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STUDIES ON INTRINSIC MICROBIAL POPULATIONS OF CONTAMINATED ENVIRONMENT AND BIODEGRADATIVE POTENTIALS

A

Thesis Submitted To

Saurashtra University

For

The Award of The Degree of

Doctor of Philosophy

In

Microbiology

By

Mr. MANISH MANSUKHLAL JANI

Registration No: 2400 **Date of Registration: 18th January 2000**

Supervising Guide: Dr. S. J. Pathak

Department of Biosciences
Saurashtra University
Rajkot – 360 005

March 2006

Department of Biosciences
Saurashtra University
Rajkot – 360 005
Gujarat, India

Telephone: 91-281-2586419 Fax: 91-281-77633, 77885

No:
Date:

CERTIFICATE

This is to certify that the work and data embodied in this thesis entitled; **"STUDIES ON INTRINSIC MICROBIAL POPULATIONS OF CONTAMINATED ENVIRONMENT AND BIODEGRADATIVE POTENTIALS"** is an original piece of research work carried out, which submitted to Saurashtra University, Rajkot by **Mr. Manish Mansukhlal Jani** (Registration No: 2400, dated 18th January, 2000, Department of Biosciences) in fulfillment of requirement for the degree of Doctor of Philosophy.

It is further certified that this work has not been submitted for the any degree or diploma to any other university or institution.

Date:

Place: Rajkot

Forwarded Through

Dr. S. J. Pathak
Supervising Guide
Department of Biosciences
Saurashtra University
RAJKOT-360 005

Prof. S. P. Singh
Head
Department of Biosciences
Saurashtra University
RAJKOT- 360 005

DECLARATION

I, **Mr. Manish Mansukhlal Jani,** the undersigned, hereby solemnly declare that the work presented in the thesis entitled**, "STUDIES ON INTRINSIC MICROBIAL POPULATIONS OF CONTAMINATED ENVIRONMENT AND BIODEGRADATIVE POTENTIALS"** is original and independent. I declare further that this work has not been submitted for any degree or diploma to any other university or institution.

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Date:

Place: Rajkot [Manish Jani]

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"To get profit without risk,
experience without hard work,
is as impossible as it is to
live without being born"
So, take an appropriate risk,
Work hard and live long with pleasure.

"Any man can make a mistake,
None but the fool will stick to it"
This is the beginning not end…

(Manish M.Jani)

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CHAPTER I: INTRODUCTION

I.1 An Overview

During the last few decades' man in his entire culture history has faced one of the most tremendous ecological crises of environmental pollution and this brings about major harmful changes in the environment. Rapid environment changes are profoundly altering the relationships between humans and the ecosystems in which they live. Environmental implications due to pollution are in various aspects. These produce serious problem for human being to maintain its existence, protection, and survival and for the improvement of general standard **(Shukla, R.S., 2001)**.

Environment pollution is the unfavorable alteration of surrounding, wholly or largely as byproducts of man's action through direct or indirect effects of changes in energy patterns, radiation levels, chemical and physical constitutions and abundance of organisms. These changes may affect man directly or through his supplies of water and agricultural and other biological products, his physical objects or possessions, or his opportunities for recreation and appreciation in nature **(Environmental pollution panel, 1965)**.

India today is one of the first ten industrialized countries of the world. Today the nation has a good industrial infrastructure in the core industries like metals, chemicals, dyeing, fertilizers, petroleum, food, etc. What has come out of these? Pesticides, detergents, plastics, solvents, fuel, paints, dyes, food additives etc. are some examples. Pollution is a necessary evil of all development **(Sharma, P.D., 1993)**. Any product, by product or residue that cannot be used profitably is called a waste. A waste product is regarded as a pollutant when it damages the environment **(Singh, B.D., 1998)**. Pollution is an undesirable change in the physical chemical or bio biological characteristics of air, water and soil that may harmfully affect the life or create a potential health hazard of any living organism **(Sharma, P.D., 1993)**.

Pollutants are contaminating the major ecological components like air, water and land. Among these I had selected the problem of water contamination through dyeing and printing industries by releasing colorant into the water resources. The major environmental problem of "colorants", therefore, is the removal of dyes from effluents.

Most xenobiotic compounds are recalcitrant and some of them are biomagnified to dangerous/toxic levels. A xenobiotic compounds are man made compound either not found in nature or found in a far lower concentration than that liberated by man. Recalcitrance of xenobiotics in the environment is usually caused by a lack of enzymes that recognize and transform the compounds of interest. The narrow substrate range and limited selectivity often restrict the biocatalytic applicability of enzymes.

The major sources of colored effluents are from textile, dye stuff industries particularly where the process of bleaching, dyeing, and printing and finishing in textile operations import huge amount of colored effluents. Untreated effluents from dyestuff production and dyeing mills may be highly colored and thus particularly objectionable if discharged into open waters. Among the chemical industries in India, about 27% from the class Dyes, pigments and colors. As a number of chemicals are used as raw materials in the manufacturing processes of these compounds, they find their way through waste effluents into the environment. Approximately 10,000 different dyes and pigments are used industrially and over 0.7 million tons of synthetic dyes are produced annually **(Padmavati, S., 2003)**.

Dye containing effluent is tough to be degraded because it has complex aromatic molecular structure and synthetic origin. Because the color in dye effluent is highly visible and effects aesthetics water transparency and gas solubility in water, and especially because many dyes are made from known carcinogens like benzidine, dye effluent treatment becomes inevitable. In this research study, the novel bacteria has been tried to degrade the azo dyes.

I.2 History of Dyes

In 1771 Woulfe prepared picric acid by the action of nitric acid on indigo and showed that it dyed silk in bright yellow shades. Laurent in 1842 converted phenol into picric acid. An 18 year old chemistry student William H.Perkin working in August Wilhelm von Hofmann’s laboratory in London attempted to synthesize quinine and instead discovered how to make mauveine, a purple dye. Perkin was experimenting with a compound called aniline in a possible first step in the pathway to quinine. He obtained a black tarry mess, but when he removed it from the flask with alcohol he observed a purple color in the dilute solution. When he dipped a piece of silk into this mixture, the silk was dyed reddish purple. Through the help

of his father, Perkin started a factory for making synthetic mauveine near London, 1957, this way the first synthetic dye to be manufactured.

Faraday discovered benzene in 1825 and Hoffmann isolated it from coalter in 1845. By 1869 kekule established the structure of benzene. It paved the way for the systematic study of aromatic compound. During last 125 years synthetic dyes have been prepared in bewildering number and variety. At least 7500 dyes are recognized now and thousand of patents have been granted in various countries for the synthesis and application of dyes. Reports of new dyes are published every two weeks in the possibilities of further synthesis are unlimited.

I.3 Classes of Dyes

All aromatic compounds absorb electromagnetic energy but only those that absorb light wavelengths in the visible range (350 -700nm) are colored. Dye contain chromophores, delocalised electron system with conjugated double bonds and auxochromes, electron-withdrawing or electron donating substituents that cause or intensity the color of the chromophore by altering the overall energy of the electron system. Visual chromophers are -C=C-; -C=N-C=O; -N=N-; -NO_2 and quinoid rings visual auxochromes are -NH_3 , -COOH, -SO_3H and -OH. Based on chemical structure or chromophore, 20-30 different groups of dyes can be discerned. Azo (monoazo, diaazo, triazo, polyazo) , anthraquinone , phthalocyanine and triarylmethane dyes are quantitatively the most important gruops. Other groups are triarylmethane , indigoid , azine , oxazine, thiazine,xanthene, nitro, nitrose, methine, thiazole, indamine, indophenol, lactone, aminoketone and hydroxyketone dyes and dyes of undetermined structure (stilbene and sulphur dyes). The vast array of commercial colorents is classified in terms of color, structure and application method in the color index (C.I), which is edited since 1924 (and revised every three months) by the society of dyers and colorists and the American Association of Textile Chemists and Colorist. Each different dye is given a C.I. generic name determines by its application characteristics and its color. The color index discerns is different application classes:

I. 3. 1 Acid dyes

The largest class of dyes in the color index is referred to as Acid dyes (~2300 different acid dyes listed, ~40 % of them are in current production). Acid dyes are anionic compounds that are mainly used for dyeing nitrogen -containing fabrics like wool, polyamide, silk and modified acryl. They bind to the cationic NH_4^+ -ions of those fibers. Most acid dyes are azo (yellow to red, or a broader range colors in case of metal complex azo dyes), anthraquinone or triarylmethane (blue and green) compounds. The adjective 'acid' refers to the pH in acid dye dyebaths rather than to the present of acid groups (sulponate, carboxyl) in the molecular structure of these dyes.

I.3. 2 Reactive dyes

Reactive dyes are dyes with reactive groups that form covalent bonds with OH-,NH-, or SH-groups in fibers (cotton ,wool , silk , nylon). The reactive group is often a heterocyclic aromatic ring substituted with chloride or fluoride, e.g. dichlorotriazine. Another common reactive group is vinyl sulphone . The use of reactive dyes has increased ever since their introduction in 1956, especially in industrialized countries. In the color index, the reactive dyes from the second largest dye class with respect to the amount of active entries: about 600 of the ~1050.

I.3.3 Metal complex dyes

Among acid and reactive dyes, many Metal complex dyes can be found (not listed as a separate category in the color index). These are strong complexes of one metal atom (usually chromium, copper, cobalt or nickel) and one or two dye molecules, respectively 1: 1 and 1: 2 metal complex dyes. Metal complex dyes are usually azo compounds. About 1/6 of all azo dyes listed in the color index are metal complexes but also phthalocyanine metal complex dyes are applied.

I. 3. 4 Direct dyes

Direct dyes are relatively large molecules with high affinity for especially cellulose fibers. Van derwaalls forces make them bind to the fiber. Direct dyes are mostly azo dyes with more than one azo bond or phthalocyanine, stilbene or oxazine compounds. In the color index, the direct dyes from the second largest dye class with respect to the amount of different dyes: About 1600 direct dyes are listed but only ~30% of them are in current productio n

I. 3. 5 Basic dyes

Basic dyes are cationic compounds that are used for dyeing acid-group containing fibers, usually synthetic fibers like modified polyacryl. They bind to the acid groups of the fibers. Most basic dyes are diarylmethane , triarylmethane , anthraquinone or azo compounds. Basic dyes represent ~5% of all dyes listed in the color index.

I. 3. 6 Mordant dyes

Mordent dyes are fixed to fabric by the addition of a mordant, a chemical that combines with the dye and the fiber. Though mordant dyeing is probably one of the oldest ways of dyeing, the use of mordant dyes is gradually decreasing: only ~23% of the ~600 different mordant dyes listed in the color index are in current production. They are used with wool, leather, and silk, paper and modified cellulose fibers. Most mordant dyes are azo, oxazine or triarylmethane compounds. The mordants are usually dichromates or chromium complexes.

I. 3. 7 Disperse dyes

Disperse dyes are scarcely soluble dyes that penetrate synthetic fibers (cellulose acetate, polyester, polyamide, acryl, etc.). This diffusion requires swelling of the fiber, either due to high temperatures (>120 ^{oC}) or with the help of chemical softeners. Dying takes place in dye baths with fine disperse solutions of these dyes. Disperse dyes from the third largest group of dyes in the color index: about 1400 different compounds are

listed, of which ~40% is currently produced. They are usually small azo or nitro compounds (yellow to red), anthraquinones (blue and green) or metal complex azo compounds (all colors).

I. 3. 8 Pigment dyes

Pigment dyes (i.e. organic pigments) represent a small but increasing fraction of the pigments, the most widely applied group of colorants. About 25% of all commercial dye names listed in the color index are pigment dyes but these ~6900 product names stand for less than 800 different dyes. These insoluble, non-ionic compounds or insoluble salts retain their crystalline or particulate structure throughout their application. Pigment dyeing is achieved from a dispersed aqueous solution and therefore requires the use of dispersing agents. Pigments are usually used together with thickeners in print pastes for printing diverse fabrics. Most pigment dyes are azo compounds (yellow, orange and red) or metal complex phthalocyanines (blue and green). Also anthraquinone and quinacridone pigment dyes are applied.

I. 3. 9 Vat dyes

Vat dyes are water insoluble dyes that are particularly and widely used for dyeing cellulose fibers. The dyeing method is based on the solubility of vat dyes in their reduced (leuco) form. Reduced with sodium dithionite, the soluble leuco vat dyes impregnate the fabric. Next, oxidation is applied to bring back the dye in its insoluble form. Almost all vat dyes are anthraquinones or indigoids. Indigo itself is a very old example of a vat dye, with about 5000 years of application history. ‘Vat’ refers to the vats that were used for the reduction of indigo plants through fermentation.

I. 3. 10 Anionic dyes and ingrain dyes

Azoic dyes and Ingrain dyes (naphthol dyes) are the insoluble products of a reaction between a coupling component (usually naphthols, phenols or acetoacetylamides; listed in the color index as C.I. azoic coupling components) and a diazotised aromatic amine (listed in the color index as C.I. azoic diazo components). This reaction is carried out on the fiber. All naphthol dyes are azo compounds.

I. 3. 11 Sulphur dyes

Sulphur dyes are complex polymeric aromatics with heterocyclic S-containing rings. Though representing about 15% of the global dye production, sulphur dyes are not so much used in Western Europe. Dyeing with sulphur dyes involves reduction and oxidation, comparable to vat dyeing. They are mainly used for dyeing cellulose fibers.

I. 3. 12 Solvent dyes

Solvent dyes (lysochromes) are non-ionic dyes that are used for dyeing substrates in which they can dissolve, e.g. plastics, varnish, ink, waxes and fats. They are not often used for textile – processing but their use is increasing. Most solvent dyes are diazo compounds that underwent some molecular rearrangement. Also triarylmethane, anthraquinone and phthalocyanine solvent dyes are applied.

I. 3. 13 Fluorescent brighteners

Fluorescent brighteners (or bluing agent) mask the yellowish tint of natural fibers by absorbing ultraviolet light and weakly emitting visible blue. They are not dyes in the usual sense because they lack intense color. Based on chemical structure, several different classes of fluorescent brighteners are discerned: stilbene derivatives, coumarin derivatives, pyrazolines, 1,2-ethene derivatives naphthalimides and aromatic or heterocyclic ring structures. Many fluorescent brighteners contain triazinyl units and water solubilising groups.

I. 3. 14 Other classes of dyes

Apart from the dye classes mentioned above, the color index also lists Food dyes and Natural dyes. Food dyes are not used as textile dyes and the use of natural dyes (mainly anthraquinone, indigoid, flavenol, flavone or chroman compounds that can be used as mordant, vat , direct, acid or solvent dyes) in textile-processing operations is very limited.

I.4 Azo Dyes

It is the largest group of dyes, with -N=N- as a chromophore, in an aromatic system. Almost all azo dyes are made by diazotization of primary aromatic amine in an acidic solution by using nitrous acid in the present of ice. The diazo compound so formed is coupled with a suitable component such as an aromatic amine, nepthol or other phenolic substance to form an azo compound. Depending upon the number of azo groups present they are called as monoazo, diazo, triazo, tetrakisazo and polyazo dyes. All types of azo dyes amount to over one thousand commercially most important class of synthetic coloring compound. It has wide variety of application.

Synthetic dyes are recalcitrant to microbial attack because they contain subtituents such as azo, nitro or sulfo groups. Reactive azo dyes are very soluble by design and as a result, not all are used up by textile fibers during the dyeing process and therefore end up with the discharge from dye houses **(Nattapun, S. and et al., 2001)**. A wide variety of dyes are used by industry and released into the environment in industrial effluents. These dyes have to be highly stable in everyday use and resistant to microbial degradation. Azo dyes are the largest class of dyes used in industry. In general, bacteria are not able to degrade azo dyes. However, some anaerobic bacteria in intestinal micro flora have been demonstrated to degrade a few azo dyes. Under these conditions the azo linkage is reduced to generate aromatic amines that are colorless but can also be toxic and potentially carcinogenic. Azo dyes coupled with the fact that azo dyes are not readily degraded in conventional aerobic treatment systems makes this class of xenobiotics a significant environmental problem **(Pogga, U. and et al., 1986)**. Generally physico chemical methods are applied for the treatment of this kind of wastewaters achieving high azo dye removal efficiencies. However, the main drawback of these methods is the cost on the other hand, biological systems could be the most cost-effective method to treat azo dye bearing wastewaters. The mineralization or complete biodegradation of an organic molecule in water is always a consequent of microbial activity **(Alexander, M. and et al.,1966)**. Bacterial degradation is usually initiated by anaerobic reduction of the azo linkage to generate aromatic amines **(Brown and Laboureur, 1983)**. The aromatic amines are generally not metabolized further in anaerobic conditions **(Brown et al., 1983; Field et**

al.,1985). Consequently an aerobic post treatment step would be required for the complete mineralization of azo dyes **(Brown et al., 1987; Field et al., 1995)**.

I. 4. 1 Properties and uses of azo dyes

Azo dyes are used by a wide number of industries. While textile mills predominantly use them, azo dyes can also be found in the food, pharmaceutical, paper and printing, leather and cosmetics industries. It is not surprising that these compounds have become a major environmental concern. Many of these dyes find their way into the environment via wastewater facilities. Because these compound retain their color and structural integrity under exposure to sunlight, soil, bacteria and sweat, they also exhibit a high resistant to microbial degradation in wastewater treatment systems. There is a continual demand to develop longer lasting applicable dyes. Azo dyes are second only to polymers in terms of the number of new compounds submitted for registration in the U.S. under the Toxic substance control Act (TSCA) **(Brown and DeVito, 1993)**. The development of synthetic fabrics such as nylon, lycra, rayon and polyester has required the production of new dyes that can effectively bond to these materials. The U.S. department of commerce has predicted a 3.5 fold increase in textile manufacturing between 1975-220 **(Ganesh, 1992)**. Azo dyes must be continually updated to produce colors that reflect the trends dictated by changing social ideas and styles.

Among the synthetic dyes, which are widely used for textile dyeing and other industrial applications, those containing an azo chromophore constitute the largest class **(Zollinger, 1987)**. According to **Pogga** and **Brown (1986);** and **Shaul et al. (1991)**, these dyes are designed in such a manner that they remain to be resistant to various physical, chemical, and biological attacks. Therefore, they remain, mostly, undegraded by the conventional processes of wastewater treatment. Further, **Chung** and **Stevens (1992)** and **Pierce (1994)** reported that the percentage of unchanged dye after such treatment estimated ranged between 50 and 90%. This fact raises environmental problems.

Azo (N=N) bridge is an efficient p- electron segment. Chromophores containing the donor p- electron acceptor (where p represent electron conjugated bridge) change transfer structure

usually exhibit large molecular first hyperpolarizability. Electron conjugated bridge, donor and acceptor play important role in this kind of system **(Ling Qiu et al.,2004)**.

I.4.2 Dyes: Recalcitrant xenobiotic compounds

I.4.2.1 Chemical properties of dyes

- A dye is used to impart color to materials of which it becomes an integral part. An aromatic ring structure coupled with a side chain is usually required for resonance and thus to impart color.

- Resonance structure that causes displacement or appearance of absorption bands in the visible spectrum of chemical structure with color has been accomplished in the synthesis of dye using a chromogen chromophore with auxochrome. Chromogen is the aromatic structure containing benzene, naphthalene, or anathracene rings. Chomophore group is a color giver and represented by the following radicals, which from the chemical basis of classification of dyes when coupled with chromogene: azo (-N=N-); carbonyl (=C=O); carbon (=C=C=); carbon-nitrogen (>C=NH or –CH=N-) or (CH=N-); nitroso (=NO or N-OH); niro (NO2 or =NO-OH); sulfur (>C=S and other carbon sulfur groups).

- The chromogen-chromophore structure is often not sufficient to impart solubility and causes adherence of dye to fiber. The auxochrome or bounding affinity, and sulfonic radicals or their derivatives **(Anonymous, 1980)**.

- Electrophilic aromatic substitution or EAS is an organic reaction in which an atom, usually hydrogen, in an aromatic system is replaced by an electrophilie. The most important reaction of this type that take place aromatic nitrogen, aromatic halogenations, aromatic sulfonation, and acylation and alkylating Friedel - craft reactions.

- The principal problem to overcome in aromatic degradation is the chemical stability of the aromatic ring.

