suspension was transferred in the cuvette and OD was taken at 595 nm by taking DMSO as blank. Graph is plotted by taking concentration of the drug on X axis and relative cell viability on Y axis.

Cell viability (%) = Mean OD/Control OD x 100

Analysis of degradation products by HPLC

The decolorized culture medium was centrifuged at 12,000 rpm for 15 min and supernatant was collected and extracted with ethyl acetate. The extracts were allowed to evaporate till dryness and redissolved in HPLC grade methanol. The samples were filtered through 0.2 µm filter and then analyzed using column RP C18 (250 x 4.6 mm, 5 micron particle size, Phenominex). The mobile phase consisted of 20mM Amonium acetate (in water) : methanol (60:40) with flow rate 1 ml/min. The purity was checked by PDA detector, isocratic system and 12 min run time.

RESULTS AND DISCUSSION

Repeated addition of dye aliquots

This study was carried out to test the ability of *Lysinibacillus fusiformis* JTP-23 to decolorize repeated additions of dye RB5 aliquots (100 mg l^{-1}) by individual as well as consortium form. Strain JTP-23 showed 97% decolorization of first dye aliquot (Figure 7.1) in 26 h and second aliquot of dye was added, which was 79% decolorized in next 9 h. The culture showed 93% decolorization of third aliquot at 70 h and only 45% decolorization of 4th aliquot at 105 h. Consortium did not show more than 77% decolorization in 3rd aliquot even on extended incubation. The 4th aliquot consortium did not show any significant decolorization.

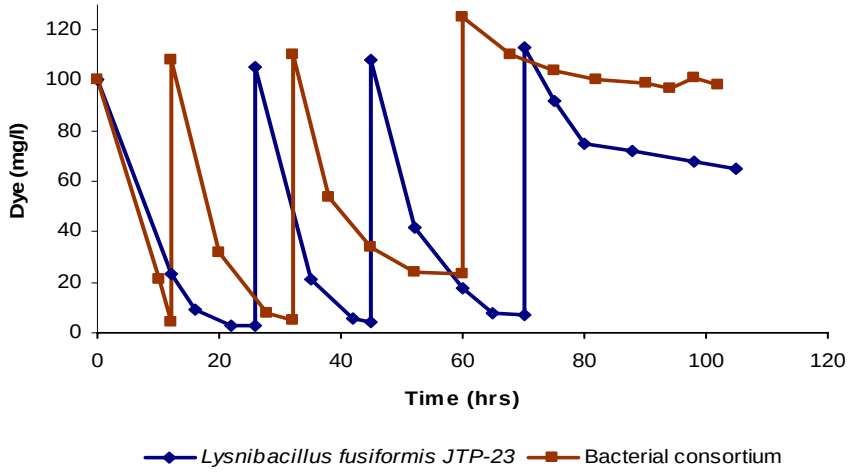


Figure 7.1: Repeated decolorization of RB5 by *Lysinibacillus fusiformis* JTP-23 and its consortium in fed batch process under static culture condition at 35 ^{o}C

In both sets of experiments, the decolorization efficiencies of *Lysinibacillus fusiformis* JTP-23 and consortium were decreases from 4th aliquot and 3rd aliquot respectively. Results indicated that when JTP-23 was used individually, it had better ability, while in the presence of other organisms (consortium), the overall efficiency decreased. This is attributed to starvation and accumulation of toxic products by other organisms present in the culture. A great amount of dead cells were found settled at the bottom of the flasks in last aliquot operation. The hostile and stressful environment inhibited the normal growth and metabolism of

bacteria (Liu *et al.*, 2007). The culture exhibited the ability to decolorize repeated additions, which is significant for its commercial application.

Toxicity on *Lysinibacillus fusiformis* JTP-23

Microbial toxicity study showed the growth inhibitory zone (0.4 cm) surrounding the well containing dye, while degradation products well did not show inhibitory zone. It confirmed non toxic nature of the extracted metabolites. These findings suggested non-toxic nature of the products formed. Previous reports showed that colorless products were also equally toxic to native dye compound (Lamia *et al.,* 2009).

Thin Layer Chromatography

Figure 7.2 shows separated spots of dye while complete disappearance of dye in treated sample. Any new spot was not found in treated samples. This indicated decolorization and complete degradation of Reactive Black 5 by *Lysinibacillus fusiformis* JTP-23 and its consortium.