Biological dye removal techniques are based on microbial transformation of dyes. Though the chemical structure of dyes designed in such away that they are stable and log-lasting colorants, but many reports have been published demonstrated partial or complete biodegradation of dyes by pure and mixed cultures of bacteria.

I.4.2.2 Impact of azo dyes on health and the environment

The potential for toxic effects to the environment and humans, resulting from the exposure to dyes and dye metabolites is not a new concern. As early as 1895 increased rates in bladder cancer were observed in workers involved in dye manufacturing. Since that time, many studies have been conducted showing the toxic potential azo dyes. As mentioned previously azo dyes primarily composed of aromatic amines. Substituted benzene and naphthalene rings are common constituents of azo dyes and have been identified as potentially carcinogenic agents. While most azo dyes themselves are non-toxic a significantly larger potion of their metabolites are **(Ganesh, 1992)**. An investigation of several hundred commercial textile sample revealed that nearly 10 percent were mutagenic in the Ames test **(MeCarthy, 1997)**. Another study conducted on 45 combined effluents from textile finishing plants showed that 27 percent of the wastewater samples were mutagenic in the Ames test **(MeCarthy, 1997)**. Other concerns are the impurities within commercial dye products and the additives used during the dyeing process. Many textile effluents contain heavy metals that are complexed in the azo dyes. High concentrations of salt often used to force Fiber-reactive dyes out of solution and onto substrates **(Zolllinger, 1991)**. These compounds can cause high electrolyte and conductivity concentrations in the dye wastewater, leading to acute and chronic toxicity problems. Understanding the dye structures and how they are degraded is crucial to understanding how toxic by products are created. **Brown** and **DeVito (1993)** have compiled a three-part list of the biological mechanisms thought to be responsible for carcinogenic activation of azo dye compounds. This list is based on an extensive review of the literature regarding azo dye toxicity and places each mechanism in order of their frequency of citation. **Brown** and **DeVito (1993)** postulate that: Azo dyes may be toxic only after reduction and cleavage of the azo linkage, producing aromatic amines. Azo dyes with structures containing free aromatic amine groups that can be metabolically oxidized without azo reduction may cause toxicity. Azo dye toxic activation may occur following direct oxidation of the azo linkage producing highly reactive electrophilic diazonium salts.

CHAPTER II: REVIEW OF LITERATURES

II.1 Review of the present status on degradation of xenobiotics

II.1.1 Microbes as biodegraders

Microbes (bacteria and fungi) found in natural waters and soils between them a very broad ability to utilize (catabolism) virtually all naturally occurring compounds as their sources of carbon and energy, thus recycling the fixed, organic carbon back into harmless biomass and CO_2. The breadth of this catabolic ability of microbes far exceeds the hugely more limited abilities of higher organisms. Which can only use a relatively small range of compounds as foodstuffs. This capability of microbes have evolved over 3 billion years of the planets history and is responsible for the balance between photosynthesis, fixing CO_2 into biomass and respiration, converting organic compounds back to CO_2 by oxidation. This allows the limited amount of carbon available for life to be recycled (the carbon cycle). In more recent times microbial degradation has been channeled into technologies for the degradation of natural household wastes by the use of cesspits, septic tank and sewage works. Biological treatment system may have promising applications for the removal of azo dye compounds since it is widely reported that the azo dyes are gratuitously reduced by anaerobic sludges, anaerobic bacterial enrichment cultures **(Brown,D. et al., 1987)**. On the other hand azo dye compounds are resistant to oxygenolytic attack. **Pogga** and **Brown (1986)** and **Shaul et al. (1991)** tested the degradation of more than 100 dyes in aerobic activated Sludge systems and found that only a few of them were actually biodegraded.

Microorganism have the capability of degrading all naturally occurring compounds this is known as the "Principal of microbial infallibility" proposed by **Alexander** in **1965**. Microorganisms are also able to degrade many of the xenobiotic compounds, but they are usable to degrade many other. The compounds that resist biodegradation and thereby persist in the environment are called recalcitrant. Physical, chemical and physiochemical methods are available to treat these colored effluents are extensive and do not provide satisfactory results. Biological treatment methods are cheap and offer the best alternative

with proper analysis and environmental control. Almost all wastewater can be treated biologically.

II.2 Concept of Biodegradation

Biodegradation is the term used to describe the breakdown of organic molecules by biological action of a living thing. In the environmental context, generally microorganisms are the most important and powerful agents for biodegradation. The heterotrophic microbes are often considered to be of prime importance. Autotrophic algae and cyanobacteria can catalyze some biodegradative processes but are probably of limited importance. Varying degrees of biodegradation can occur according to how much the compound has been modified.

II. 2.1 Primary biodegradation

This term is used to describe processes in which a compound lose its characteristic properties but may be little altered in terms of size or complexity. The compound may loss some characteristics and may no longer respond in particular assay. For example, the reductive decolorization of an azo dye. This primary biodegradation leads to a change in color (**discolorization**) or its removal (**decolorization**), but two aromatic amines are released.

Sulphonated monoazo dye

4 H **Anaerobic process**

Sulphanilic acid

Dimethylphenylenediamine

II.2.2 Mineralization

It is synonymous with ultimate or complete biodegradation. It describes the degradation of a compound to its mineral components, viz. CH_4, CO_2, H_2O, NH_3, SO_4, NO_3, NO_2, PO_4, Cl, F etc. Generally in this reaction, out of the net mass of the chemical, 50% to 80% of the carbon is converted to CO_2.

II.2.3 Co-metabolism

This term is used (and after mis-used) to apply to a range of different types of degradative process. Perhaps the best way to describe is the degradation of a compound in the obligate presence of another compound(s). It is used to describe a situation in which a microbial culture is only able to degrade a substance in the presence of other compounds. These compounds may be specific in some cases while in others a wide variety of co-substrates may be suitable.

Specific co-substrates are required in the case of a process often called analogue enrichment. This is when the recalcitrant compound and the co-substrate are structurally related and the co-substrate has a role in inducing the production of some, or all, of the enzymes required to degrade to the recalcitrant compound which cannot itself act as an inducer. This process can only work if the degradative enzymes are sufficiently non-specific to degrade compounds other than their natural substrate.

Non-specific co-metabolism generally occurs in cases in which the degrading organisms derive no obvious benefit from the degradation of the recalcitrant compound. The degrading organism can not use energy or carbon resulting form the degradation process and therefore needs to be provided with a source of carbon and energy on which they can grow. Such processes are sometime referred to as gratuitous degradation (i.e. it is for free) or alternatively as fortuitous or accidental degradation (i.e. it occurs by chance).

II.2.4 Presence of suitable organism(s)

For a compound to be degraded the presence of suitable organisms is essential. By **suitable** it means organisms that already have some ability to degrade the target compound. These suitable organisms are often present in very small numbers and until a large population of suitable organism has developed, little degradation will occur. The lag period before degradation is observed and once the microbial population reaches to a **critical mass**, the process of degradation is observed.

II.2.5 Presence of oxygen

Oxygen plays an important role in microbial growth. Its presence in the medium can profoundly influence the metabolic activity of aerobic and facultative organisms.

- The main use of oxygen is as a terminal electron acceptor during respiration in aerobic growth. Biological oxidations often involve the removal of hydrogen ions (H^+) and electrons (e^-) [reducing equivalent] which must be coupled to an oxidizing agent – the terminal electron acceptor. In this process energy is produced and intermediate electron acceptors are regenerated so that more oxidation can occur. Strict aerobes can not grow without oxygen, and if it is in short supply, their growth will be slower than normal and metabolism may be atypical. Thus, in the absence of oxygen, biodegradative reactions that depend on strict aerobes will not occur.

- Oxygenation is a process, where oxygen reacts directly as a substrate, i.e. molecular oxygen is incorporated into an organic molecule. Such reactions are found in many living organisms, used to activate a relatively inert molecule (such as lignin or methane or ammonium) and lead to further degradation.

- At one time it was thought that O_2 was essential for degradation of most recalcitrant xenobiotic compounds – it is now known that it is not the case. Indeed many compounds are degraded preferentially, if not uniquely, in anaerobic

conditions. For example, reductive decolorization of an azo dye by anaerobic microbes.

II.3 Review of the decolorization processes/techniques

Various physical, chemical and biological pre treatment, main treatment and post treatment techniques can be employed to remove color from dye containing wastewaters **(Cooper.P., 1993; Grau,P., 1991; Hao,O.J., et al., 2000)**.

II. 3.1 Physico-chemical process of decolorization

II. 3.1.1 Membrane filtration

Nano-filtration and reverse osmosis, using membranes with a molecular weight cut-off (MWCO) below ~10,000 Dalton, can be applied as main or post treatment processes for separation of salts and larger molecules including dyes from dye bath effluents and bulk textile-processing wastewaters.

II. 3.1.2 Coagulation/ flocculation

Coagulation/flocculation is often applied in the treatment of textile-processing wastewater, either to party remove chemical Oxygen Demand (COD) and color from the raw wastewater before further treatment **(Altinbas,U., et al., 1995)** to polish the final effluents of biologically or otherwise treated wastewater **(Marmagne and Coste, 1996)** or even as the main treatment process. The principle of the process is the addition of a coagulant followed by a generally rapid chemical association between the coagulant and the pollutants. The thus formed coagulates or flocs subsequently precipitate or are to be removed from the water phase by flotation. Various inorganic coagulants are used, mostly lime, magnesium, iron and aluminum salts.

II. 3.1.3 Sorption and ion exchange

Activated carbon or other materials can be used to remove dyes from wastewater, either by adsorption (anionic dyes) or by combined adsorption and ion exchange (cationic dyes). Sorption techniques yield waste sludge, i.e. dye-saturated material that should be disposal off or regenerated.

II. 3.1.4 Electrolysis

Electrolysis is based on applying an electric current through to the wastewater to be treated by using electrodes.

II. 3.1.5 Advance oxidation processes

Advanced oxidation can be defined as oxidation by compounds with an oxidation potential (E_0) higher than that of oxygen (1.23 V), i.e. hydrogen peroxide (E_0 = 1.78 V), ozone (E_0 = 2.07 V) and the hydroxyl radical (E_0 = 2.28 V).

II. 3.2 Biological processes

Biological dye removal techniques are based on microbial biotransformation of dyes. As dyes are designed to be stable and long-lasting colorants, they are usually not easily biodegraded. Nevertheless, many researches have demonstrated partial or complete biodegradation of dyes by pure and mixed cultures of bacteria, fungi and algae.

II. 3.2.1 Role of bacteria

Aromatic compounds are susceptible to biological degradation under both aerobic and anaerobic conditions **(Field et al., 1995)**. Under aerobic conditions, the enzymes mono- and dioxygenase catalyse the incorporation of oxygen from O2 into the aromatic ring of organic compounds prior to ring fission **(Madigan et al., 2003)**. In most monooxygenases, the electron donor is NADH or NAD(P)H, even though the direct coupling to O2 is through a flavin that is reduced by the NADH or NAD(P)H donor

(Madigan et al., 2003). Although azo dyes are aromatic compounds, their substituents containing mainly nitro and sulfonic groups, are quite recalcitrant to aerobic bacterial degradation **(Claus et al., 2002)**. This fact is probably related either to the electron-withdrawing nature of the azo bond and their resistance to oxygenases attack, or because oxygen is a more effective electron acceptor, therefore having more preference for reducing equivalents than the azo dye **(Chung and Cerniglia, 1992; Knackmuss, 1996)**. However, in the presence of specific oxygen-catalysed enzymes called azo reductases, some aerobic bacteria are able to reduce azo compounds and produce aromatic amines **(Stolz, 2001)**. Examples of aerobic azo reductases were found in *Pseudomonas* species strains K22 and KF46 **(Zimmermann et al., 1982; Zimmermann et al., 1984)**. These enzymes, after purification, characterization and comparison were shown to be flavin-free. The aerobic azo reductases were able to use both NAD(P)H and NADH as cofactors and reductively cleaved not only the carboxylated growth substrates of the bacteria but also the sulfonated structural analogues. Recently, Blümel and Stolz (2003) cloned and characterized the genetic code of the aerobic azo reductase from *Pagmentiphaga kullae* K24. This strain was able to grow with the carboxylated azo compound 1-(4'-carboxyphenylazo)-4-naphtol as a sole source of carbon and energy. Furthermore, the gene encoded a protein with a molecular weight of 20,557 Da, having conserved a putative NAD(P)H-binding site in the amino-terminal region.

II. 3.2.2 Role of fungi

The capacity of fungi to reduce azo dyes is related to the formation of exoenzymes such as peroxidases and phenoloxidases. Peroxidases are hemoproteins that catalyse reactions in the presence of hydrogen peroxide **(Duran et al., 2002)**. Lignin and manganese peroxidases have a similar reaction mechanism that starts with the enzyme oxidation by H_2O_2 to an oxidized state during their catalytic cycle. Afterwards, in a mechanism involving two successive electron transfers, substrates such as azo dyes reduce the enzyme to its original form **(Stolz, 2001)**.

Eighteen fungal strains able to degrade lignocellulosic material or lignin derivatives were tested with the azo dyes Reactive Orange 96, Reactive Violet 5 and Reactive Black 5.

Only the strains of *Bjerkandera adusta*, *Trametes versicolor* and *Phanerochaete chrysosporium* were able to decolorise all azo dyes **(Heinfling et al., 1997)**. Although lignin peroxidases are able to oxidize both phenolic and nonphenolic aromatic compounds, manganese peroxidases must convert Mn+2 to Mn+3 in order to oxidize phenolic compounds **(Glenn et al., 1986)**. Phenoloxidases, which can be divided into tyrosinases and laccases, are oxidoreductases that can catalyse the oxidation of phenolic and other aromatic compounds without the use of cofactors **(Duran et al., 2002)**. Laccases are copper-containing enzymes that have very broad substrate specificity with respect to electron donors, e.g. dyes **(Abadulla et al., 2000)**. However, despite the fact that laccases from *Trametes Versicolor*, *Polyporus pinisitus* and *Myceliophthora thermophila* were found to decolorise anthraquinone and indigoid-based dyes at high rates, the azo dye Direct Red 29 (Congo Red) was a very poor substrate for laccases **(Claus et al., 2002)**. **Chivukula** and **Renganathan (1995)** cited that the azo dye must be electron-rich to be susceptible to oxidation by laccase of *Pyricularia oryzae*. This situation is suitable for the generation of a phenoxy radical, with consequent azo bond cleavage, and the release of molecular nitrogen. The addition of redox mediators has been shown to further extend the substrate specificity of laccases with regard to several dye classes, although redox mediators can also be formed from laccase oxidation of phenolic azo dyes **(Li et al., 1999; Soares et al., 2001; Claus et al., 2002)**.

II.3.2.3 Strictly anaerobic and / or facultative bio-decolorization

Under anaerobic conditions a low redox potential (< -50 mV) can be achieved, which is necessary for the effective decolorisation of dyes **(Beydilli et al., 1998; Bromley-Challenor et al., 2000)**. Colour removal under anaerobic conditions is also referred as dye reduction in which literature mostly covers the biochemistry of azo dye reduction. The azo bond cleavage - N- N- involves a transfer of four-electrons (reducing equivalents), which proceeds through two stages at the azo linkage. In each stage two electrons are transferred to the azo dye, which acts as a final electron acceptor:

$$(R_1—N{=}N—R_2) + 2e^- + 2H^+ \rightarrow R_1—HN—NH—R_2$$

(*Hydrazo intermediate*)

$$(R_1—HN—NH—R_2) + 2e^- + 2H^+ \rightarrow R_1—NH_2 + R_2—NH_2$$

(*Reductive cleavage of the azo bond*)

Anaerobic Conditions

The exact mechanism of azo dye reduction, whether occurring intracellularly or extracellularly, is still a subject of investigation, as is the role of biogenic intracellular water-soluble electron carriers such as flavins. Reduced flavins can act as an electron shuttle from nicotinamide adenine dinucleotide phosphate (NADPH)-dependent flavoproteins to azo dye as electron acceptor **(Gingell and Walker, 1971)**. Intracellular azo dye reduction cannot be responsible for the conversion of all types of azo dyes, especially for sulfonated azo dyes, which have limited membrane permeability **(Stolz, 2001)**. **Kudlich et al. (1997)** demonstrated an increase on colour removal rates of sulfonated azo dyes by cell free-extracts, as well as after addition of toluene, i.e. a membrane-active compound which increases cell lysis, thus showing the limited membrane permeability of this type of dye. The current hypothesis is that azo dye reduction mostly occurs by extracellular or membrane-bond enzymes **(Stolz, 2001)**. Reduced cytoplasmic cofactors such as reduced flavins do not contribute to the chemical dye reduction due to their inability to cross living cell membranes **(Russ et al., 2000)**. However, cell fractionation experiments demonstrated that a quinone reductase activity located in the cell membranes enhanced the reductive decolorisation of a sulfonated azo compound, and no dye cross-membrane transport was required **(Kudlich et al., 1997)**. Recently, a NADH-dependent lawsone reductase activity located in the cytosolic fraction of *Escherichia coli* also showed the capacity for azo dye reduction **(Rau and Stolz, 2003)**.

The reductive decolorisation of azo dyes under anaerobic conditions is a combination of both biological and chemical mechanisms. The biological contribution can be divided in

specialized enzymes called azo reductases, which are present in bacteria that are able to grow using only azo dye as a carbon and energy source. However, up to date there is no clear evidence of anaerobic azo reductase; or non-specific enzymes that catalyse the reduction of a wide range of electron-withdrawing contaminants, including azo dyes **(Stolz, 2001)**. Thus, a co-metabolic reaction is probably the main mechanism of dye reduction **(Figure 1.1)**, in which the reducing equivalents or reduced cofactors like NADH, NAD(P)H, $FMNH_2$ and $FADH_2$ acting as secondary electron donor, channel electrons to cleave the azo bond **(Gingell and Walker, 1971)**. The chemical contribution to the reductive decolorisation of azo dyes under anaerobic conditions may involve biogenic reductants like sulfide, cysteine, ascorbate or Fe^{2+} **(Yoo, 2002)**. **Figure 1.2** shows the electron flow preference in the presence of different redox couples involved in biological processes. Thus, oxygen is a more effective electron acceptor than azo dyes, which justify the low decolorisation rates (10-30%) under aerobic conditions.

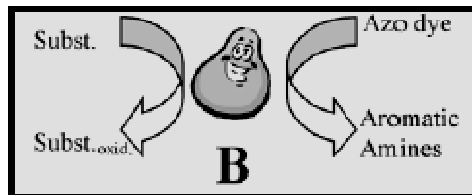


Figure II.1. Co-metabolic reactioninvolved in reductive decolorisationof azo dyes. Legend: Subst., substrateor primary electron donor; Subst.oxid,products of substrate oxidation.

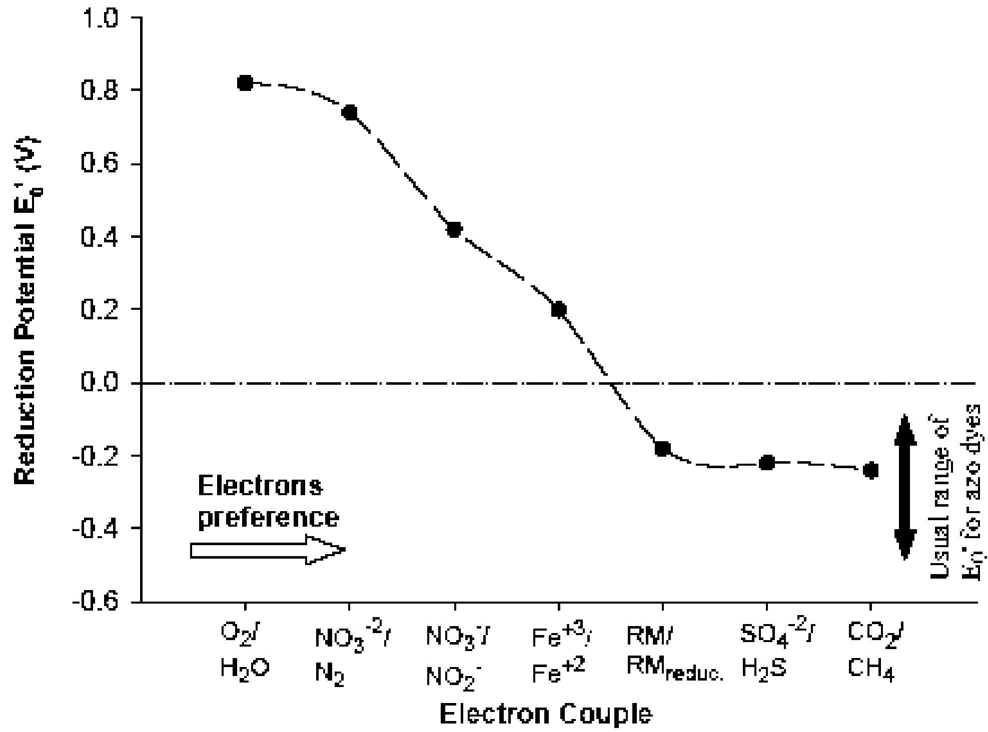


Figure II.2 Electron flow preference as a function of the different electron couples **(Adaptedfrom Dubin and Wright, 1975; Cervantes, 2002; Madigan et al., 2003)**. RM and RMreduc are theoxidized and the reduced form of the redox mediator, respectively.