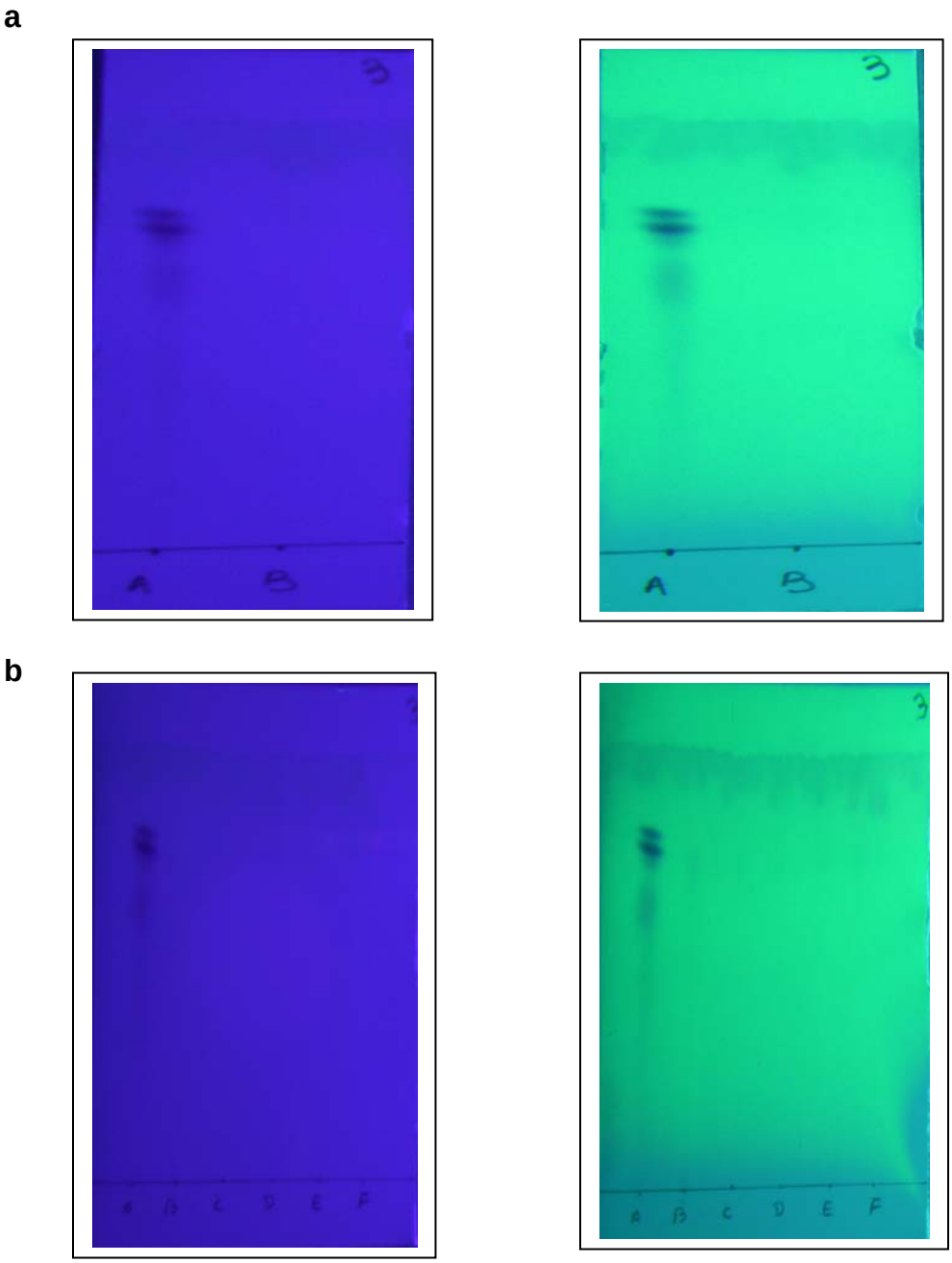


Figure 7.2: Thin Layer Chromatography of dye treated with (**a**) *Lysinibacillus fusiformis* JTP-23, where A: Original dye, B: Treated dye and (**b**) Consortium, where A: Original dye, B and C: Treated dye at 24 h; D and E: Treated dye at 48 h; F: Empty.