Decolorisation of textile wastewaters: perspectives for anaerobic biotechnology nevertheless, either by using pure cultures or granular sludge under anaerobic conditions, literature reports poor reductive decolorisation with specific dye s **(Brown and DeVito, 1993; Van der Zee et al., 2001b)**. Furthermore, the rates are extremely dependent on the type of dye, in which the azo dyes generally present the highest rates of decolorisation. On the other hand, anthraquinone and phtalocyanine dyes are shown to be rather recalcitrant. Another drawback is that some dyes are quite toxic to the anaerobic microorganisms, which in some cases, may lead to a permanent loss of the methanogenic activity even for low dye concentrations **(Brown and Laboureur, 1983; Van der Zee et al., 2001a; Fontenot et al., 2003)**. For instance, the inhibitory concentration of RB19, exerting 50% reduction in methanogenic activity (IC-value), was 55 mg/l at 30ºC **(Dos Santos et al., 2005a)**. It is important to mention that a sound comparison among investigations is extremely difficult because of the differences in type and concentrations

of dyes, sludge source and concentrations, electron donor, the way of calculating the decolorisation rates, etc.

II.3.2.4 Role of redox mediators

Redox mediators are compounds that accelerate the electron transfer from a primary electron donor to a terminal electron acceptor, which may increase the reaction rates by one to several orders of magnitude **(Cervantes, 2002)**. Redox mediators have shown to be effective not only for reductive decolorisation, but also for the reductive transformation of iron **(Lovley et al., 1998), nitroaromatics (Dunnivant et al., 1992)**, polyhalogenated compounds **(O'Loughlin et al., 1999)** and radionuclides **(Fredrickson et al., 2000)**. Recently it was found that during the aerobic degradation of naphthalene-2-sulfonate (2NS) by Sphingomonas xenophaga strain BN6, quinoid redox mediators were produced, which mediated the reduction of azo dye under anaerobic conditions **(Keck et al., 2002)**. Flavin-based compounds like FAD, FMN and riboflavin, as well as quinone-based compounds like AQS, AQDS and lawsone, have been extensively reported as redox mediators during azo dye reduction **(Semdé et al., 1998; Cervantes et al., 2000; Rau et al., 2002a; Field and Brady, 2003)**. Reductive decolorisation of azo dyes in the presence of redox mediators occurs in two distinct steps, the first step being a non-specific enzymatic mediator reduction, and the second step being a chemical reoxidation of the mediator by the azo dyes **(Figure 1.3) (Keck et al., 1997)**.

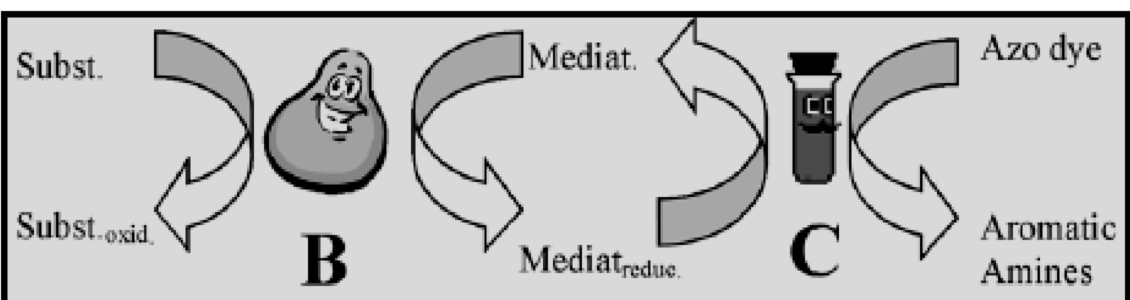


Figure II.3. Co-metabolic reaction involved in reductive decolorisation of azo dyes in the presence of redox mediators. B and C are the biological and chemical steps, respectively.

Theoretically, feasible redox mediators for biological azo dye reduction must have redox potentials between the half reactions of the azo dye and the primary electron donor **(Van der Zee et al., 2003b)**. Unfortunately, the standard redox potential (E0') for most azo dyes is unknown, but this information can be obtained by using polarography. In a screening of redox potential values for different azo dyes, it was found that E0' values are generally between –0.430 and –0.180 V **(Dubin and Wright, 1975)**. **Rau et al. (2002)** cite that the NAD(P)H cofactor, which has the lowest E0' value of –0.320 V, seems to set the limits of redox mediators application. The reason for this is that mediators with a more negative E0' value will not be reduced by the cells, and mediators with E0' greater than –0.05 V will not efficiently reduce the azo bond at high rates.

The standard redox potential value (E0') is a good indication of a compound capacity to function as a redox mediator. However, apparently other factors are of importance as well since different decolorisation rates in the presence of mediators with similar E0' values have been reported, and similar decolorisation rates with mediators with different E0' values. For instance, **Brown (1981)** tested the polymeric nitro dye Poly Y-607 and found that methyl viologen and benzyl viologen increased the decolorisation rates 4.5-fold, even though the E0' of methyl viologen is much lower than that of benzyl viologen, i.e. –0.440 V and –0.360 V, respectively **(Figure 1.4)**. **Walker and Ryan (1971)** postulated that decolorisation rates are related to the electron density in the azo bond region. They suggested that color removal rates would increase by lowering the electron density in the azo linkage. Therefore, the use of redox mediators would not only tend to accelerate the transfer of reducing equivalents to the terminal electron acceptor, i.e. the azo dye, but also to minimize the steric hindrance of the dye molecule **(Bragger et al., 1997; Moir et al., 2001)**. Thus, in estimating the theoretical decolorisation rates by using specific redox mediators, the differences in steric and electro-chemical factors between mediator and azo dye should also be considered.

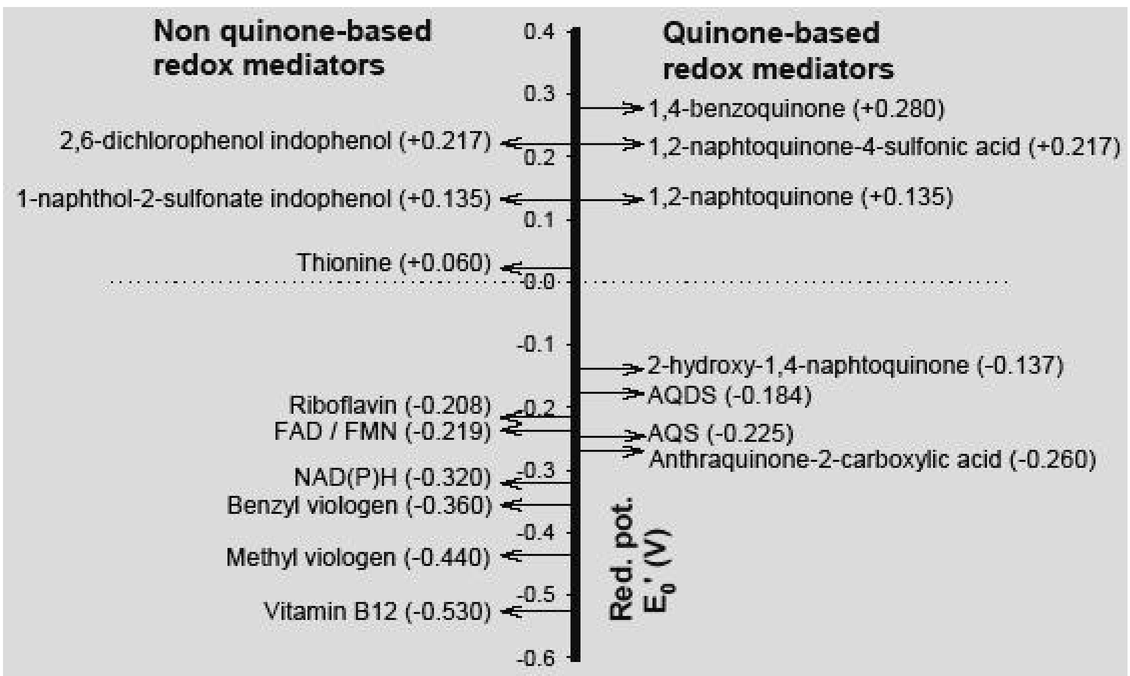


Figure II.4. E_0' values for both quinone-based and non quinone-based redox mediators (Adapted from Rau et al., 2002a; Fultz and Durst, 1982).

II.3.2.5 Dye decolorization by pure cultures and sludge

The literature extensively reports the use of pure cultures **(Table II.1)**, either whole cells or specific enzymes, for a better insight of the anaerobic azo dye reduction mechanisms, which are not fully understood yet **(Stolz, 2001; Pearce et al., 2003)**.

Table II.1 Examples of facultative and strictly anaerobic bacterial cultures, which were able to decolourise azo dyes under anaerobic conditions.

Organism	Dyes	Activity	Decolour. (%)	Comments	Ref.
		μ mol/ml/h			
Clostridium perfringens ATCC 3626	Amaranth	0.74	-	Dye concentration of 0.033 mM.	1
	Methyl Orange	0.62	-		
	Orange II	0.70	-		
	Tartrazine	0.67	-		
		μ mol/ml/h			
Bacteroides fragilis	Amaranth	0.30	66.0	After 6 h of incubation. Dye concentration of 0.1 mM.	2
	Orange II	0.20	37.0		
	Tartrazine	0.08	9.0		
Pseudomonas GM3	Acid Violet 7	-	97.4	After 72 h of incubation. Dye concentration of 100 mg/l.	3
	Reactive Blue 2	-	18.3		
	Acid Green 27	-	75.6		
	Acid Red 183	-	20.1		
	Indigo Carmine	-	69.0		
		μ mol/min/g protein			
Sphingomonas xenophaga BN6	Acid Red 27	0.10	-	Dye concentration of 0.1 mM.	4
	Acid Orange 20	0.10	-		
	Acid Orange 7	0.30	-		
	Acid Red 14	0.20	-		
	Acid Yellow 23	0.10	-		
	Acid Black 1	0.30	-		
		AU x 10^{-2} / mg protein			
Enterococcus faecalis	Methyl Red	1.81	99.4	After 20 h of incubation. Dye concentration of 0.2 mM	5
	Orange II	1.39	95.1		
	Orange G	1.20	64.1		
	Amaranth	1.37	99.5		
Eubacterium biforme	Tartrazine	-	4.0	After 150 minutes of incubation. Dye concentration of 2 mM	6
	Sunset Yellow	-	22.0		
	Methyl Orange	-	79.0		
	Orange II	-	81.0		
	Amaranth	-	19.0		
	Allura Red 40	-	11.0		

[1]Semdé et al., 1998; [2]Bragger et al., 1997; [3]Yu et al., 2001; [4]Rau et al., 2002a; [5]Chen et al., 2004; [6]Chung et al., 1978.

Microbial decolorisation requires an unspecific enzymatic capacity ubiquitously found in a wide diversity of microorganisms **(Chung and Stevens, 1993)**. This has been mainly demonstrated with microorganisms present in the intestine such as *Clostridium, Salmonella, Bacillus, Eubacterium* and *Escherichia coli*, which are able to reduce the dyes ingested through food, drugs and cosmetics **(Brown and DeVito, 1993; Rau et al., 2002a; Chen et al., 2004)**. The understanding of azo dye reduction mechanisms is important not only under a biotechnological approach toward the use of biological processes for decolorisation, but also under a medical approach to have an insight into how the intestinal microflora metabolites the ingested azo dyes **(Brown and DeVito, 1993; Semdé et al., 1998)**. Azo dyes are converted into aromatic amines because of both the presence of microflora and the anaerobic condition found in the human intestine. Aromatic amines present a mutagenic and carcinogenic character much higher than their precursor azo dyes **(Weisburger, 2002)**. Therefore, a lot of effort has been made in the production of compounds, which are resistant to these reductive transformations. Another approach has been investigated in the use of azo polymers that would be insoluble in the upper gastrointestinal track, but susceptible to degradation on the colon, acting as an oral colon-specific drug delivery system **(Bragger et al., 1997; Rau et al., 2002b)**. In this review we will focus on the biotechnological approach of azo dye reduction.

Even though anaerobic azo dye reduction could be readily achieved with different microorganisms, there is no strain reported so far that is able to decolorize a broad range of azo dyes. Therefore, the use of a specific strain or enzymes on reductive decolorisation does not make much sense in treating textile wastewater, which is composed of many kinds of dyes **(Laszlo, 2000)**. The use of mixed cultures such as anaerobic granular sludge, which is composed of stable microbial pellets with a high activity, is probably a more logic alternative. Different reactor configurations like the widely used upflow anaerobic sludge bed (UASB) system and expanded granular sludge bed (EGSB) system, are used to immobilize high concentrations of biomass **(Lettinga et al., 1980; Lettinga, 1995; Van Lier et al., 2001)**. Indeed, the different microbial consortia present in anaerobic granular sludge can carry out tasks that no individual pure culture can undertake successfully **(Nigam et al., 1996; Pearce et al., 2003)**. However, little is

known about the microbiological aspects of the reductive decolorisation of azo dyes with anaerobic consortia commonly found in wastewater treatment plants, although the applicability of the cost-effective high-rate anaerobic reactors 28 Decolorisation of textile wastewaters: perspectives for anaerobic biotechnology for azo dye reduction has been well demonstrated **(Cervantes et al., 2001; Dos Santos et al., 2003; Dos Santos et al., 2004c)**.

As previously explained, the reductive decolorisation of azo dyes by using methanogenic anaerobic granular sludge is very likely controlled by a co-metabolic reaction in the presence of different electron donors, in which the azo dye is the terminal electron acceptor of the reduced cofactors. **Table II.2** shows the main biochemical reactions involved in the conversion of organic matter in methanogenic environments.

Table II.2 Biochemical reactions involved in the conversion of organic matter in methanogenic environments

Acetogenic reactions			?G° '25 °C (kJ mol-1)	?G° '55 °C (kJ mol-1)
Glucose + 12 H_2O	?	6 HCO_3^- + 12 H_2 + $6H^+$	+ 3.2	-51.8
Glucose + 4 H_2O	?	2 Acetate$^-$ + 2 HCO3$^-$ + $4H_2$ + $4H^+$	- 206.3	-232.2
Ethanol + H_2O	?	Acetate$^-$ + 2 H_2 + H^+	+ 9.6	+1.7
Lactate- + 2 H_2O	?	Acetate$^-$ + HCO_3^- + $2H_2$ + H^+	- 4.2	-12.6
Acetate- + 4 H_2O	?	2 HCO_3^- + 4 H_2+ H^+	+ 104.2	+ 89.8
Propionate$^-$ + 3 H_2O	?	Acetate$^-$ + HCO_3^- + $3H_2$ + H^+	+ 76.5	+ 64.7
Propionate$^-$ + 2 HCO_3^-	?	Acetate$^-$ + 3 Formate$^-$ + H^+	+ 72.4	+ 61.6
Butyrate$^-$ + 2 H_2O	?	2 Acetate$^-$ + 2 H_2+ H^+	+ 48.3	+ 39.5
Butyrate$^-$ + 2 HCO_3^-	?	2 Acetate$^-$ + 2 Formate$^-$ + H^+	+ 30.6	+ 20.9
Homoacetogenic reactions				
Acetate$^-$ + 4 H_2O	?	2 HCO3$^-$ + 4 H_2 + H^+	+ 104.6	+ 90.2
2 CO_2 + 4 H_2	?	Acetate$^-$ + 2 H_2O	- 55.0	- 33.5
Methanogenic reactions				
4 H_2 + HCO_3^- + H^+	?	CH_4 + 3 H_2O	- 135.6	- 124.9
4 Formate- + H_2O + H^+	?	CH_4 + $3HCO_3^-$	- 130.4	- 118.9
Acetate$^-$ + H_2O	?	HCO_3^- + CH_4	- 31.0	- 34.7

Energy changes were calculated by using the van 't Hoff equation, standard enthalpy values of compounds **(Chang, 1977)** and Gibbs free-energy changes at 25°C **(Thauer et al., 1977)**.

II.3.2.6 The role of yeast in dye decolorization

Decolorization of azo dyes by yeasts is much less studied than the homologous process mediated by bacterial and mold species. Compared to bacteria and filamentous fungi, yeasts have many advantages. They not only grow rapidly like bacteria, but like filamentous fungi they also have the ability to resist unfavorable environments **(Zhisheng Yu, Xianghua Wen, 2005)**. Furthermore, some yeasts have been found to be efficient in treating high strength organic wastewaters such as food industry effluents **(Yang et al., 2003)**. To the present, however, the use of yeast strains in treating dye wastewater has been very limited and most of the available reports on dye decolorization by yeasts documented biosorption of dye on yeast biomass. For example, **De Angelis, F. E., and Rodrigues, G. S., 1987** have shown that species of *Candida* can remove different azo dye more than 90% (initial concentration100 mg/L) by biosorption from culture media within 2 h. Similarly thermo tolerant yeast, *K. marxianus* IMB3 exhibits biosorption of diazo dye, Ramazol Black-B under aerobic condition at acidic pH and room temperature **(Meehan, C., et al., 2000). Safarik, I., et al., (2002)** revealed biosorption of various azo dyes and triphenyl methane dyes on magnetically labeled *Saccharomyces cerevisiae.* While **Donmez, G., (2002)** reported bioaccumulation of the reactive textile dyes by *C. tropicalis* growing in molasses medium.

However, few authors also reported decolorization of azo dyes by different species of yeasts with probable involvement of an azo reductase enzyme for the reduction of azo linkage. For instance, **Kakuta, A., et al., (1998)** had purified two different azo reductases from *Candida curvata* that were capable of azo dye decolorization, and **Ramalho, P. A., et al., (2004)** characterized azo reductase activity in *Issatchenkia occidentalis* responsible to decolorize several monoazo dyes under microaerophilic conditions**.** Most yeast capable of dye decolorization requires aerobic or microaerophilic environment for azo bond reduction, but rejects anaerobic environment to complete dye decolorization process.

Several authors in their recent reports on dye decolorization described this situation by yeast. **Martins, A. M., et al., (1999);** and **Ramalho, P. A., et al., (2002)** shown that *Candida zeylanoides* can degrade various azo dyes under appropriate aerobic conditions. **Marco S. L., et. al. 2005** isolated phenolic acid assimilating non-conventional yeast *Candida oleophila* that completely decolorized Reactive Black 5 up to 200 mg L^{-1} within 24h of aerobic incubation. **Zhisheng Yu** and **Xianghua Wen (2005)** have isolated two yeast species *Pseudozyma regulosa* and *Candida krusei* that were capable of reducing azo dye; Reactive Brilliant Red K-2BP (200mg L^{-1}) and other six azo dyes completely within 24 h but in microaerophilic conditions.

Yeasts also exhibit its degradation versatility for textile dyes. Because aside azo dye decolorization, certain species of yeast can also decolorize anthraquinone dyes and others. **Yang, Q., et al., 2003** have reported that manganese-dependent peroxidase (MnP) activity of yeast strains, *Debaryomyces polymorphus* and *Candida tropicalis* is responsible for decolorization of Reactive Black B and other azo dyes as well as anthraquinone dye completely within 48 h and **Itoh, K., et al., (1996)** have elucidated the pathway for the biodegradation of an anthraquinone dye C.I. Dispersed Red 15, by yeast strain *Pichia anomala*. **Kwasniewska, K., (1995)** had reported biodegradation of tryphenyl methane dye, crystal violet (hexamethyl – p – ros- aniline chloride) by oxidative red yeast.

Even yeast culture, in immobilized form had also been tested for the treatment of azo dye wastewater in cost-effective manner (**Kakuta, T., et. al. 1992),** albeit the strain must be non-pathogenic. However, most literature related to the role of yeast in dye decolorization, represents *Candida* and other pathogenic yeast strains that may cause health related problems for humans if applied in the treatment of textile dye containing effluents. And as per our informations there is no detailed report on the decolorization of azo dyes by simple, nonpathogenic and novel ascomycete yeast *S. cerevisiae*. Although **Ramalho P. A., et. al. (2005),** using gene cloning approach had tried to elucidate the involvement of ferric reductase of *S.cerevisiae* in decolorization of azo dye.

CHAPTER III: AIMS AND OBJECTIVES

III.1 Aims

Many microbial strains have been studied for decolorization of azo dyes. But their mechanism to attack and reduce azo bond to decolorize the dye is quite different. Bacteria being prokaryotes operates their electron transport chain on their plasma membrane for respiration and production of ATP at the expense of proton motive force (PMF). When any bacterium is engaged in reducing azo bond of dye, the reduction equivalent is generated by the proteins of their respiratory chain and donated to azo bond of the dye. This is generally possible in microaerophilic or anaerobic growth conditions. However if the redox potential of their proteins is not sufficient enough, they recruit the help of redox mediator like riboflavin, quinone etc. to parcel the hydrogen up to azo dye.

From the previous studies in our laboratory, it has been shown that several gram positive and gram negative bacteria were capable of decolorizing azo dyes under facultative growth conditions with microaerophilic environment. Further, the whole cell studies also indicated that to decolorize azo dye(s), carbon substrate (common hexose sugars) and growth factors (yeast extract or riboflavin) were essential in the reaction assay mixtures. Also it was noted during the experiments that the conditions were favoring only decolorization and not the growth of the organisms (i.e. increase in biomass). The entire play has been considered as the flux of electrons from the metabolic pools were not directed to the natural electron acceptors but, has been mediated through the redox mediator(s) that ultimately passed on to azo bond of the dye and reduced it. Keeping in view of the above findings the present study was carried out. There are numerous reports, which describe the reduction of azo compounds by bacteria under anaerobic condition. The main interest of this research is to focused on bacteria from the CETP which are involved in the metabolisms of azo dyes

III.2 Objectives

Looking to the aim of decolorization of sulphonated azo dyes and other textile dyes using yeast cultures, following objectives were set in this work.

- To isolate and enriched the biodegrading microorganisms from contaminated environments
- To determine the biodegradation of various xenobiotic compounds (azo dyes)
- To check out the biodegrading potentiality of organisms
- Observe the effect of higher concentration of pollutants on growth kinetic of organisms
- Determination of most optimum factor for potential biodegradation scenarios.
- Process optimization
- The development of skills and techniques for the functional and structural analyses of microbial communities
- To investigate the different process of xenobiotic compounds (azo dyes) degradation

CHAPTER IV: MATERIALS AND METHODS

IV.1 MATERIALS

IV.1.1 Samples for isolation of microbes

Effluent samples for isolation of microorganisms were obtained from Common Effluent Treatment Plant (CETP) of Jetpur and from the dyeing industries located in Jetpur.

IV.1.2 Chemicals

Chemicals and reagents were obtained from laboratory supplier such as Sigma, E.Mercks, Hi-Media laboratory Ltd., Qualigens Fine Chemicals, SRL and Spectrochem Ltd. All the chemicals were of the highest purity and commercially available. None of the chemicals were purified further. Dyes were obtained from the local markets. Azo dyes obtained were measured for their $?_{max}$ and available C.I. name and structures are given in **Table IV.1**.

Table IV.1 Azo dyes employed in the present study

No.	Dye name	$?_{max}$	C.I. Name
1	RS Red H5BL	515	Reactive Red 35
2	RS Red H5RL	560	Not available
3	Red H5 B1	550	Not available
4	Golden Yellow R	425	Not available
5	Sunset yellow	480	Food yellow 3
6	Congo red	480	Laboratory dyes
7	Ponceau 4R	485	Food red 7
8	Orange G	470	Laboratory dyes
9	Violet 5R	560	Not available
10	Reactive Black B	600	Reactive Black 5
11	Biebrich scarlet	500	Ponceau BS
12	Sunzol Violet 5R	560	Reactive violet 5
13	Methyl red	528	Laboratory dyes

Table IV.2 Chemical structures of the azo dyes with C.I. numbers and name

No	C.I. No	C.I. Name	Chemical Structure
1	16230	Orange G	
2	53907	Food yellow 3	
3	16255	Food red 7	
4	13020	Acid red 2 (Methyl Red)	
5	22120	Congo Red	

6	20505	**Reactive Black 5**	
7	16250	**Ponceau BS**	
8	18097	**Reactive Violet 5 (Sunzol Violet 5R)**	
9		**Reactive Red 35 (RS RED H5BL)**	

IV.1.3 Instruments used for studies

- Spectrophotometer 106 Systronics
- Digital Spectrophotometer 166 Systronics
- Research centrifuge R-24 Remi & Laboratory centrifuge R4C Remi
- Cooling centrifuge Remi
- Orbital environmental shaker
- Microscope Zess Axiovision
- UV-Visible spectrophotometer UV-1601 Shimadzu
- UV cabinet Nova

IV.2 METHODS

IV.2.1 Physico-chemical characteristics of the samples

Total five to six samples **(Table IV.3)** were collected from the various sites of the industries in autoclaved BOD bottles for microbial analysis and polythene bottles for physical and chemical analysis. Some common characteristics of effluent like pH, COD, BOD etc. were tested as per **APHA (Andrew et al., 1995)**.

Table IV.3 Showing physico-chemical properties of the effluent samples

Samples	Color of effluents	pH	COD mg/L	BOD mg/L
1	Red	8	610	315
2	Green	9	595	308
3	Light Green	8.5	580	295
4	Reddish Brown	9.2	608	302
5	Light Green	8.7	576	282
6	Reddish Brown	8.5	582	291

IV.2.2 Enrichment and isolation of dye decolorizing microorganisms

Collected samples were inoculated in the enrichment medium, containing; gl^{-1} : KH_2PO_4 10; yeast extract 5; peptone 5; glucose 2; $MgSO_4.7H_2O$ 0.02; pH 7.0 .1% dye solution was prepared (i.e. 1g in 100 ml D/W) and separately autoclaved with enrichment medium. 200 µl of the sterile dye solution added in to 100 ml enrichment medium. 1 ml of sample was inoculated in to the medium and incubated for 24 h under shaking condition (110 rpm) at $30^{0\ C}$. The flasks in which growth was observed were further used. A loop-full from the growth medium was streaked onto nutrient agar plates and then incubated at $30^{0\ C}$ for 24 h. Colonies from plates were observed. Each differentiating colonies from plates were subculture and then pure cultures were obtained. The isolates were grouped on the basis of cell morphology, Gram's reaction, cultural characteristics, and colony characteristics. Thus, thirty-three pure isolates were obtained from different samples.

IV.2.3 Primary screening and selection of dye decolorizing microorganisms

Organisms were grown in 250 ml flasks containing 100 ml of N-broth. The dye (RS Red H5 BL) stock solutions (1g of dye per 100 ml of D/W) were prepared, autoclaved and separately added at 2 mg final concentration to the autoclaved medium. The flasks were inoculated (5%) with young culture (OD 0.9) of each thirty-three isolated organisms. Flasks were incubated at 30^{0} C for 24 h under static condition. Reaction mixture (after 24 h) from the flasks was transferred in to centrifuge tube, centrifuge at 10,000 RPM for 10 min. and decolorizations of dye in each flask were measured specrophotomatrically, Among the thirty three isolated employed, only four isolates (having highest percentage of decolorization) were considered for further detailed studies.

IV.2.3.1 Biomass

Biomass was measured by turbidity reading of appropriately diluted culture samples at 530 nm against a blank prepared with the same dilution of the supernatant in DW.

IV.2.4 Maintenance of microorganisms

Out of thirty-three, the four selected isolates were maintained. Stock cultures of the four isolates were maintained at $4^{0\ C}$ in refrigerator. Stock cultures were sub-cultured every 2-10 weeks. The maintenance medium used in this case was synthetic medium SM6 containing dye **(Table IV.5)**.

IV.2.5 Tentative characterization of the isolates

Pure cultures of four isolates maintained on the slants were used for the identification by studding following phenotypic characteristics as mentioned in **Bergey's manual of Systematic Bacteriology (1994)**.

IV.2.5.1 Colony characteristics

A loop-full culture of all isolates from slants was inoculated in nutrient broth. All flasks were incubated at $30^{0\ C}$ fro 24 h and then colony characteristics on nutrient agar plates were examined.

IV.2.5.2 Gram's reaction and cell morphology

Gram's reaction of all the isolates was performed and on the bases of microscopic observation, cell morphology study was carried out.

IV.2.5.3 Biochemical tests

Media for biochemical tests as listed **(Table IV.4)** were prepared and as mentioned above, young culture were prepared at $30^{o\ C}$ for 24 h and results were examined.

Table IV.4 Biochemical tests

No.	Tests	Results			
		Isolate - 1	Isolate - 2	Isolate - 3	Isolate - 4
1.	Growth on MacConkey agar	+	-	+	+
2.	Indole Test	-	-	-	-
3.	Methyl Red Test	-	-	-	-
4.	Voges proskauer test	-	-	-	-
5.	Citrate Utilization	+	+	+	+
6.	Gas production from glucose	-	-	-	-
7.	Casein hydrolysis	-	-	-	-
8.	Starch hydrolysis	-	+	-	-
9.	Urea hydrolysis	-	-	-	-
10.	Nitrate Reduction	+	+	+	+
11.	Nitrite Reduction	+	+	+	+
12.	H_2S Production	+	+	+	+
13.	Cytochrome Oxidase	-	-	-	-
14.	Catalase Test	+	+	+	+
15.	Oxidation / Fermentation (O/F)	F	F	F	F
16.	Gelatin Hydrolysis	-	-	-	-
17.	Arginine dihydrolase	+	+	+	+
18.	Lysine decarboxylase	-	-	-	-
19.	Ornithine decarboxylase	±	±	±	±

IV.2.6 Decolorization of various dyes by the four isolates

The four isolates were selected further screened, employing 13 dyes to obtain the most efficiently potentially decolorizing isolates. It was aimed to find out efficient microorganisms that would decolorize maximum amount and numbers of dyes within shorter period of time. The experiment was conducted using 13 dyes (2 mgl^{1}) in the 100 ml nutrient broth medium inoculated by 5 ml young culture of all the four isolates and time duration were noted down for dye decolorization.

IV.2.7 Decolorization of three dyes in anaerobic condition by the four isolates

Synthetic medium SM6 was transferred in 300 ml BOD bottles and separately autoclaved stock solution of dye (1%) Biebrich scarlet, Reactive Black B and Sunzol violet 5R (2 mg) added in the respective bottle and they were incubated in BOD incubator at $30^{0\ C}$ for 24 h. After incubation, 100 ml of the sample from each bottle was harvested, centrifuged at 10,000 RPM for 20 min. The supernatants obtained were further filtered through 0.4 µm Millipore filter and IR spectra were measured on IR spectrophotometer (shimadzu).

IV.2.8 Optimization of synthetic media

Initially during enrichment, screening and maintenance of organisms for dyes, nutrient broth/agar was used. Since nutrient broth is a complex medium, congaing ingredients of comp lex nature, it is necessary to employing synthetic medium with known amount of ingredients. So, the following ingredients were tested to optimize growth and dye decolorization (**Table IV.5**).

Table IV.5 Compositions of various synthetic media

Sr. No.	Ingredients (gL^{-1})	*SM1	SM2	SM3	SM4	SM5	SM6
1	Dextrose	10	10	10	10	5	10
2	$(NH_4)_2HPO_4$	5	--	--	--	--	--
3	$(NH_4)_2SO_4$	--	--	--	1	2	2
4	Urea	--	5	--	--	--	--
5	KH_2PO_4	--	5	10	5	1	2
6	K_2HPO_4	--	--	--	5	5	--
8	NH_4NO_3	--	--	5	--	--	--
9	$MgSO_4.7H_2O$	0.5	--	--	0.1	--	0.2
10	Na_2HPO_4	--	--	--	--	--	2
11	NaCl	--	--	--	--	--	2
12	Trace element solution (ml)	1	1	1	1	1	--
13	Dye	0.20	0.20	0.20	0.20	0.02	0.20

*** Synthetic medium (SM)**

Trace element solution contain: gI^1 $FeSO_4.7H_2O$, 0.5; $ZnSO_4.7H_2O$, 0.5; $MnSO_4.7H_2O$, 0.5; MgSO4, 0.5; $CuSO_4$, 0.1; D/W, 1 liter.

IV.2.8.1 Assays of decolorization

Decolorization of dye assays were performed in 250 ml conical flasks with 100 ml volume of sterile medium (mostly in SM6) concentration dye solution were separately prepared and autoclaved. All the young cultures were routinely using the medium containing the respective azo dye. Thus, the exponential growth phase were harvested after 8 –10 h, by centrifugation at 10,000 RPM (Refrigerated at $15^{0\ C}$), washed and resuspended in such a way to obtain OD approximately 1.0.

IV.2.8.2 Influence on medium pH in shake and stationary cultures by the Isolate I to IV

To examine any changes of the medium pH when the four isolates grown under shaking (aerobic) and stationary batch cultures. The four isolates were inoculated individually in separate flasks in the medium containing SM6 + Dye (BS) and incubated at $30^{0\ C}$. At regular interval 0.0, 8.00, 12.0, 24.0 and 32.0 h, biomass and % dye removed were noted.

IV.2.9 Effect of various carbon sources on dye decolorization and growth

10ml of SM6 medium was transferred to test tube. Each carbon sources was added in the respective tubes to achieved 1.0% concentration (glucose, sucrose, mannitol, fructose, starch, lactose, maltose, casein, and gelatin. 100µl of riboflavin (3mM), azo dye solution (0.2mg) and 200µl of young culture of Isolate-I were added. Tubes were incubated at $30^{0\ C}$ for 48 h. Results were noted at every 24 h intervals.

IV.2.10 Effect of varying concentration of glucose on decolorization and growth

The SM6 medium (100ml) transferred in the flasks contains 1.0ml riboflavin (3mM). For each concentration-separated flask in triplicate along with control were taken. Glucose solutions of varying range of concentration (0.0 mg/ml to 25.0 mg/ml) were transferred to each set. Activated culture of Isolate-I was added in such a way that gave initial OD of the reaction mixture about 0.07. The flasks were incubated at 30^{0C} under static condition and results were noted was the dye decolorized after 24 to 40 h.

IV.2.11 Comparison of dye decolorization rates and growth in N-broth and synthetic medium SM6

Young cultures (OD, 0.625) of all four isolates were inoculated (1 ml) in 100 ml nutrient broth with dye RS Red H5BL (2 mg/100 ml). Flasks were incubated under static condition at $30^{0\ C}$, and at every intervals of 2 h, 2 ml samples were withdrawn aseptically, centrifuged in 1.5 ml vial at 10,000 rpm for 20 min. Supernatant was taken for dye decolorization study and sediment cell in vial were filled with normal saline (2.0 ml). Vials were properly shaken and tested for growth. Dye decolorization was measured at 515 nm and growth measured at 530 nm.

IV.2.12 Effect of riboflavin on dye decolorization and biomass

SM6 broth was transferred in each flask was prepared and containing 1.0ml sunzol violet 5R dye (2 mg). Two sets were prepared, one containing 1.0 ml of riboflavin (3mM) and the other without riboflavin. Young culture (0.9 OD) of the four isolate were prepared and added in the respective flaks in such a way to obtain initial OD about (0.04). Flasks were incubated at $30^{0\ C}$ under static conditions for 40 h. Results were observed at regular interval of 12 h.

IV.2.13 Effect of potassium nitrate (KNO_3)

To SM6 **(Table IV.5)** medium, sunzol violet 5R (0.04 mg/ml) was added and then activated young culture (OD 0.7) of Isolate-I and Isolate-II was inoculated to 100ml SM6 in the respective flasks.

Set no.	SM6 (ml)	KNO_3 mg/100ml	Dye (mg)	Inoculum (OD 0.7)	Remarks
1	100	-	4	-	Without inoculum
2	100	-	-	1 ml	Without dye and KNO_3
3	100	5	4	1 ml	Treatment
4	100	-	4	1 ml	Without KNO_3
5	100	5	4	1 ml	Without $(NH4)_2SO_4$ in SM6
6	100	10	4	1 ml	Treatment

IV.2.14 Comparison of dye decolorization in presence and absence of riboflavin and KNO_3 by shaking cultures

Following sets were prepared for both the condition:

Shaking condition (inoculated at 30^{0C} for 24 hours)

Set no.	SM6	KNO_3 mg/100ml	Riboflavin (3mM)	Dye (mg)	Inoculum	Remarks
1	100	-	1 ml	2.0	-	Without inoculum but with Riboflavin
2	100	-	-	-	1 ml	Control-2
3	100	10	-	2.0	1 ml	Control-4 without RF
4	100	-	1 ml	2.0	1 ml	Control-3 without KNO_3
5	100	10	1 ml	2.0	1 ml	SM6 without $(NH_4)_2SO4$
6	100	10	1 ml	2.0	1 ml	Treatment: RF + KNO_3

IV.2.15 Comparison of dye decolorization in presence and absence of riboflavin and KNO_3 under static condition and shaking cultures

Experimental conditions were same as in **IV.2.14** but with the dye Biebrich Scarlet and under static condition.

IV.2.16 Influence of varying concentration of the dye on decolorization

Varying concentration (0.5 mg to 50 mg) of the dye Sunzol violet 5R into respective flasks was added in the medium SM6 with one ml of riboflavin (3 mM) in respective flasks. Young culture of isolate-I was prepared in same medium. Inoculums size was 1 ml and OD- 0.721. All flasks were incubated statically at $30^{o\ C}$ for 24 h.

IV.2.17 Effect of successive loading of the dye in the course of prolonged incubation

2.5 liter of synthetic medium SM6 was prepared. 10 ml 3mM riboflavin and 5 ml Biebrich Scarlet (1%) dye were aseptically added into medium. 100 ml young inoculums (isolate-I, OD - 0.525) were inoculated. Flask was incubated at $30^{0\ C}$ for 24 h. Next day percentage decolorization and biomass were measured. Again 5 ml dye was reloaded and flask was incubated for next 24 h. Results were noted.

Same experiment was repeated. After decolorization each time before reloading a dye, pH of medium was adjusted with sterile 0.1 M NaOH solution and incubated for 24 h. After complete decolorization 5–20 ml of dye was reloaded each time. Biomass, percentage dye decolorization and pH were measured during frequent time interval.

IV.2.18 Decolorization of dyes in sequential anaerobic and aerobic Condition

The experimental set up was designed to study the transformation of dyes, first in static condition until complete dye was decolorized. In the next step, the same broth (after 24 h.) was placed on orbital shaker (100 RPM) and further degradation of product was examined as increase in biomass.

Step-1 The set up was as follows:

Set- 1 SM6 100ml + Inoculum (5ml) of Isolate (O.D. 0.7) + Riboflavin 1 ml (3mM) + sunzol violet 5R (2.5 mg); incubated at $30^{0\ C}$ for 24 h in static conditions.

Set- 2 same as a given in set 1expect the dye was Biebrich Scarlet.

Step-2 After complete decolorization (100%) of both the dyes, the same flasks were transferred on orbital rotary shaker (100RPM) and incubated for 48 h. Biomass was considered as the indicator parameter.

IV.2.19 Confirmation of complete metabolism of the products formed after decolorization

The objective of the experiment was to find out whether the decolorized products of the azo dyes were further metabolized or not. To investigate the above objective, following set up steps were performed

Set -1

Step-I SM6 broth (100ml) in 250 ml flask (in triplicate) comprising; 2 mg dye (sunzol violet 5R and Biebrich scarlet separately) + 1 ml RF (3.0mM) + 5 ml inoculum (0.7 OD). The flask was incubated at $30^{0\ C}$ for 24 h in static condition. Results were examined after the incubation period.

Step-II The decolorized broth was further centrifuge or filtered (0.4µm) and the supernatant to filtrate was transferred in 1-liter conical flask. The broth pH measured was 5.6 and therefore adjusted to 7.0 before autoclaving. Agar powder was added in the above both to make 2% agar, and autoclaved at $120^{0\ C}$ for 15 min. Petriplates were prepared pouring the autoclaved medium and the same Isolate-I was streaked, and incubated at $30^{0\ C}$ for 72 h. The plates examined at every 24 h intervals.