Cytotoxicity study

In order to assess the effect of xenobiotic compounds on cell, it is important to determine the cytotoxicity concentration of the dye or its degraded compound. Cytotoxicity tests define the upper limit of the extract concentration, which is non-toxic to the cell line. Bioremediation processes must ensure the concentration of

xenobiotic compound in treated sample which is nontoxic to the cells. After the addition of the samples, cell death and cell viability was estimated. Table 7.2 explains the results of cytotoxicity of control dye and its degradation extracted metabolites (Figure 7.4). Results indicated that toxic effect of dye is decreased with removal of its color. This means degradation metabolites are less toxic to VERO cell line. This is suggestive of good pattern of breakdown of RB5 by Lysinibacillus fusiformis JTP-23, which does not produce more toxic degradation metabolites. The result was confirmed by additional metabolic intervention experiment such as MTT assay.

MTT test

MTT assay is called as (3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide. MTT assay was first proposed by Mossman in 1982. MTT is cleaved by mitochondrial dehydrogenase in viable cells, yielding a measurable purple product formazan. Formazan production is proportionate to viable cell number and inversely proportional to the degree of Cytotoxicity (Mosmann, 1983). Figure 7.3 shows the result of MTT test.

HPLC analysis

Figure 7.5 shows the result of HPLC analysis on the decolorization metabolites of Green B by *Lysinibacillus fusiformis* JTP-23 culture. After anaerobic incubation for 24 h, the intensity of peaks at retention time 3.9 and 6.0 increased significantly, which were not present in control Green B chromatogram. It is thus reasonable to suggest that these both peaks represent degradation metabolites. When such metabolites were degraded aerobically they formed less aromatic, more polar compounds, since the metabolites peak area decreased and shifted towards lower retention time at 3.7 and 5.6, respectively.

Decolorization product analysis of Reactive Black 5 by HPLC was performed. RB5 was treated with bacterial consortium under static and static-shaking sequential incubations. New peaks in chromatogram of sample extracted at static incubation 24 h indicated newly synthesized degradation metabolites (Figure

7.6). After shaking, the intensity of the peak was reduced and it moved to lower retention time, indicating further degradation of the dye metabolites.

Table 7.2: Cytotoxicity effect of RB5 and extracted metabolites on VERO cell line (African Green Monkey Kidney Cell Line).

Sr. No.	Reactive Black 5			Extracted metabolite		
	Dilution	*Absorbance*	*Cell viability*	*Dilution*	*Absorbance*	*Cell viability*
1	Neat	0.11	16.92	Neat	0.25	38.46
2	1:1	0.19	15.38	1:1	0.34	52.30
3	1:2	0.22	33.84	1:2	0.42	64.61
4	1:4	0.29	44.61	1:4	0.48	73.84
5	1:8	0.36	55.38	1:8	0.52	80.00
6	1:16	0.44	67.69	1:16	0.55	84.61
7	1:32	0.49	75.38	1:32	0.59	90.76
8	1:64	0.54	83.07	1:64	0.61	93.84
9	1:128	0.58	89.23	1:128	0.64	96.92
10	-	0.65	100	-	0.65	100

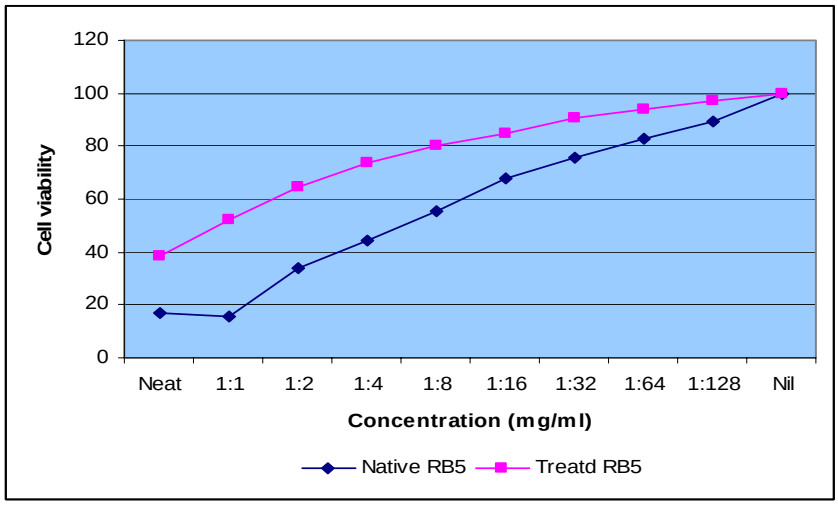


Figure 7.3: Cell viability estimated by MTT test on exposure of native RB5 and treated RB5 to VERO cell line.

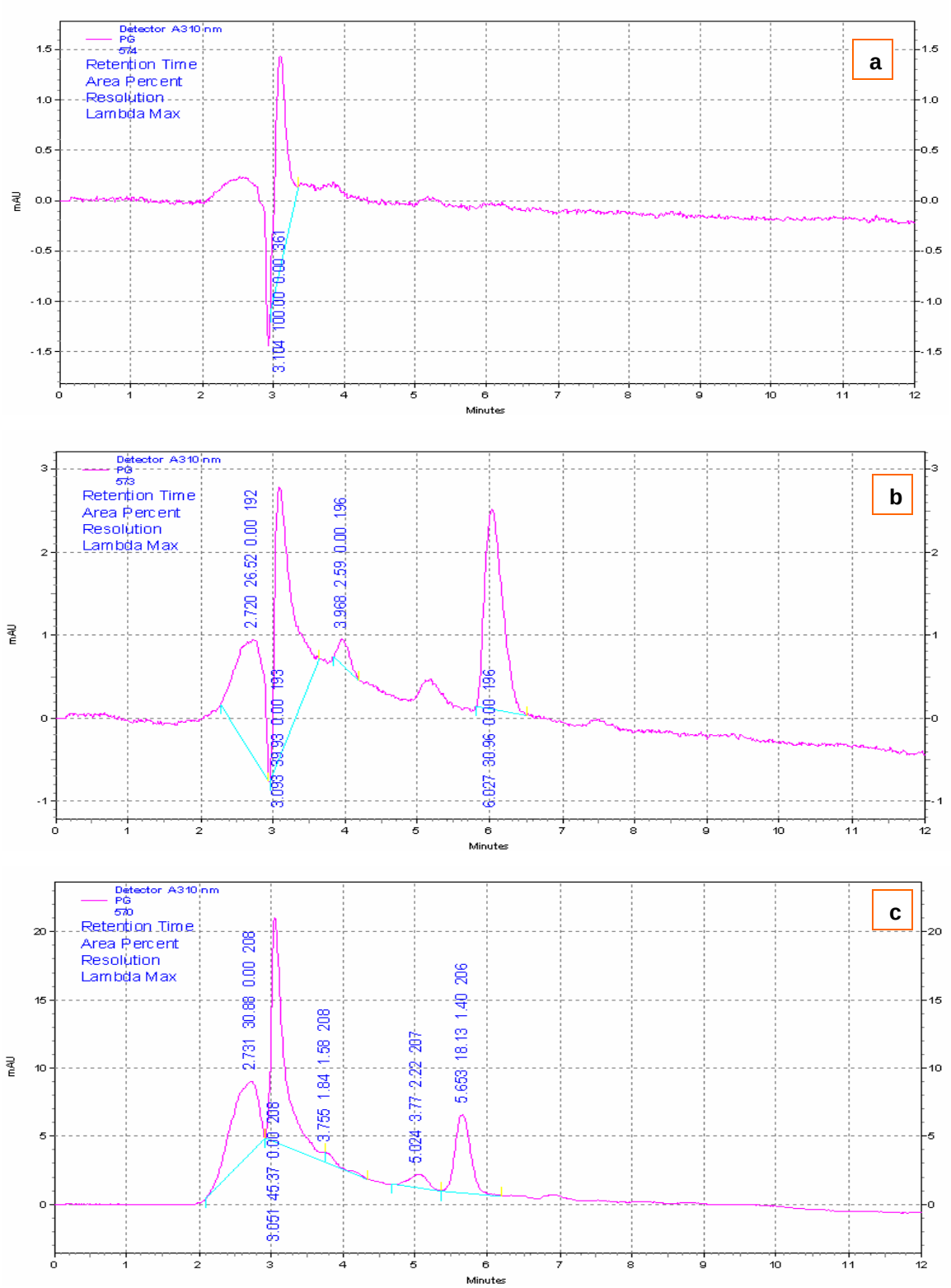


Figure 7.5: **a**, control dye Green B; **b**, static incubation; and **c**, static-shaking sequential incubation respectively by *Lysinibacillus fusiformis* JTP-23 growing on complete medium containing Green B dye analyzed by HPLC