Set- 2

Step-I The condition was same as described in set I, step-I. The flasks were incubated at $30^{0\ C}$ in static condition for 48 h. As usual, there was 100% dye decolorization. Biomass was measured.

Step-II All the flasks of the step- I were placed on orbital rotary shaker at 100 RPM and further incubated for further 48 h. OD of the incubated broth was measured spectophotometrically at 530 nm for biomass.

Step-III Cultured broth of the step- II was centrifuged at 10,000 RPM for 20 min. and supernatant was transferred 1000ml flask (pH adjusted to 7.0) and agar powder was added to obtain 2.0% agar medium. The supernatant of the broth with agar was autoclaved and transferred on petriplates. The plates were streaked with young culture of Isolate-I, incubated at $30^{0\ C}$ for 72 h. Every 24 hours interval plates were examined for colony development.

CHAPTER V: RESULTS

V.0 RESULTS

In the present work, a study on decolorization of azo dyes by certain prokaryotes has been investigated. From the previous studies on the subject of decolorization, it was concluded that facultative bacteria have found capable of transforming azo dyes to colorless under anaerobic (oxygen-depleted) conditions. The bacterial strains isolated being the best facultative organisms, have been employed to understand the process of decolorization under laboratory conditions.

In the present study, experiments were conducted to understand the medium conditions essential for the "suitable bacterial isolates" to decolorize the azo dyes. The experiments performed were:

1. Enrichment, isolation, screening, and tentative characterization of dye decolorizing bacteria
2. Selection of the suitable Isolates
3. Selection of azo dyes
4. Optimizing the suitable physiological conditions (with respect to environmental parameters and medium ingredients)
5. Decolorizatiuon and further metabolism of decolorized products

To achieve the aims, the above stated experiments were performed showing that given the right process conditions; color due to azo dye(s) can be removed that mediated by biological entity inoculated in the assay medium.

V.1 Enrichment, isolation and screening of suitable organisms

To fulfill the objectives, a complex medium containing certain minerals was employed. When the sample(s) the sludge, enriched in the complex medium in presence of azo dye(s), it was

observed that diverse population of microorganisms developed during the course of incubation. To isolate the microorganisms from the sludge sample, the enrichment medium **(refer section IV.2.2)** was used. In view of the heterotrophic microbial requirements such as C, N, P, S etc. for their growth, the components of enrichment medium was selected (KH_2PO_4, Yeast extract, Peptone, Glucose, $MgSO_4.7H_2O$). The final results obtained show cultural characteristics of the thirty-three isolates, as given in **Table V.1**.

During the primary screening experiments, results clearly indicate that shake culture (aerobic) conditions did not support the process of dye decolorization significantly and extended (time) incubation up to 120 h also showed the same results. Thus, the results of primary screening shows decolorization process more susceptible under stationary batch cultures toward several azo dyes.

Table V.2 shows the results obtained for the ability of the thirty-three isolates to decolorize azo dyes **(RS Red H5BL)** in the complex medium (N-broth) when subjected to stationary batch culture incubation. All dyes in the range of 50% - 100% were decolorized within 24 h. Isolate-I to Isolate-IV showed the highest level of decolorization, i.e. 100%. Also, it was noted that these isolates had high growth (OD at 530 nm = 1.382 to 1.728) in the given incubation conditions.

The results were confirmed by repeating the primary screening experiment of the isolates for three times. The isolates showing fairly high growth and also decolorizing the azo dye have been considered to be the suitable organisms and therefore employed for further study.

V.2 Characterization of bacterial isolates

The four isolates (I to IV) were studied to characterize their gram reaction, colony and cell morphology (**Table V.3**). Some physiological tests were performed for the Isolate I to IV and the results are given in **Table V.4**.

From the above-mentioned tests and biochemical test (not shown here), the four isolates have been tentatively attempted to identify **(Table V.5)**. Also the isolate-I has been confirmed by **IMTECH, Chandigarh, (India) as *Cirtobacter freundii* (MTCC No. 6736).**

Table V.1 Colony characteristics of thirty-three isolates

Micro organisms	Colony Characteristics						
	Size	Configuration	Margin	Elevation	Texture	Pigment	Opacity
*Iso-1	Medium	Round	Entire	Flat	Rough	Colorless	Translucent
Iso-2	Small	Round	Entire	Raised	Smooth	Colorless	Opaque
Iso-3	Medium	Round	Serrate	Raised	Dry	Colorless	Opaque
Iso-4	Large	Round	Entire	Flat	Smooth	Gray	Opaque
Iso-5	Medium	Round	Filamentous	Raised	Smooth	White	Transparent
Iso-6	Large	Rhizoids	Entire	Flat	Rough	Colorless	Transparent
Iso-7	Small	Round	Serrate	Raised	Smooth	Colorless	Transparent
Iso-8	Medium	Round	Filamentous	Flat	Smooth	Colorless	Transparent
Iso-9	Medium	Filaments	Entire	Flat	Smooth	White	Opaque
Iso-10	Small	Round	Serrate	Flat	Rough	White	Transparent
Iso-11	Small	Round	Serrate	Flat	Smooth	White	Transparent
Iso-12	Small	Round	Serrate	Flat	Dry	Colorless	Transparent
Iso-13	Large	Filaments	Entire	Raised	Rough	White	Transparent
Iso-14	Medium	Irregular	Filamentous	Raised	Smooth	Colorless	Opaque
Iso-15	Medium	Filaments	Serrate	Flat	Wrinkle	White	Transparent
Iso-16	Large	Round	Entire	Flat	Rough	White	Transparent
Iso-17	Small	Filaments	Entire	Raised	Smooth	White	Opaque
Iso-18	Pin point	Round	Serrate	Flat	Smooth	White	Transparent
Iso-19	Medium	Round	Entire	Raised	Rough	Green	Transparent
Iso-20	Medium	Rhizoid	Entire	Raised	Smooth	Colorless	Transparent
Iso-21	Small	Round	Entire	Raised	Smooth	Colorless	Transparent
Iso-22	Medium	Irregular	Serrate	Raised	Mucoid	Colorless	Transparent
Iso-23	Pin point	Round	Filamentous	Raised	Smooth	Yellow	Transparent
Iso-24	Medium	Round	Entire	Raised	Rough	White	Transparent
Iso-25	Small	Round	Entire	Raised	Smooth	Colorless	Transparent
Iso-26	Small	Round	Entire	Raised	Smooth	Colorless	Transparent
Iso-27	Medium	Filaments	Entire	Raised	Smooth	Colorless	Transparent
Iso-28	Small	Round	Entire	Flat	Smooth	White	Transparent
Iso-29	Medium	Round	Entire	Raised	Smooth	White	Opaque
Iso-30	Small	Filaments	Serrate	Flat	Mucoid	Colorless	Transparent
Iso-31	Large	Round	Entire	Flat	Smooth	Green	Opaque
Iso-32	Medium	Round	Entire	Raised	Smooth	White	Transparent
Iso-33	Medium	Round	Serrate	Raised	Smooth	Colorless	Opaque

*** Iso:** Isolate

Table V.2 Results showing potentialities of microorganisms to decolorized dye

Microorganisms	Growth	Decolorization	Microorganisms	Growth	Decolorization
Iso-1	1.421	100%	Iso-15	1.249	80%
Iso-2	1.382	100%	Iso-21	1.285	80%
Iso-3	1.501	100%	Iso-12	0.841	75%
Iso-4	1.728	100%	Iso-20	0.998	75%
Iso-25	1.343	95%	Iso-31	0.821	75%
Iso-26	1.352	95%	Iso-14	1.178	75%
Iso-5	1.123	90%	Iso-33	0.988	70%
Iso-23	1.381	90%	Iso-19	0.838	70%
Iso-24	1.325	90%	Iso-13	1.289	70%
Iso-30	0.995	90%	Iso-11	1.109	60%
Iso-9	1.325	85%	Iso-18	0.838	60%
Iso-22	1.298	85%	Iso-17	1.255	60%
Iso-27	1.211	85%	Iso-29	0.701	50%
Iso-28	1.212	85%	Iso-32	0.601	50%
Iso-8	0.981	85%	Iso-16	1.267	50%
Iso-6	0.931	80%	Iso-10	1.218	50%
Iso-7	1.211	80%			

Table V.3 Characterization of colony and cell morphology

No.	Tests	Results			
		Isolates-I	Isolates -II	Isolates -III	Isolates -IV
1.	Colony morphology				
	Configuration	Round	Round	Round	Round
	Margin	Entire	Entire	Serrate	Entire
	Elevation	Convex	Convex	Flat	Convex
	Texture	Rough	Smooth	Dry	Smooth
	Opacity	Translucent	Opaque	Opaque	Opaque
	Pigments	-	-	-	Gray
2.	Gram's Reaction	-ve	-ve	+ve	-ve
	Size & Shape	Rods	Small Rods	Rods	Small Rods
	Arrangement	Single	Single	Single	Single
3.	Spore				
	Endospore	-	-	+	-
	Position	-	-	Terminal	-
	Shape	--	-	Round	-
	Sporangia Bulging	--	-	--	-
4.	Motility	+	+	+	+
5.	Fluorescence(UV)	-	-	-	+

Table V.4 Physiological tests

No.	Tests	Results			
		Isolate-I	**Isolate -II**	**Isolate -III**	**Isolate -IV**
1.	Growth Temp.				
	4°C	-	-	-	-
	10°C	-	-	-	-
	15°C	+	+	+	+
	25°C	+ +	+ +	+ +	+ +
	30°C	+ + +	+ + +	+ + +	+ + +
	37°C	+ +	+ + + +	+ + + +	+ + +
	42°C	+ +	+ +	+ + + +	+ +
	55°C	+	-	+ + +	-
	65°C	-	-	-	-
2.	Growth at pH				
	5.0	+ +	+ +	+ +	+ +
	5.7	+ +	+ +	+ +	+ +
	6.8	+ + + +	+ + + + +	+ + + +	+ + + +
	8.0	+ +	+ +	+ + +	+ +
	9.0	+	+	+ +	+
	11.0	+	+	+	+
3.	Growth on NaCl (%)				
	2.5	+ + +	+ + +	+ + + +	+ + +
	5.0	+	+	+	+
	7.0	+	+	+	+
	8.5	-	-	-	+
	10.0	-	-	-	-

\+ = 0.2 to 0.35 OD at 530 nm, initial OD= 0.07

\+ + = 0.4 to 0.6

\+ + + = 0.6 to 0.8

\+ + + + = 0.8 to 1.2

Table V.5 Characterization (tentative) of the four isolates

Serial no.	Strain Designation	Identity	MTCC Number
1	Isolate- I	***Citrobacter freundii***	6736
2	Isolate- II	***Enterobacteriaceae***	---
3	Isolate- III	***Bacillus sp.***	---
4	Isolate- IV	**Gram –ve small rods** ***Pseudomonas sp.***	---

V.3 Selection of azo dye(s)

There are several numbers of azo dyes, which are manufactured and applied to dyeing and printing operations in textile industries. It was aimed to select dye(s) having more or less recalcitrant property towards the action of the suitable organism to become decolorized.

Table V.6 shows the results of different azo dyes (13) against the four isolates. It can be seen that the isolates were growing actively in presence of these dyes. Also, that all dyes were susceptible to the isolates that transformed and decolorized, almost 100% (incubated under static conditions at $30^{0\ C}$ for 24 h).

Any of the following azo dyes were used in the following experiments

1. Biebrich Scarlet - (chemically defined)
2. Sunzol Violet 5R - (chemically defined)
3. Reactive Black B - (chemically defined)
4. RS Red H5BL - (chemically defined)
5. RS Red H5RL - (Structure not available)

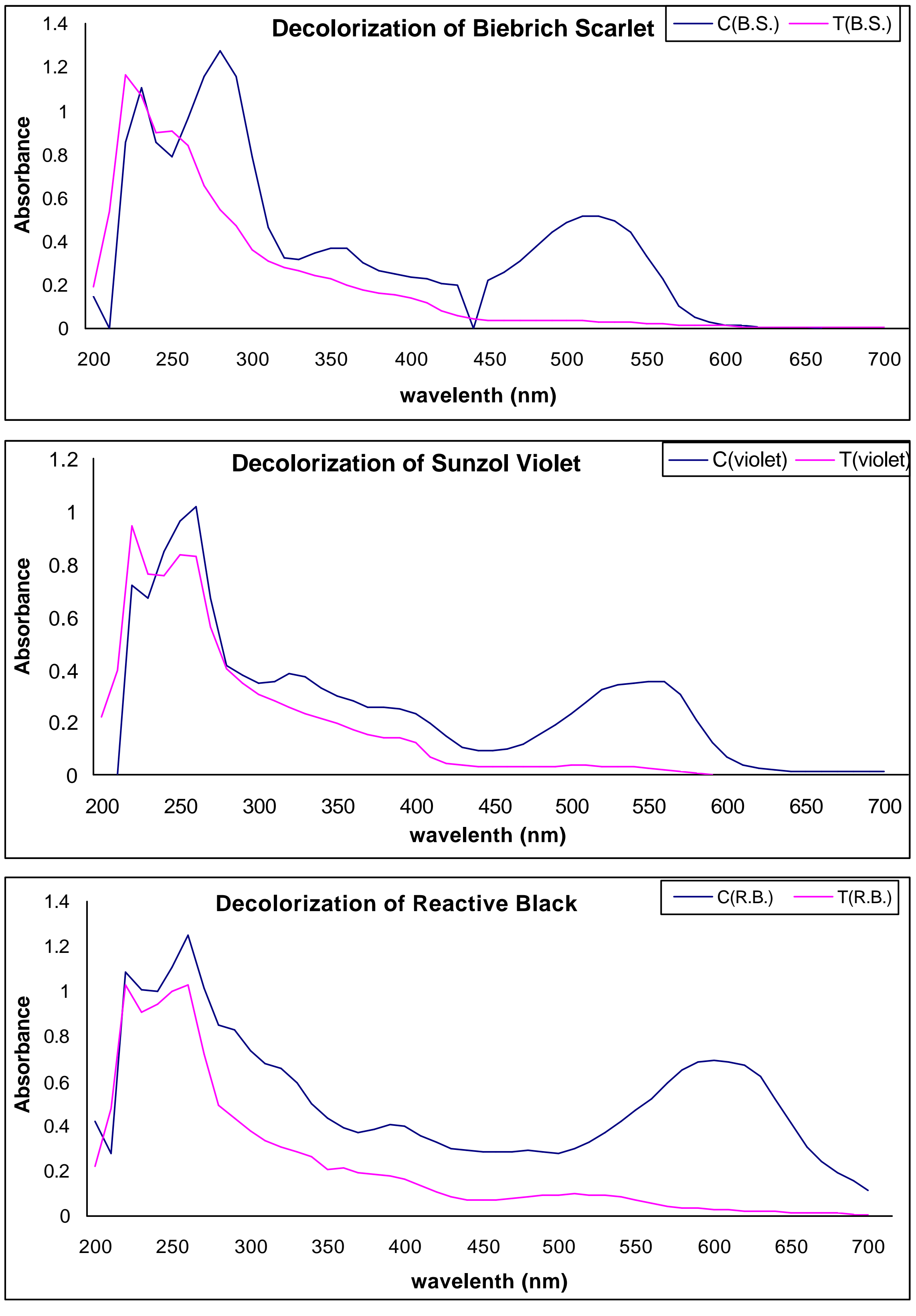
Table V.6 Decolorization of various dyes by selected isolates

	Dyes	Isolates-I		Isolates -II		Isolates -III		Isolates -IV	
		Growh	Decolo rization	Growth	Decolo rization	Growth	Decolo rization	Growth	Decolo rization
1	RS Red H5BL	1.215	100 %	1.307	100 %	1.285	100 %	1.261	100 %
2	RS Red H5RL	1.105	100 %	1.266	100 %	1.262	100 %	1.289	95%
3	Red H5 B1	1.201	100 %	1.232	100 %	1.223	100 %	1.245	100 %
4	Golden Yellow R	1.001	100 %	1.185	100 %	1.201	95%	1.234	95%
5	Sunset yellow	0.985	100 %	1.105	100 %	1.098	95%	1.112	100 %
6	Congo red	1.195	100 %	1.211	100 %	1.167	100 %	1.189	100 %
7	Ponceau 4R	1.208	100 %	1.222	100 %	1.020	100 %	1.266	100 %
8	Orange G	1.092	100 %	1.225	100 %	1.108	100 %	1.266	100 %
9	Violet 5R	1.126	100 %	1.186	100 %	1.078	100 %	1.111	100 %
10	Reactive Black B	0.935	100 %	0.989	100 %	1.002	100 %	1.042	100 %
11	Biebrich scarlet	1.145	100 %	1.089	100 %	1.165	100 %	1.189	100 %
12	Sunzol Violet 5R	1.208	100 %	1242	100 %	1.231	100 %	1.268	100 %
13	Methyl red	1.211	100 %	1.258	100 %	1.232	100 %	1.263	100 %

V.3.1 UV-Visible profile of the assay mixture before and after incubation(24h)

From the assay mixtures of the 13 selected dyes, three azo dyes were further selected to determine their UV-Visible spectra. 5 ml of the mixtures were withdrawn, centrifuged and supernatants used to determine UV-Visible spectra (200 nm to 700 nm). **Figure V.1** and **following table** shows the peaks (absorbance) of the samples (0.004 and 24 h) at different wavelength:

Wavelength nm	Biebrich Scarlet (Absorbance)		Wavelength nm	Sunzol Violet 5R (Absorbance)		Wavelength nm	Reactive Black B (Absorbance)	
	0 h	24 h		0 h	24 h		0 h	24 h
210	0.0	0.544	220	0.72	0.943	230	1.011	0.908
280	1.279	0.553	260	1.018	0.833	260	1.249	1.03
350	0.37	0.225	320	0.383	0.255	390	0.406	0.181
520	0.521	0.032	560	0.353	0.019	600	0.696	0.029
530	0.500	0.029						



C: Control (without inoculum); T: Treated

Figure V.1 UV-Visible spectra of decolorization of three dyes in anaerobic condition by the isolate- I

V.4 Optimization of suitable (favourable) physiological conditions with respect to growth (nutrient) requirement

Potential organisms (Isolate – I to IV) have been tentatively identified **(Table V.5)**. These organisms also had very high potential to decolorize azo dyes **(13 dyes, Table 3.6)** and therefore considered as suitable isolates.

All the four isolates were further studied for their requirements of different minerals (N, S, P, sources). The objective was to standardize a medium conditions that favors the process of decolorization along with optimum yield of their growth.

In addition, the optimized synthetic medium was compared with a complex medium i.e. Nutrient broth. Also, various carbon sources were employed to find out which carbon source would be more favourable (two azo dyes were used, Sunzol Violet 5R and Biebrich Scarlet).

V.4.1 Optimization of synthetic medium

Table V.7 presents the results observed when the four isolates were subjected to different nitrogen, phosphorous, sulfur, trace elements and NaCl in the media SM1 to SM6. The reaction mixtures were incubated up to 36 hours at room temperature (30^{0} ^{C}) under static conditions. The results show that the synthetic media SM5 and SM6 having 100% decolorization (Sunzol Violet 5R) and maximum growth in all four isolates. Glucose concentration in all the media was 1.0%. Thus, the SM5 and SM6 media were found to be very favorable in the process of decolorization and $(NH_4)_2SO_4$ has been confirmed as the best nitrogen source.

Table V.7 Optimization of synthetic media

No.	Media	Isolate-I		Isolate -II		Isolate -III		Isolate -IV	
		Growth	% dye Decolo-rization	Growth	% dye Decolo-rization	Growth	% dye Decolo-rization	Growth	% dye Decolo-rization
0	Blank	0.028	0	0.028	0	0.029	0	0.031	0
1	SM1	0.028	0	0.028	0	0.029	0	0.031	0
2	SM2	0.028	0	0.028	0	0.525	8	0.588	10
3	SM3	0.408	10	0.431	10	0.442	10	0.501	15
4	SM4	0.581	15	0.592	20	0.601	23	0.622	25
5	SM5	0.902	100	0.911	100	0.936	100	0.988	100
6	SM6	0.988	100	0.995	100	1.205	100	10.86	100

V.4.2 Comparison of decolorization and growth in N-broth and SM6

The four selected isolates were tested to examine and compare the biomass production and dye decolorization employing the two broths, i.e. Nutrient broth (complex) and chemically defined mineral based (glucose = carbon source) **Figure V.2** shows that in both the media growth and color removal were not significantly affected. Decolorization was achieved within 24 h by all the isolates. Biomass reached to stationary phase within 20 h that persisted for longer period (34 to 38 h). The rate of color removal increased with the increase in biomass.