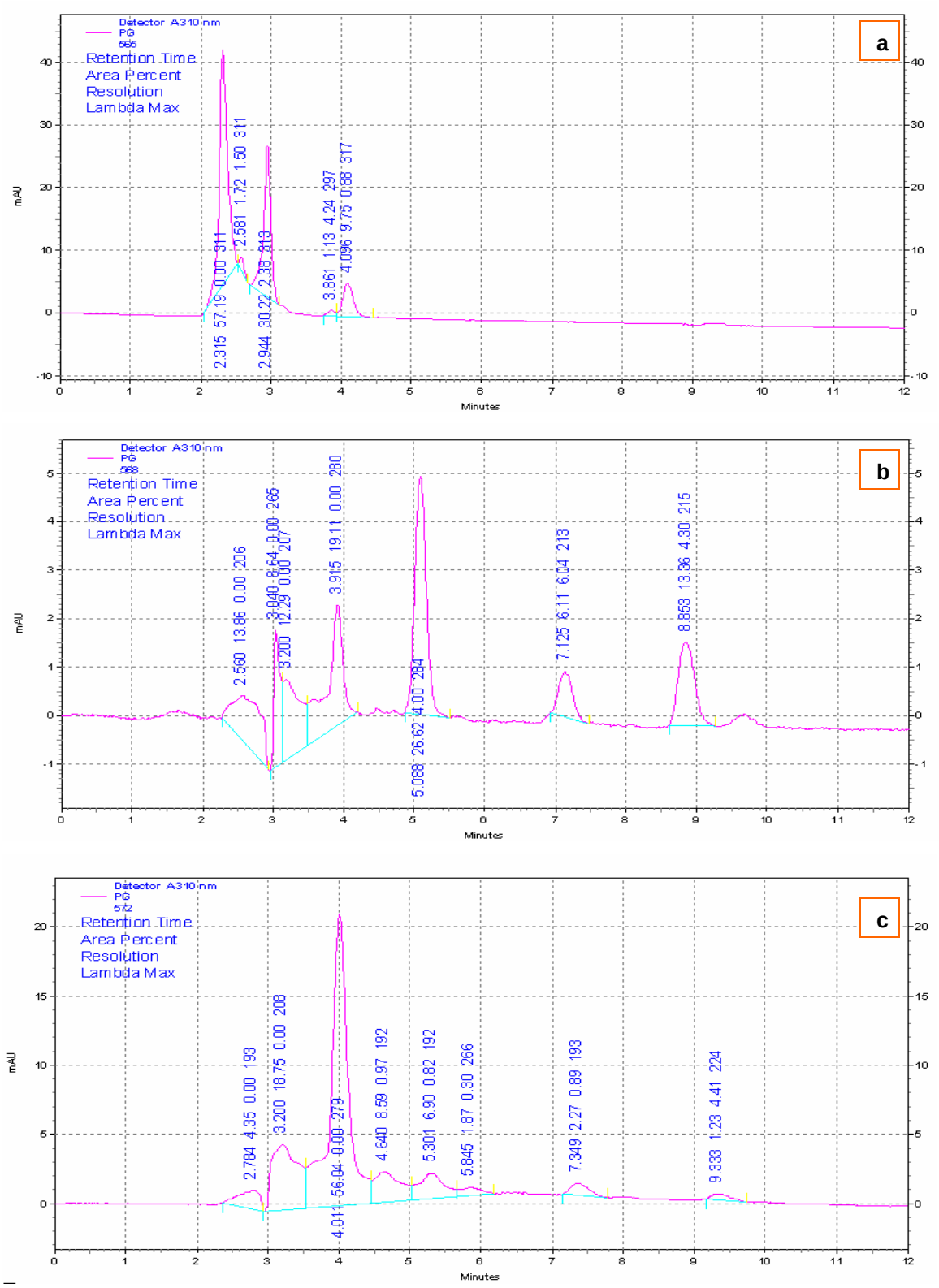


Figure 7.6: **a**, Control dye RB5; **b**, static incubation; and **c**, static-shaking sequencial incubation respectively by bacterial consortium growing on complete medium containing RB5 dye analyzed by HPLC

CONCLUSION

Though benzidine based dye are occupational carcinogens, they are also found widely among the general population and indeed. It's likely that many more people are exposed to these substances outside than inside the occupational environment. They are not readily degradable under natural conditions and are typically not removed from waste water by conventional waste water treatment systems (Puvaneswari *et al.,* 2006).

Decolorization of repeated dye aliquot suggested the suitability of *Lysinibacillus fusiformis* JTP-23 for large scale application. Decolorization efficiency was decreased from 4^{th} and 3^{rd} aliquot of dye by strain JTP-23 and its consortium respectively. The single culture has better efficiency but it was reduced in the presence of other organisms. This might be due to starvation and accumulation of toxic products by other organisms present in culture.

The degradation product was less toxic to VERO cell line as compared to native dye structure. TLC analysis did not show any spot of static-shaking sequentially degraded dye. This supported the dye degradation. HPLC analysis indicated structural changes by the organisms during its degradation. On extended incubation with organisms, the newly synthesized metabolites were degraded.

8

Summary

Textile effluent and sludge produced by effluent treatment plant is rich source of dye decolorizing bacterial population. Total of 37 isolates were selected on the basis of their Gram reaction, colony characteristics, growth pattern and cell morphology. Samples contained both the types of bacteria, with the dominance of Gm +ve members. Initially, all 37 isolates were tested for their ability to decolorize two azo dyes, Reactive Black 5 and Green B. Six potential isolates were then selected on the basis of their dye decolorizing ability and were further tested with FF sky Blue, Black E, Black BT and Reactive Orange M_2R. Differences in structures and complexity of dye led to the variable percentage of decolorization of different dyes by same organisms.

Thirty seven bacterial isolates were considered for their characterization, based on Gram's reaction, cell morphology, colony characteristics, growth patterns in nutrient broth and biochemical tests. Triple Sugar Iron agar was used to check sugar fermentability of individual bacteria. Three important bacterial species were identified as Alcaligenes faecalis JTP-07, Lysinibacillus fusiformis JTP-23, and Pseudomonas aeruginosa JTP-37 by 16 s rDNA sequencing. Phylogenetic analysis was performed for identified cultures.

Various aspects of environmental and cultural parameter were studied for decolorization of Reactive Black 5 by *Lysinibacillus fusiformis* JTP-23. The results indicated that the new species had high capacity for rapid decolorization of azo dyes. *Lysinibacillus fusiformis* JTP-23 produced abundant biomass under shaking condition. However, decolorization capacity was sharply reduced under

this condition. On the other hand, anoxic conditions produced less biomass with greater decolorization. Maximum decolorization was observed in static condition, while COD was decreased in subsequent shaking condition. Further, with increasing dye concentrations, efficiency of decolorization decreased.

The suitable initial pH for decolorization of RB 5 was in the range of 6-9 under static condition and 6-10 under anoxic condition. Optimum temperature for decolorization was 30-35^{o}C. The results on the effect of size of inoculum indicated that under anoxic condition 2% v/v was ideal for rapid decolorization of RB 5 dye.

Textile industries use wide variety of structurally different dyes; therefore, effluents are extremely variable in dye composition. Treatment of such effluent and decolorization of dye, therefore, is difficult. Wide range of pH, salt concentrations and heterogeneous hazardous chemicals often add to the difficulties for the treatment.

The newly isolated bacteria, *Lysinibacillus fusiformis* JTP-23, efficiently decolorized dye under varied cultural and environmental conditions, highlighting the significance of this strain in large scale treatment of textile effluent.

Nutritional parameters were examined for their effect on decolorization of RB5 by *Lysinibacillus fusiformis* JTP-23. The results showed that lactose, tryptone and glucose were most suitable. Organic nitrogen sources in the form of peptone, yeast extract and meat extracts were most appropriate as compared to other inorganic nitrogen sources.