V.4.3 Comparison of the biomass development of four cultures under stationary and shake batch system (with RF)

All the four isolates were studied to determine their biomass development when grown in SM6 medium and incubated at $30^{0\ C}$ under stationary and shake (aerobic) conditions. Figure clearly demonstrates that the biomass developed much faster when incubated under shake conditions (100 RPM). Initial OD of the medium (SM6, 100 ml) was adjusted to almost of similar values (0.038 to 0.04 OD) without dye and riboflavin. Measurements of OD were read at 4 h intervals for 24 h (**Table V.8 and Figure V.3**).

Table V.8 Comparison of the biomass development of four cultures under stationary and shake batch system (with RF)

Time (hrs.)	Isolate - I		Isolate - II		Isolate - III		Isolate - IV	
	OD? Stationary	OD? Shake	OD? Stationary	OD? Shake	OD? Stationary	OD? Shake	OD? Stationary	OD? Shake
0	0.039	0.040	0.042	0.041	0.039	0.041	0.043	0.043
4	0.108	0.118	0.099	0.143	0.096	0.182	0.098	0.163
8	0.296	0.466	0.300	0.643	0.223	0.531	0.321	0.486
12	0.493	0.890	0.582	0.932	0.564	0.941	0.586	0.893
16	0.708	1.180	0.795	1.053	0.791	1.243	0.963	1.203
20	0.988	1.261	1.032	1.382	1.036	1.512	1.189	1.495
24	1.240	1.321	1.102	1.424	1.105	1.568	1.234	1.528

? Mean of triplicate samples

At each interval the shake cultures showed almost double the growth then the stationary cultures of the four isolates. Maximum biomass was yielded in case of the four isolates within 24 h (**Table V.9**).

V.4.4 Effect of riboflavin (RF) on dye decolorization and biomass (stationary)

The SM6 medium was used to assay the effect of riboflavin (RF) on the process of color removal. The four isolates were inoculated in separate conical flasks (250ml, 100ml SM6) having two different conditions (presence and absence of RF) in the flasks to estimate biomass and color removal.

The results revealed the followings:

(i) Conditions one, absence of RF (SM6 + Dye + Inoculum) = Biomass increased to optimum level with 20 h (OD 0.945) which subsequently entered in stationary growth phase and the biomass increment from 20 to 30 hours time interval was comparatively insignificant. The dye decolorized only 55% (20 h), while in presence of RF biomass level almost similar to that in control, but percentage of color removed was more (i.e. 70%). Further, incubation

showed that the amount of dye decolorized was 100% (26 h) in case of RF presence whereas in case of assay medium-lacking RF was observed 100% at 30 h.

(ii) Studies on the other three isolates showed the similar results.

(iii) Presence of RF did not have any significant effect both on biomass as well as the dye decolorization.

(iv) Both the processes, increases in biomass and color removal were linear to time.

The SM6 medium was used to assay the dye decolorization in presence of RF. The observations presented in **Table V.8** and **Figure V.3** for increase in biomass and removal of dye color was not significantly different in all the four isolates. Presence of RF slightly reduced to course of time for dye decolorization (28 h w/o RF, 24 h with RF).

The experiment when further extended to examine UV – Vis spectra of control-1 (only medium SM6), control-2 (SM6 + dye) and treated (SM6 + Dye + RF) at zero hours and after incubation (24 hours). From and **Figure V.4** it is evident that dyes Biebrich Scarlet (BS) and Sunzol Violet 5R have been completely reduced. The peak at 500 nm (A 1.076) for the control with dye BS has been disappeared. The similar way for Suzol Violet 5R, the peak at 560 nm also disappeared. Thus, the results are similar to that obtained in the section (3.3) which were noted without RF. The following table shows the values of peaks:

Effect of riboflavin on Biebrich Scarlet decolorization:

Control / Treatment	**? of peaks / Absorbance**					
	? 200	**? 230**	**? 250**	**? 370**	**? 440**	**? 500**
Set – 1	2.635	1.331	1.226	0.638	0.658	0.047
Set – 2	2.872	1.746	1.153	0.726	0.656	1.076
Set – 3	3.311	1.900	2.101	0.431	0.329	0.071

Effect of riboflavin on Sunzol Violet 5R decolorization:

Control / Treatment	**? of peaks / Absorbance**		
	? 200	**? 270**	**? 560**
Set – 1	2.216	0.658	0.007
Set – 2	2.709	0.881	0.350
Set – 3	0.389	1.347	0.034

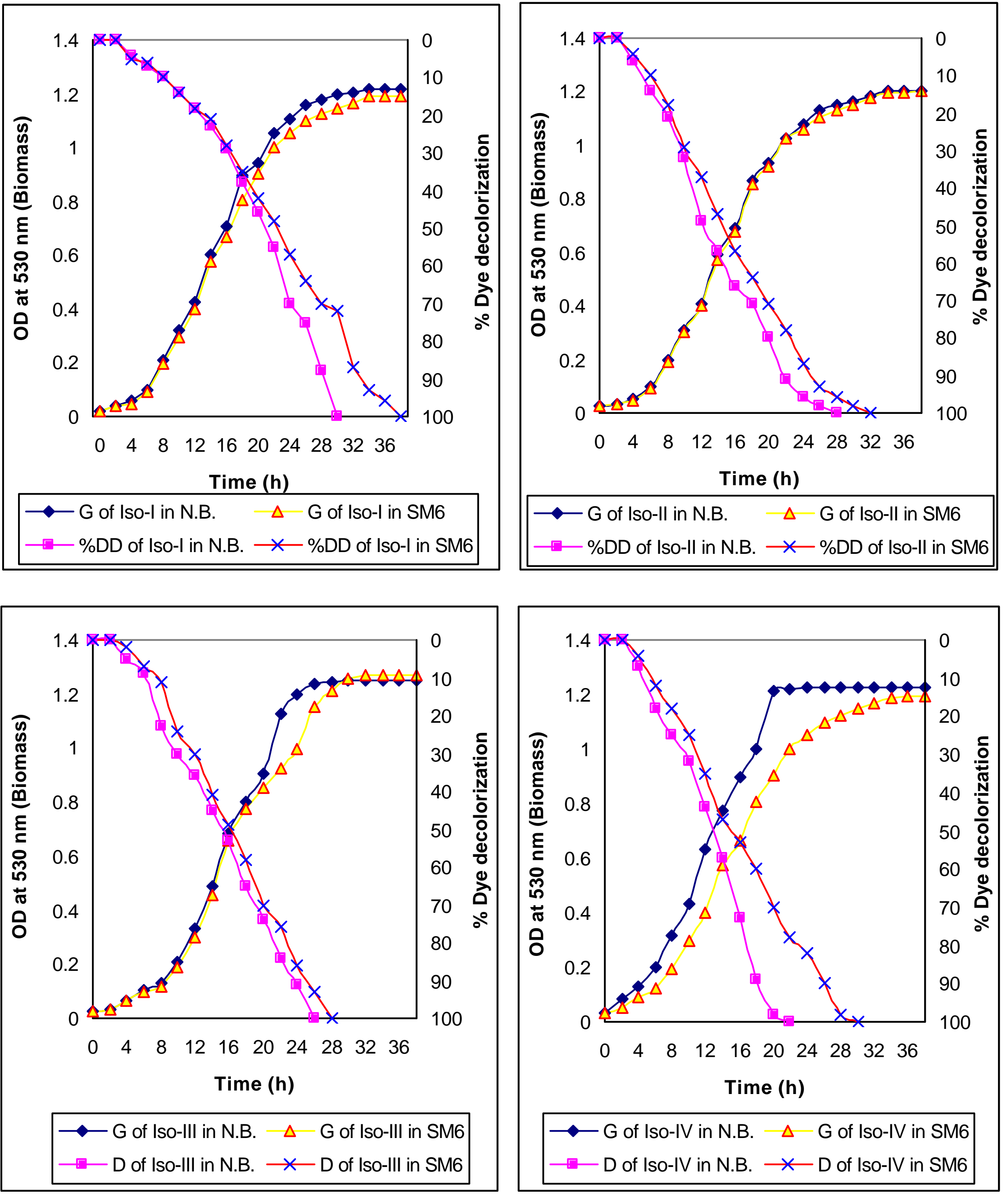


Figure V.2 Comparative study of growth and decolorization of four isolates in N-broth and SM6

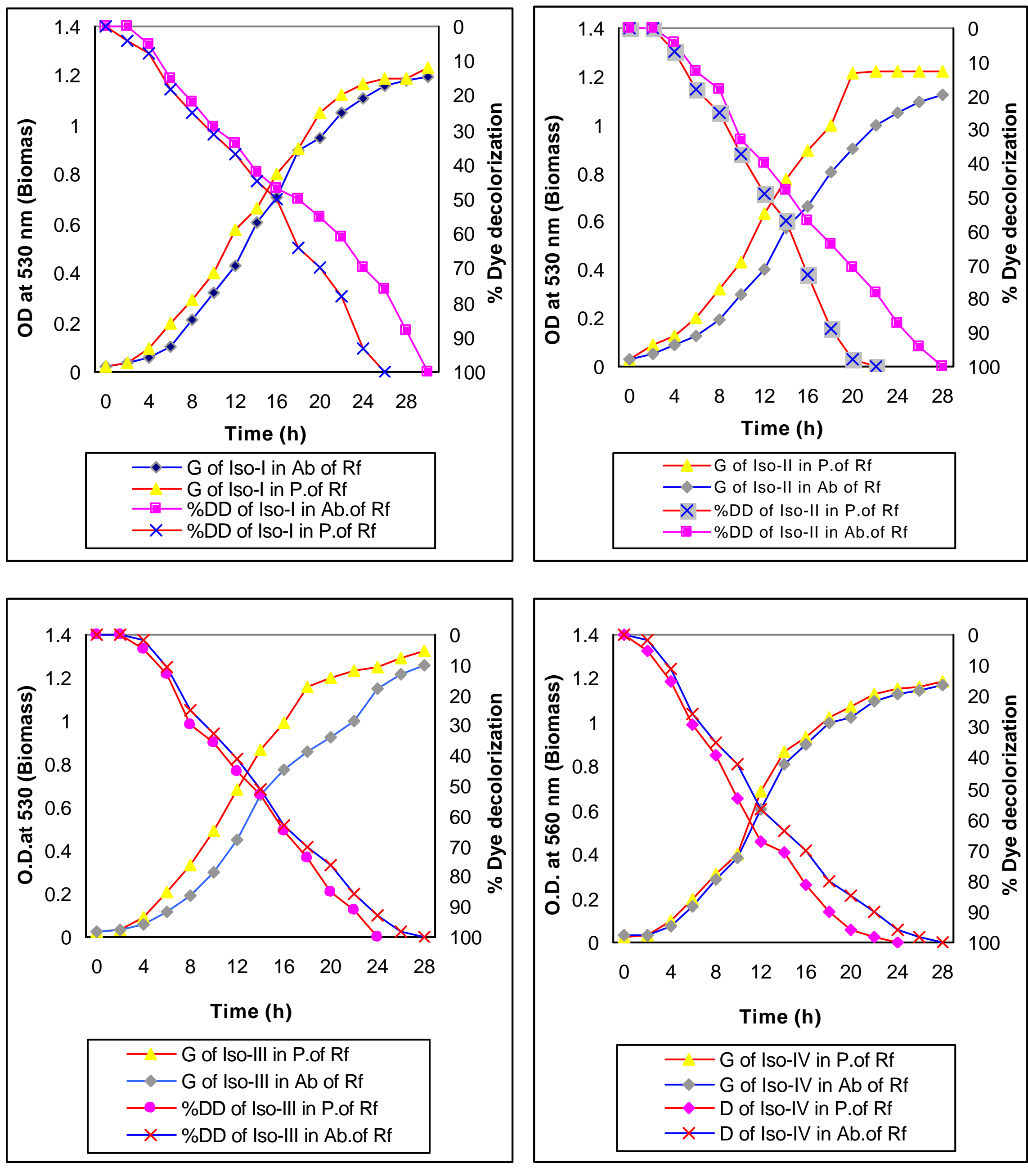
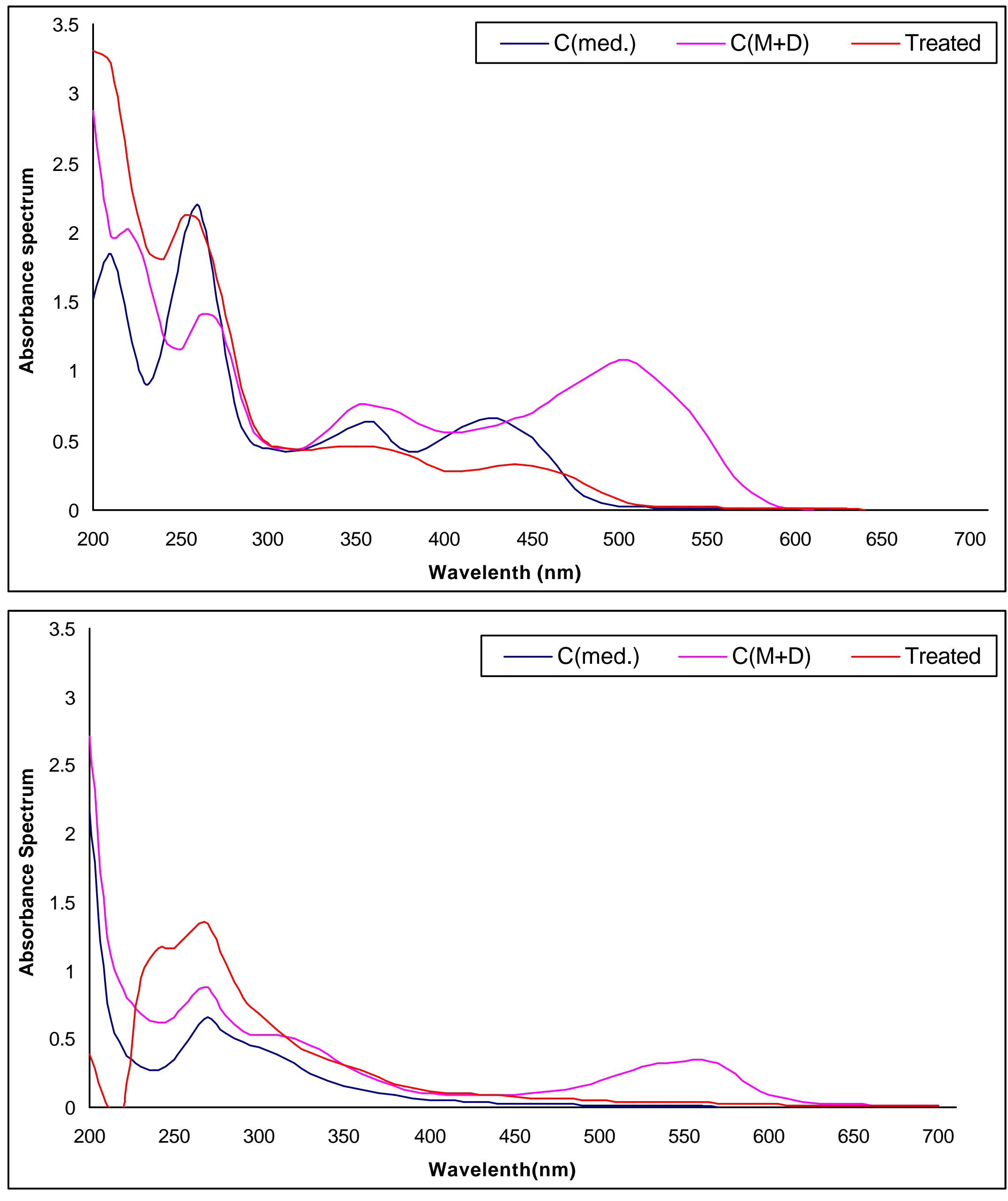


Figure V.3 Effect of riboflavin (w/o) on growth and decolorization of four isolates under stationary batch culture



C(med): Control of SM6; C(M+D): Control of SM6 with dye; T: Treated

Figure V.4 UV-Visible spectra of decolorization of Biebrich Scarlet and Sunzol Violet

V.4.5 Comparison of change in the medium pH under shake and stationary batch system (without riboflavin)

Growing cultures of the four isolates showed 100% decolorization only under static condition. The cultures incubated under shaking conditions were unable to decolorize any significant level (**Table V.9**). The results also confirmed that the growing cultures of all the isolates under static growth changed the pH of the medium while the shake growth medium showed very less change in the pH. The values of dye decolorization, under static conditions did not change with the change in pH.

Table V.9 Comparison of change in the medium pH under shake and stationary batch system (isolate – I and isolate – II)

Time	Isolate-I						Isolate-II					
	% Dye decolorization		Biomass		pH		% Dye decolorization		Biomass		PH	
	St	Sh	St	Sh	St	Sh	St	Sh	St	Sh	St	Sh
0	0	0	0.045	0.045	7.0	7.0	0	0	0.039	0.039	7.0	7.0
8	0	0	0.490	0.608	6.8	6.8	0	0	0.518	0.692	6.8	6.8
12	10	0	0.730	0.981	6.5	6.8	18	0	0.840	1.132	6.2	6.7
24	100	08	0.983	1.341	6.1	6.6	100	10	0.998	1.411	5.9	6.6
32	-	16	0.900	1.332	5.3	6.6	-	13	1.118	1.461	5.8	6.6
Time	**Isolate-III**						**Isolate-IV**					
	% Dye decolorization		Biomass		pH		% Dye decolorization		Biomass		Ph	
	St	Sh	St	Sh	St	Sh	St	Sh	St	Sh	St	Sh
0	0	0	0.043	0.043	7.0	7.0	0	0	0.048	0.048	7.0	7.0
8	0	0	0.578	0.898	6.7	6.9	0	0	0.618	0.928	7.0	7.0
12	40	11	0.720	1.081	6.1	6.6	32	0	0.830	1.182	6.2	6.8
24	100	18	0.910	1.481	5.4	6.6	100	4	1.230	1.482	5.6	6.8
32	-	22	1.240	1.392	5.1	6.4	-	8	1.230	1.511	5.4	6.7

St: Stationary, Sh: Shake

V.5 Influence of different carbon sources on dye decolorization (Isolate - I dyes Sunzol Violet 5R and Biebrich Scarlet)

Isolate – I has been employed to determine the effects of different carbon sources in the SM6. Results obtained are presented in **Table V.10** showing that Sunzol Violet 5R dye was decolorized 75% (24 h) and 100% (48 h) in presence of carbon sources – glucose, fructose, and starch by the Isolate – I. Almost similar results were observed for the dye Biebrich Scarlet **(Table V.11)**. Thus glucose, fructose, mannitol, sucrose, maltose and starch in both cases of the dyes, casein and gelatin, though nitrogenous complex protein, have been also utilized as carbon sources. However, in the subsequent experiments only 1.0 % glucose has been considered as the suitable carbon source.

Table V.10 Effect of various carbon sources on Sunzol Violet 5R dye decolorization and growth

Carbon sources	% Dye decolorized (24 h)	% Dye decolorized (48 h)
Glucose	75 %	100 %
Fructose	80 %	100 %
Mannitol	13 %	26 %
Sucrose	65 %	100 %
Lactose	31%	50 %
Maltose	49 %	100 %
Starch	100 %	100 %
Casein	24 %	40 %
Gelatin	49 %	100 %

Table V.11 Effect of various carbon sources on decolorization of Biebrich Scarlet and growth

Carbon sources	% Dye decolorized (24 h)	% Dye decolorized (48 h)
Glucose	88 %	100 %
Fructose	85 %	100 %
Mannitol	79 %	100 %
Sucrose	81 %	100 %
Lactose	20 %	30 %
Maltose	51 %	100 %
Starch	100 %	100 %
Casein	88 %	76 %
Gelatin	42 %	100 %

V.6 Effects of varying concentrations of the carbon source (glucose) (Figure V.5)

Glucose being the most preferred carbon source, its effect has been worked out applying varying concentrations, on percentage of dye decolorized, at 6, 12, 24 and 35 h. At higher concentrations (2100 mM and 1350 mM) there was decolorization of the dye, but that delayed (35 h). There was almost 100% decolorization from the range between 27.5 mM to 825 mM glucose levels within 24 h of incubation. Thus, the lowest level, i.e. 27.5 mM and 55 mM, there was complete decolorization (24 h). From the results, it was possible to conclude that higher concentrations, though favors the process of decolorization, but it would be wasteful to employ these concentrations in the assay medium. Therefore, 1.0% or 1mg/ml or 55 mM concentration has been taken into consideration for later experiments.