Decolorization of dye remained unaffected under different salt concentrations. However, at higher than 5% (w/v) salt, the biomass synthesis was adversely affected with the sharp reduction in dye decolorization. It was observed that glucose was unavoidable in small amount for better decolorization. However, the increased glucose concentrations retarded the decolorization process.

Metals are commonly found in waste water from dying industry. It was observed that increased concentration of metal reduced decolorization. Prior exposure of the organism to the dye did not improve decolorization, indicating the constitutive nature of the enzymes involved in the process.

Analysis of Plackett-Burman statistical design for the optimum nutritional requirement for decolorization revealed that peptone favored decolorization, while glucose and K_2HPO_4 had negative effect under anoxic condition. However, both, glucose and K_2HPO_4, displayed positive effect under the static culture condition.

Lysinibacillus fusiformis JTP-23 decolorized Reactive Black 5 and other textile dyes optimally under employed nutritional parameters, displaying the ability of the isolate to decolorize various textile dyes under varied cultivation conditions.

With the static-shaking sequential treatment along with designer consortium, successful decolorization and degradation of the dyes was achieved. This was supported by sharp reduction in toxicity of degradation metabolites on the germination and early seedling growth in *Triticum aestivum* and *Phaseolus mungo*.

Decolorization of repeated dye aliquot suggested the suitability of *Lysinibacillus fusiformis* JTP-23 for large scale application. Decolorization efficiency decreased from 4^{th} and 3^{rd} aliquot of dye, by JTP-23 and consortium, respectively. The single culture has better efficiency.

Though benzidine based dye are occupational carcinogens, they are also found among the general population. The degradation product was less toxic to VERO cell line as compared to native dye. TLC analysis did not show any spot of static-shaking sequentially degraded dye, supporting dye degradation. HPLC analysis indicated structural changes during degradation. It was revealed that on extended incubation with the organism, the newly synthesized metabolites were degraded.

There are few reports on the biological decolorization and degradation of textile and dyestuff industrial wastes containing azo dyes. The reports so far available are on a small scale and no large scale data on biological decolorization and degradation is available Biological treatment is the only way for ultimately controlling pollution generate by textile and dyes stuff industries. However, further research would be required to develop a viable alternative process for the treatment of dye wastewaters.

9

Conclusion

Continuous dumping of dye stuffs and dye waste water has created environmental pollution as well as medical and aesthetic problems associated with human health and agriculture, thus bioremediation of contaminated site is of prime importance. Due to adaptation of various bacterial species, an effluent had high count of Gm +ve and Gm –ve dye decolorizing bacteria. The difference in decolorization capacity of different azo dyes by individual bacteria was due to dissimilarity in specificities, structure and complexity, and the interaction with azo bond with different dyes.

Gm +ve bacteria dominated in different samples collected from CETP. Anoxic condition was most suitable for rapid decolorization of different dyes by potential isolate *Lysinibacillus fusiformis* JTP-23. The major drawback with this cultural condition was no reduction in COD of the reaction mixture. Best growth and decolorization was achieved in mesophilic range of temperature, while alkaline pH (8 to 10) favored decolorization.

Increased concentration of metal had reduced the decolorization capacity. Higher concentrations of glucose and K_2HPO4 decreased decolorization under anoxic condition while increased decolorization under static culture condition was observed. The decolorization of dye was favored by variety of carbon and nitrogen sources to different degree.

Use of anaerobic-aerobic sequential treatment by consortium was successful decolorization and degradation of azo dyes. The reduced toxicity of extracted metabolites on *Triticum aestivum* and *Phaseolus mungo* indicated the degradation of azo dyes. The extracted metabolites of degraded dye were safer not only for plant germination and seedlings growth but also for animal cell line as it was tested on African green monkey kidney cell line (VERO).

TLC results supported breakdown of the native dye structure. HPLC analysis showed dye native structure was degraded to its intermediate structures that were further degraded on extended incubation of reaction mixture. Degradation of aromatic amines was evidenced by reduction in height and size of peak in chromatogram.

The isolated bacteria, *Lysinibacillus fusiformis* JTP-23, could effectively be used as an alternative to physical and chemical process used for textile effluent treatment.

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