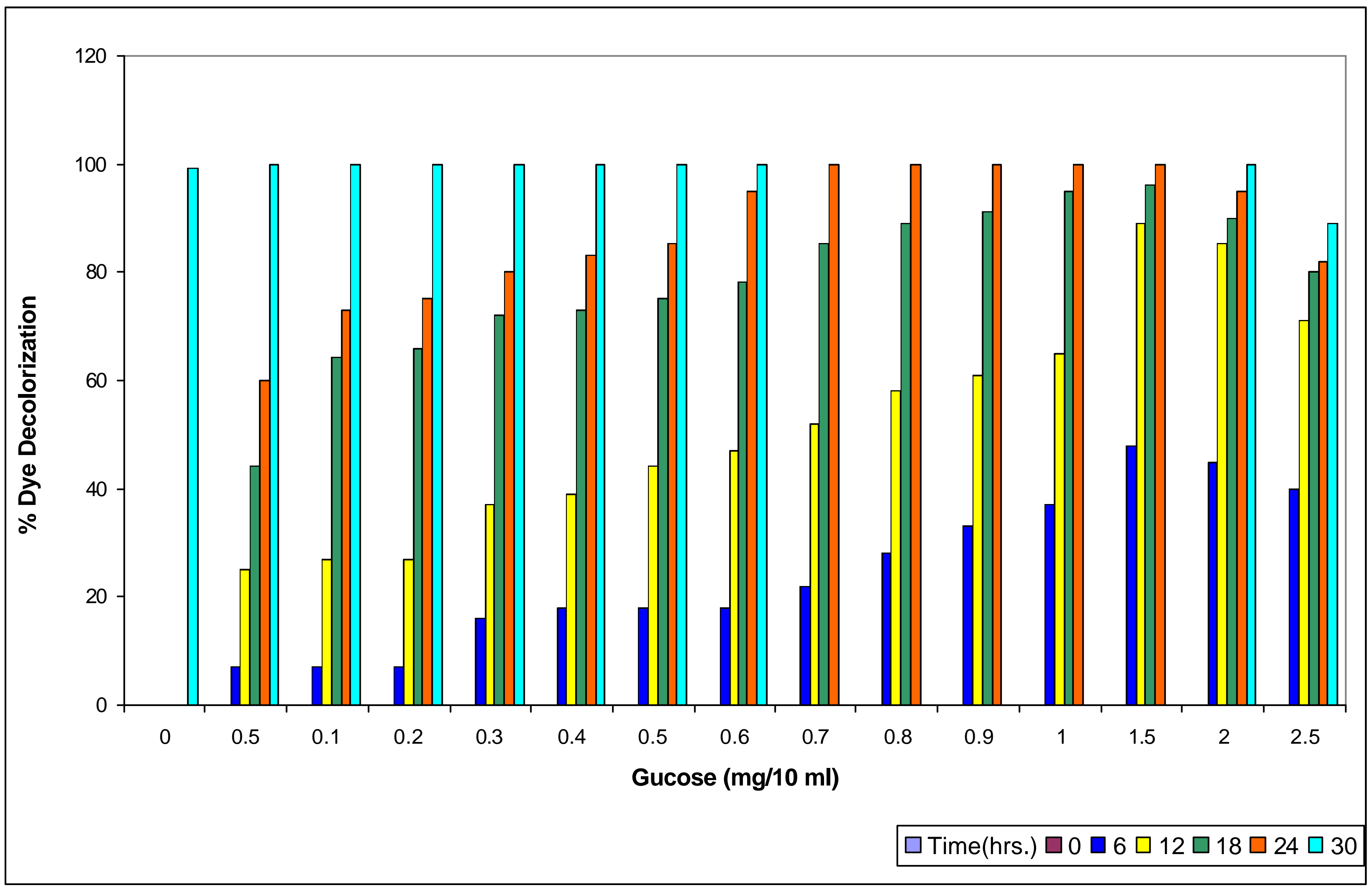


Figure V.5 Effect of varying concentration of glucose on decolorization and growth

V.7 Influence of inorganic nitrogen sources on the dye decolorization by Isolate-I and II

Table IV.5 shows that four different nitrogen sources have been tested to optimize the growth medium. It was decided to use Ammonium sulfate as nitrogen source in the synthetic medium (SM6). Since several bacteria utilize NO_3 as final electron acceptor when they grow in oxygen limited conditions. In the present study, effect of absence and presence of KNO_3 on dye decolorization.

V.7.1 Effects of KNO_3 on dye decolorization

As explained in the section **(IV.2.13)**, six sets were observed to compare results of KNO_3 (at 5 mg/100ml and 10mg/100ml). Results **(Figure V.6)** show that the presence of KNO_3 in addition to ammonium sulfate in the medium **(Set 5 in Figure V.6)** did not decolorize the azo dye more than 40% when compared with set 4 (only ammonium sulfate as nitrogen source) in which 100% color removed within 24 h. The biomass remained the same in all conditions. In the set 3 where KNO_3 was only source of nitrogen and decolorization occurred more than 60%.

Further, increase in the level of KNO_3 (i.e. 10 mg/ml, set 6) had remained (40%) the amount of dye color removal **(as in set 5)**. Thus, the evidence clearly shows that KNO_3 inhibited the process of decolorization (about 50%) while the biomass was not affected. The set 3 results indicate that Isolate-I could utilize KNO_3 as nitrogen source.

V.7.2 Combined effects of RF/KNO_3 under stationary and shake cultures

V.7.2.1 Shake Culture (Table V.12)

The assays were designed employing six different sets and the process of decolorization and biomass estimated at regular intervals 0, 12, 24, 48 and 72 h (in triplicates).
Set - 1 contained only SM6 + RF + dye. There was no growth and biomass formed.

Set - 2 in which assay mixture comprised only SM6 + RF + Inoculum (Isolate-I). Highest biomass observed at 24 h time (0.948 OD).

Set– 3 composed of SM6 + Dye + KNO_3 (5 mg/100ml) did not show significant decolorization (only 31%) within 72 h.

Set - 4 contained all components except KNO_3. The level of color removal was 27%.

Set - 5 all the ingredient except ammonium sulfate of SM6 was excluded. 18% of the dye was decolorized.

Set 6 which was included all the ingredients could remove color only 15%. The lowest value observed compared to set 3, set 4 and set 5. The biomass had not any significant difference.

V.7.2.2 Stationary conditions (Table V.13)

When all these six sets were incubated under stationary batch system, there was not much variation in biomass. However, in the set 4, the cultural conditions favored 100% dye decolorization. In case of set 5, since KNO_3 utilized as nitrogen source, it supported about 50% of color removal. Thus reduction in dye decolorization did have effect of KNO_3, which might have competed for electron channelisation as a terminal electron acceptor

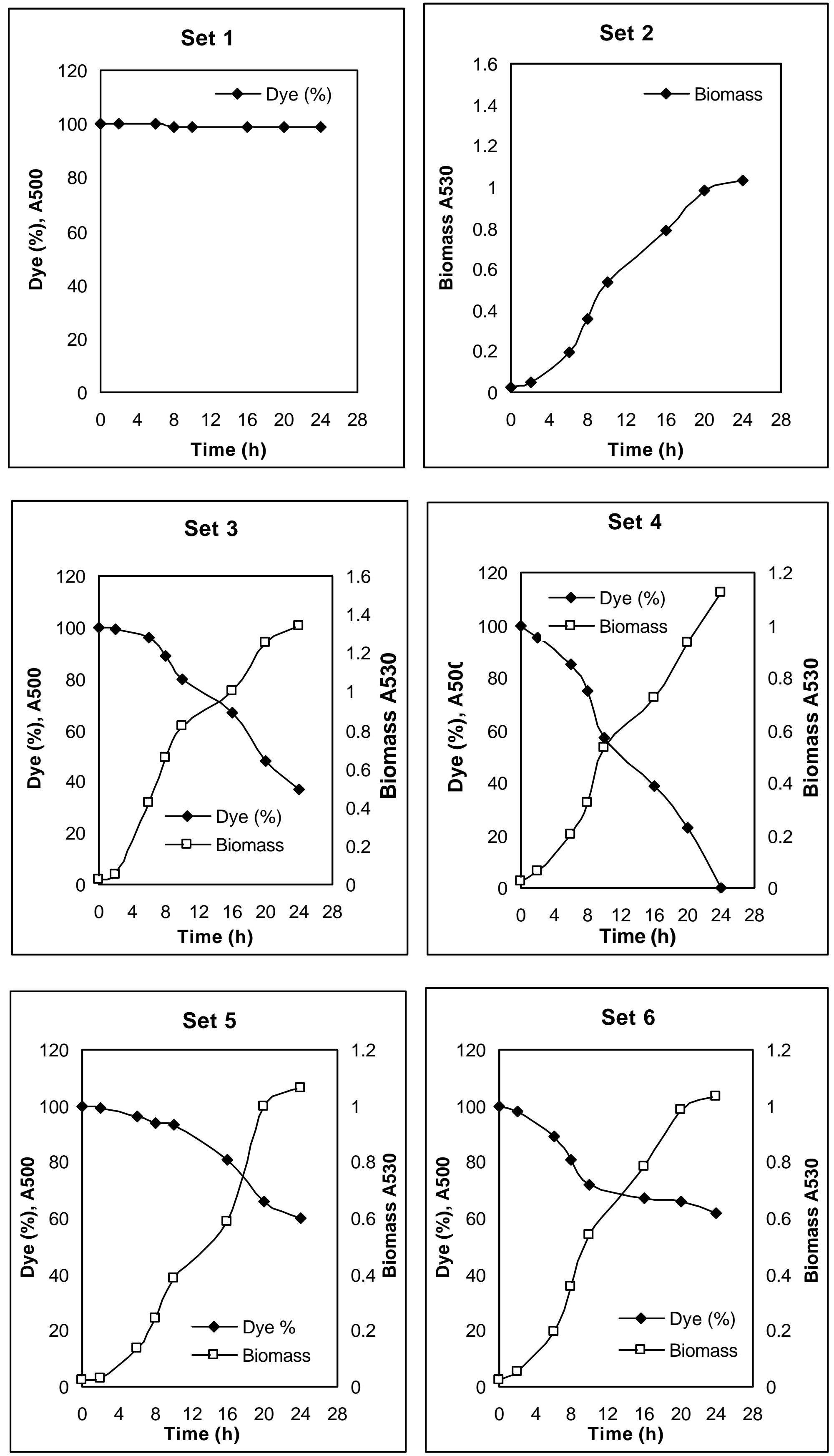


Figure V.6 Effects of KNO_3 on dye decolorization

Table V.12 Effect of riboflavin and KNO_3 on decolorization of Sunzol Violet 5R by Isolate-1 in shaking incubations

Time (h)	Set 1		Set 2		Set-3		Set-4		Set 5		Set 6	
	BM	Dye (%)	BM	Dye (%)	BM	Dye	BM	Dye	BM	Dye (%)	BM	Dye (%)
0	-	100	0.031	100	0.033	100	0.031	100	0.031	100	0.033	100
12	-	100	0.965	98	0.911	95	0.912	93	0.922	96	1.083	94
24	-	99	1.381	97	1.101	88	1.121	87	1.201	89	1.262	85
48	-	98	1.321	93	1.301	75	1.285	75	1.293	83	1.293	85
72	-	98	1.305	92	1.295	69	1.325	73	1.152	82	1.308	85

Table V.13 Effect of riboflavin and KNO_3 on decolorization of Sunzol Violet 5R by Isolate-1 in static incubations

Time (h)	Set 1		Set 2		Set 3		Set 4		Set 5		Set 6	
	BM	Dye (%)	BM	Dye (%)	BM	Dye (%)	BM	Dye (%)	BM	Dye (%)	BM	Dye (%)
0	-	100	0.034	-	0.038	100	0.034	100	0.037	100	0.038	100
12	-	97	0.734	-	0.611	86	0.585	55	0.611	91	0.645	91
24	-	98	0.982	-	0.942	76	0.991	8	0.840	73	0.865	71
48	-	98	1.083	-	1.086	68	0.901	0.0	0.958	54	0.992	51
72	-	98	1.002	-	1.105	63	0.915	0.0	1.082	53	1.140	50

V.8 Effect of varying amounts of dye on decolorization (Figure V.7)

Figure V.7 shows the results obtained on the decolorization process when the assay mixtures were subjected to different concentration of the dye (0.5 mg to 50 mg /100 ml) incubated at $30^{0\ C}$ up to five days under static conditions. Percentage of dye decolorization measured at every 24 h and biomass was measured at the end of the experiment (120 h). It shows that for 0.5. 1.0 and 2.0 mg / 100 ml levels, there was complete decolorization of the dye within 24 h, while in 3.0 mg dye concentration, it took 48 h. As the concentration of the dyes increases, the process of decolorization decreased and above 10 mg / ml level there was small amount of dye decolorization even after 120 hours. Biomass estimated of each level after 120 h (o.5 to 10 mg /100 ml) was 1.01 OD, whereas in case of 15 to 50 mg / 100ml, biomass was within 0.25 OD.

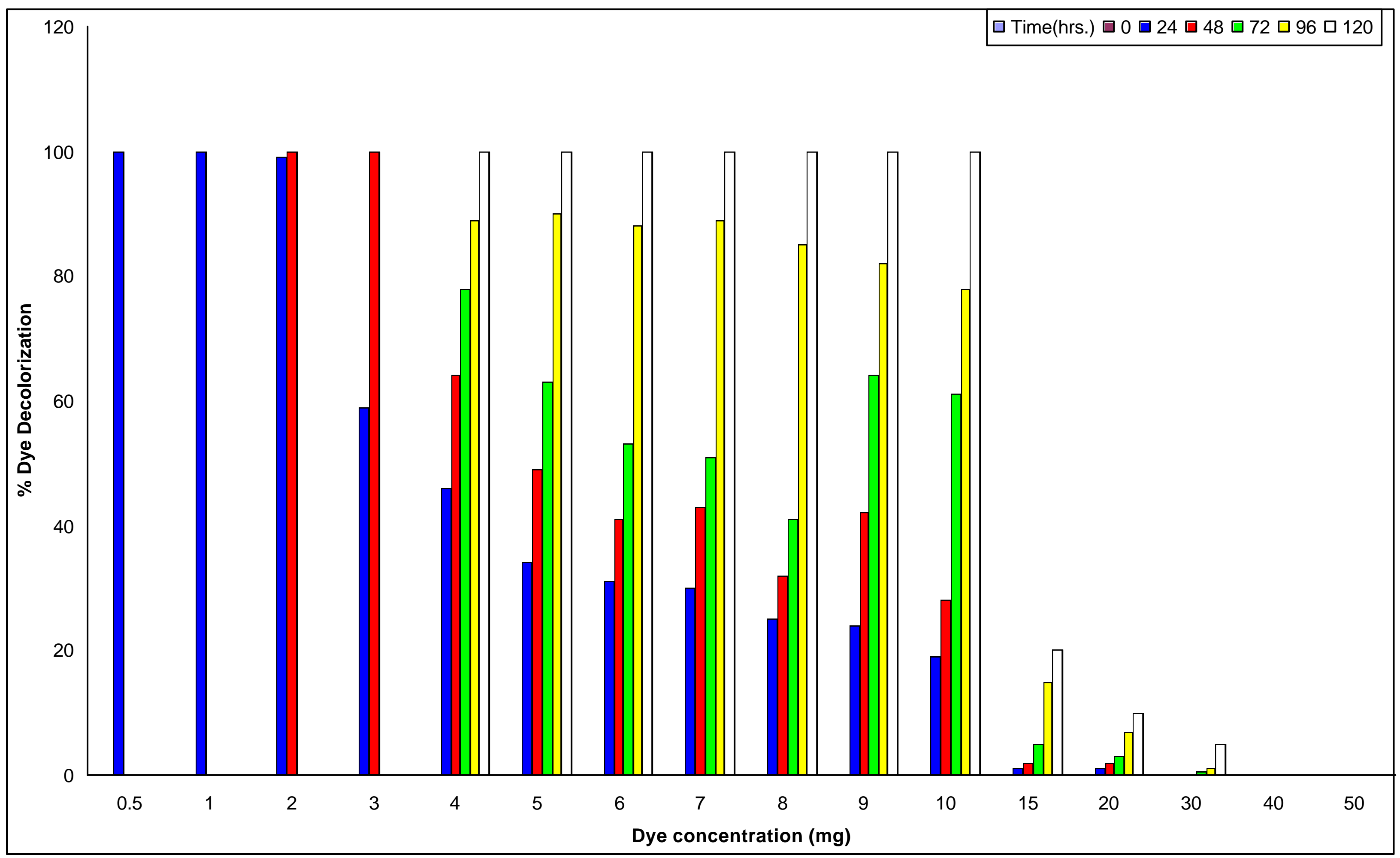


Figure V.7 Influence of varying concentration of Sunzol Violet 5R on decolorization activity of isolate – I

V.9 Fed batch analysis of dye decolorization on successive addition of the dye

The most widely used technique for many results is fed-batch process. In this mode there is no true steady state and evaluation of the state variables will locate the position of the system on a trajectory through the operation cycle. In the present experiment, all the cultural parameters, the SM6 medium + carbon source (glucose) + biomass + incubation conditions were maintained constant. The only parameter, i.e. addition of fresh solution of the dye that added at the certain time intervals. The successive loading of the dye was done in the assay medium when 100% color removal **(table V.14)** and also pH adjusted to 7.0. Changes in biomass were measured at every successive interval. Extended course of incubation was up to 128 h and in total 820 mg of dye could decolorize. More than 50% biomass increase within 24 h and further incubation showed almost 8 – 10 % increment, up to 104 h.

Table V.14 Fed batch analysis of dye decolorization on successive addition of the dye

Total Incubation time (h)	Loaded dye (mg)	Cumulative values of loaded dye (mg)	Biomass	pH	pH*
0	50	50	0.045	7.0	7.0
24	50	100	0.625	4.1	7.0
39	50	150	0.721	5.5	7.0
42	50	200	0.958	6.2	7.0
45	50	250	1.101	5.6	7.0
60	170	420	1.205	5.8	7.0
80	200	620	1.255	6.2	7.0
104	200	820	1.348	5.8	--

*pH adjusted before addition of fresh dye solution

V.10 Decolorization and further degradation in sequential anaerobic – aerobic batch system (Table V.15)

The experiment commenced (step I) in 250 ml flask (in triplicates) the SM6 medium (150 ml) + 5ml inoculum (0.5 ml OD – 0.7) + riboflavin 1 ml (3mM) dye(s) (set- 1 Sunzol Violet 5R; set – 2 Biebrich Scarlet; each 2.5 mg), incubated under static condition for 24 h 100% of dye decolorization was observed at 16 h.

In step – 2, these flasks were transferred on rotary shaker (100 RPM) at $30^{0\,C}$ and further incubated up to 72 h. Results were compared with control for both the dyes. The biomass when compared with control, the controls showed slightly higher value (0.895 OD) while in the treated cultures biomass was (0.668 OD for Biebrich Scarlet and 0.692 OD for Sunzol Violet 5R). During the incubation in step – 2, the biomass of treated cultures were raised from 0.7 OD to 1.7 OD, while in cases of controls, the biomass increased was less (0.895 to 1.34 OD).

Table V.15 Decolorization of dyes in sequential anaerobic and aerobic system

Time (h)	Control (w/o dye)	Biebrich scarlet		Sunzol Violet 5	
		Percentage decolorization	Biomass	Percentage decolorization	Biomass
0	0.094	0	0.092	0	0.095
5	0.228	41	0.208	40	0.218
10	0.810	67	0.838	55	0.659
16	1.132	100	1.068	100	0.992
After decolorization, growth on aerobic condition					
Time (h)	**Control (w/o dye)**	**Biomass in Biebrich scarlet flasks**		**Biomass in Sunzol Violet 5 flasks**	
16	1.133	1.068		0.992	
22	1.361	1.288		1.298	
48	1.422	1.651		1.689	
72	1.481	1.650		1.685	

V.11 Measurement of color removal of the dye and degradation by UV–Visible spectra

SM6 medium seeded (OD) with isolate – I, and the stationary culture that reached maximum biomass and during the batch process 100% dyes have been reduced. The assay medium was withdrawn (5.0 ml) at zero hour, 24 and 48 hours. The first 24-hour interval sample was centrifuged (10,000 RPM). The supernatant then obtained was employed for measurement of the absorption spectra by scanning between 200 to 700 nm **(Figure V.8 and V.9)**. The spectra obtain for the anaerobic (stationary) and aerobic (shaking) demonstrate decrease in peak height and shifting.

Biebrich Scarlet: The first peak at 200 nm was 2.8 in control had not decreased significantly within 16 hours, while after 48 h there was an increase in the absorbance (2.8 to 4.0). Again at 240 – 245, the same pattern observed except that of 48 hours absorbance was significantly more than 2.5 A. At 270 – 273, there was increase in absorbance after 24 h. There was high absorbance peak noted at 385 nm (0.657 A) that reduced to 0.5 (16 h) and 0.354 (48 h). A new peak was developed (0.604 A) at 450 nm within 16 h that again decreased to 0.288 A. The $?_{max}$ for this dye is 500 nm and the same was noted in the Figure 10 that totally disappeared after 16 and 48 h. The decolorization of Biebrich Scarlet was clearly evident by the loss of color in the visible region (480 to 530) and the increase in UV absorption (200 – 260 nm). The same pattern was examined in case of Sunzol Violet 5R.

V.12 Confirmation of further metabolism of the product(s) generated during anaerobic reduction

Firstly, the stationary batch culture (in six replicates and control) that consisted SM6 (150 ml), Inoculum, dye Biebrich Scarlet, and was incubated at $30^{0\ C}$ for 24 h. After the complete decolorization, three flasks harvested and centrifuged aseptically, supernatant was autoclaved after adding 2.0% agar and plates were prepared and streaked with the same isolate–I (from young culture). Incubation for 24 to 48 h was examined for development of colonies on the plates. There was development of colonies on the streaked areas while in control (stationary

culture without dye) no colony formation observed. Remaining three flasks along with control were further incubated aerobically (shaking condition i.e. at 100 RPM) on rotary shaker for 48 h. The steps of agar plating were repeated in the same manner. It was found that there was no colony formed on plates (after 48 h) even in control also.

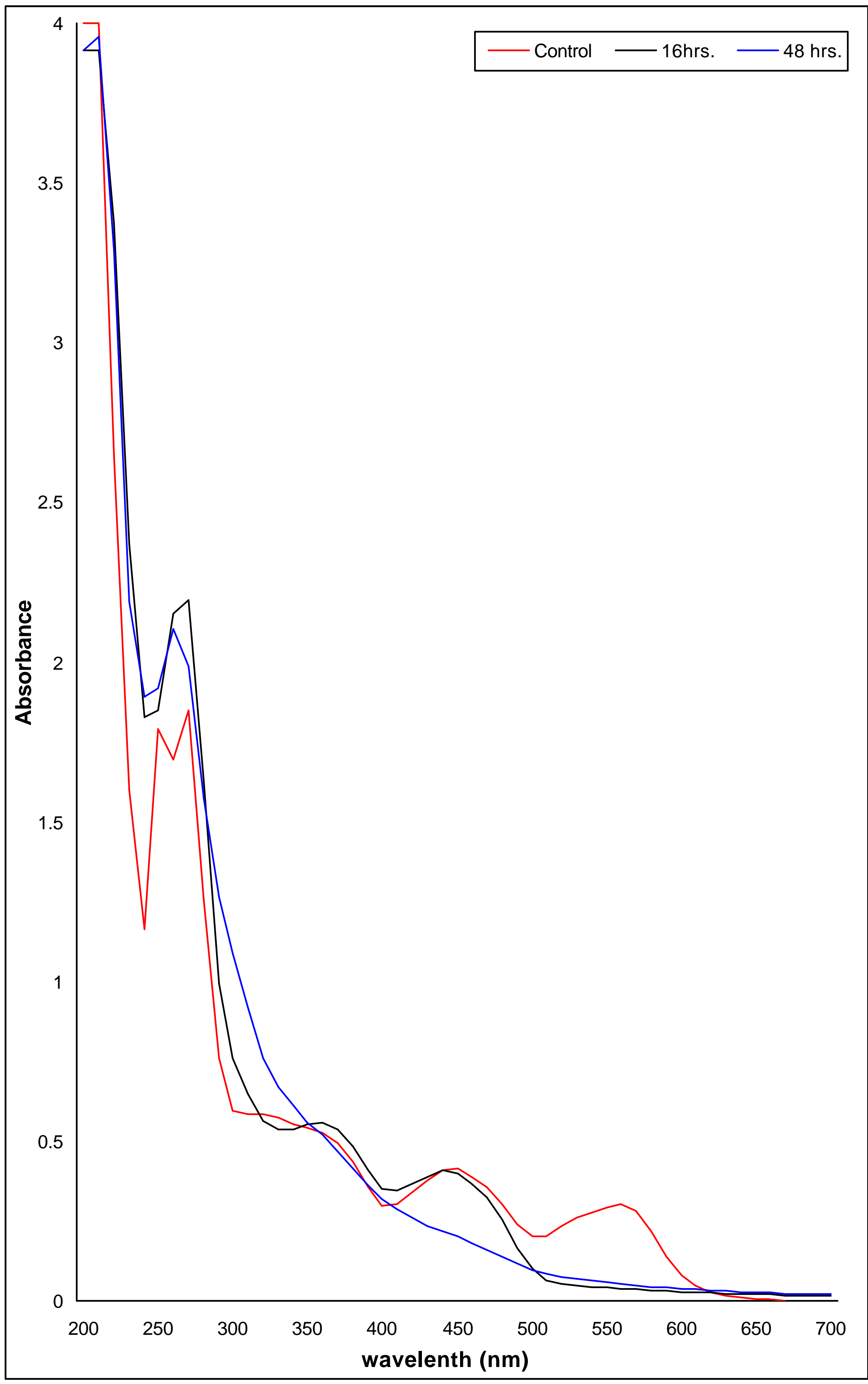


Figure V.8 Decolorization and further degradation of Sunzol Violet 5R in sequential anaerobic - aerobic batch system

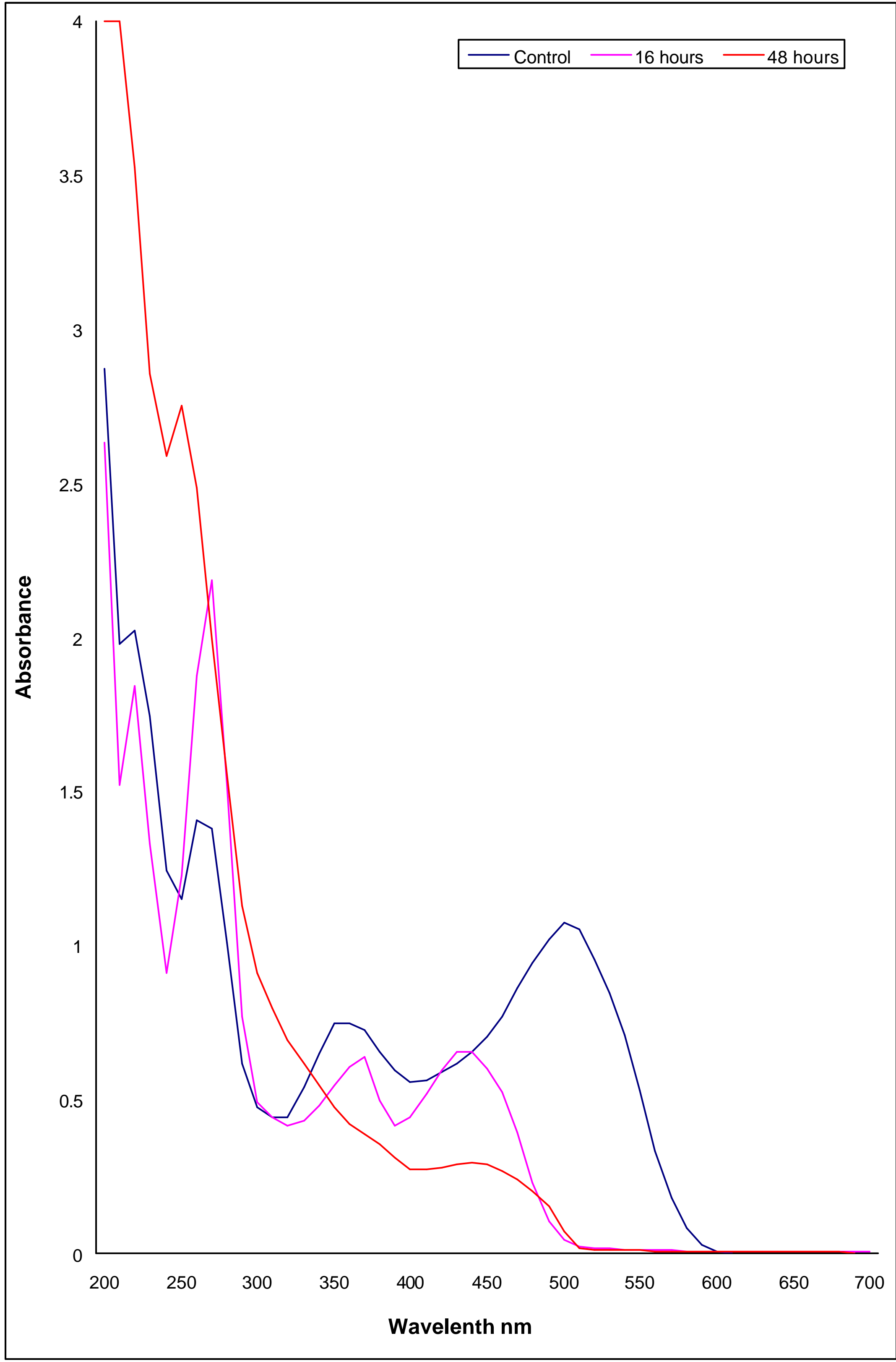


Figure V.9 Decolorization and further degradation of Biebrich Scarlet in sequential anaerobic - aerobic batch system

CHAPTER VI: DISCUSSION

VI.1 General Discussion: Understanding of general physiology of the bacterial cultures and important parameters

The diversified prokaryotes are able to survive and grow (sustain) under every varying environmental condition wherever they occur. Variability and adaptations (acclimation) are the crucial characteristics of prokaryotes. The most adaptable organisms contain a large reservoir of genetic information encoding alternative biochemical pathways/reactions designed to cope with variety of environmental situations. Organism has the genetic capability to respond to altered conditions does so when stimulated by specific transcriptional or behavioral response is the 'essence of life'.

In view of the present study that mainly focuses on the variety of nutritional parameters and the batch culture system where the dynamic medium conditions assumed to have several influence on the physiological manifestations during the growth stages.

Firstly, the discussion involves understanding of the physiological processes that how a bacterium, in general, performs its metabolism during normal medium conditions and the conditions generated in course of incubation by the growing organism, an over view of metabolic network of bacterial central carbon metabolism **(FigureVI.1)**.

Secondly, to relate the conditions with the bacterial metabolic action(s) on the process of dye decolorization. As a matter of fact that (i) Why a bacterium transfer the reducing equivalents to the azo bond(s)? (ii) What are the metabolic (physiological) properties of a bacterium that continues to grow in facultative environment (may be at slower rate than the aerobic conditions) and maintain its energy levels for the supply of **ATP** and reoxidized **NAD^+** and (iii) What is the mechanism(s) that force the organisms to transfer the reducing equivalents to azo bonds.

A brief discussion on the present status of knowledge of the subject(s) mentioned above is necessary.

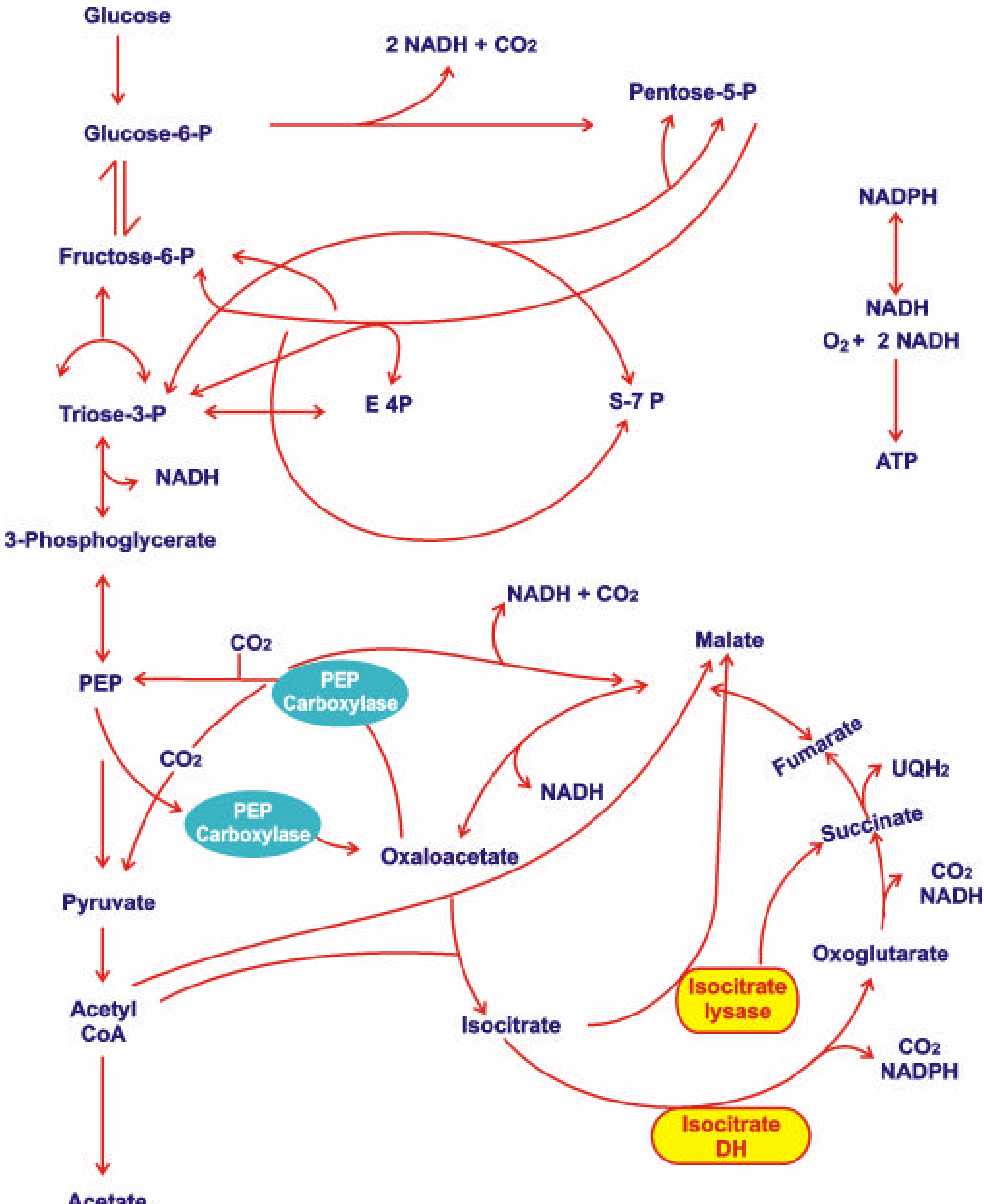


Figure VI.1 Bioreaction network of *E.coli* central metabolism

VI.1.1 Factors that influence the transformations of xenobiotics

In order to understand why xenobiotic molecules (not all) can persist for a prolonged time in the environment, or survive wastewater treatment operation, it is essential to understand and study the factors that influence their transformation/degradation.

VI.1.1.1 Factors

[A] Chemical – specific factors

State	? Gas, liquid or solid surface area
Solubility	? Aqueous
Hydrophobicity	? Ability to dissolve in hydrophobic (lipophilic) solvents, hydrophobic compounds will have relatively low water solubility
Absorbability	? Ability to adsorb to and complex with organic and inorganic fraction in soil, sediments and water
Size and Shape	? Can affect ability to permeate cell membrane and to interact with enzymes
Change	? Can affect ability to permeate cell membrane and to interact with enzymes
Toxicity	? May be of specific effect or a general one
Detailed molecular structure	? Important factor include: a) Presence of an "easily metabolically" structural unites b) Presence of a "difficult to metabolize" structural unites c) Presence of an "un-natural (xenobiotic)" structural unites d) Degree of branching e) Nature of substituants f) Number of substituants g) Position of substituants
Concentration	? Too high or too low

[B] Environment specific factors

Biotic factors	? Presence of suitable or potentially suitable organims
Abiotic factors	? Physical: temperature ? Chemical: (i) Nutrients = minerals and growth factors (ii) Presence of oxygen as: - terminal electron acceptor - substrate - inhibitor ? Presence of alternative electron acceptors: (i) Nitrate (ii) Sulphate (iii) Carbon dioxide ? pH ? Inhibitory materials ? Soil type ? Moisture level ? Type of water – fresh, brackish, saline

The environment specific factors, with respect to the present investigation have been experimentally studied under laboratory conditions and reported here.

VI.1.2 Oxygen diffusion in culture media

The standard laboratory media became saturated with air within a very few days after sterilization, partly through diffusion, partly through convection currents. The solubility of oxygen from the air in these media is not much different from that in water. The oxygen content in the deep layers, e.g. near the bottom of a completely filled flasks will be below saturation point.

A medium saturated with at **$30^{0\ C}$** is depleted of all its oxygen when the bacteria have multiplied **2** to **10** million cell per ml. The maximal population reached in these cultures when grown in test tubes or flasks is about **500** to **2000** million. In other words the oxygen becomes exhausted when only about one percent of the maximal population reached.

VI.1.3 Aerobic-anaerobic switch

In aerobic growth, energy can be generated through oxidative phosphorylation and molecular oxygen is available as an electron acceptor in a variety of enzymatic reactions. In anaerobic growth, energy is generated solely through fermentation or anaerobic respiration. To efficiently regulate cellular metabolism in these alternative routes and physiological states, a large numbers of genes are differently expressed in response to oxygen **Figure VI.2** and **VI.3** depict energy sensing mechanisms and metabolic network during facultative / anaerobic conditions.

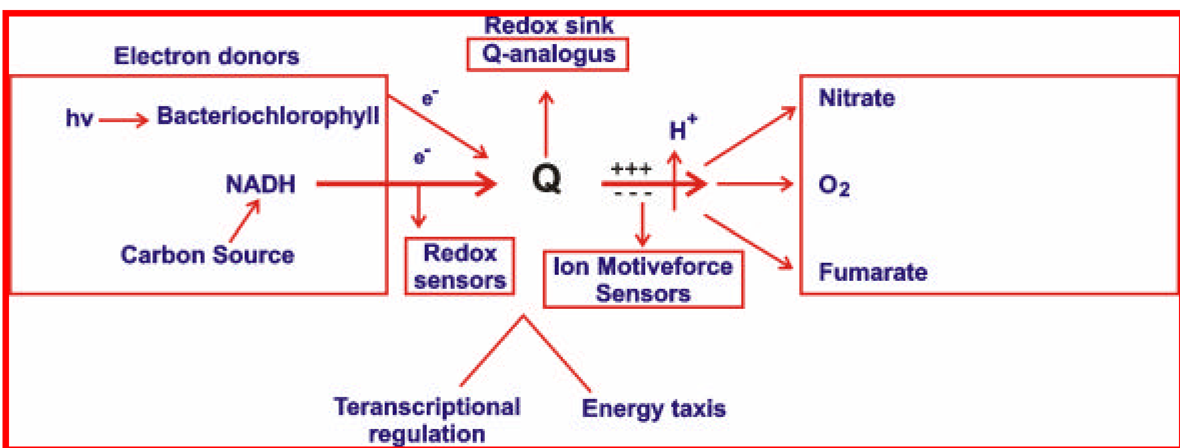


Figure VI.2 Scheme showing energy sensing through the electron transport system (Taylor et al., 1999).

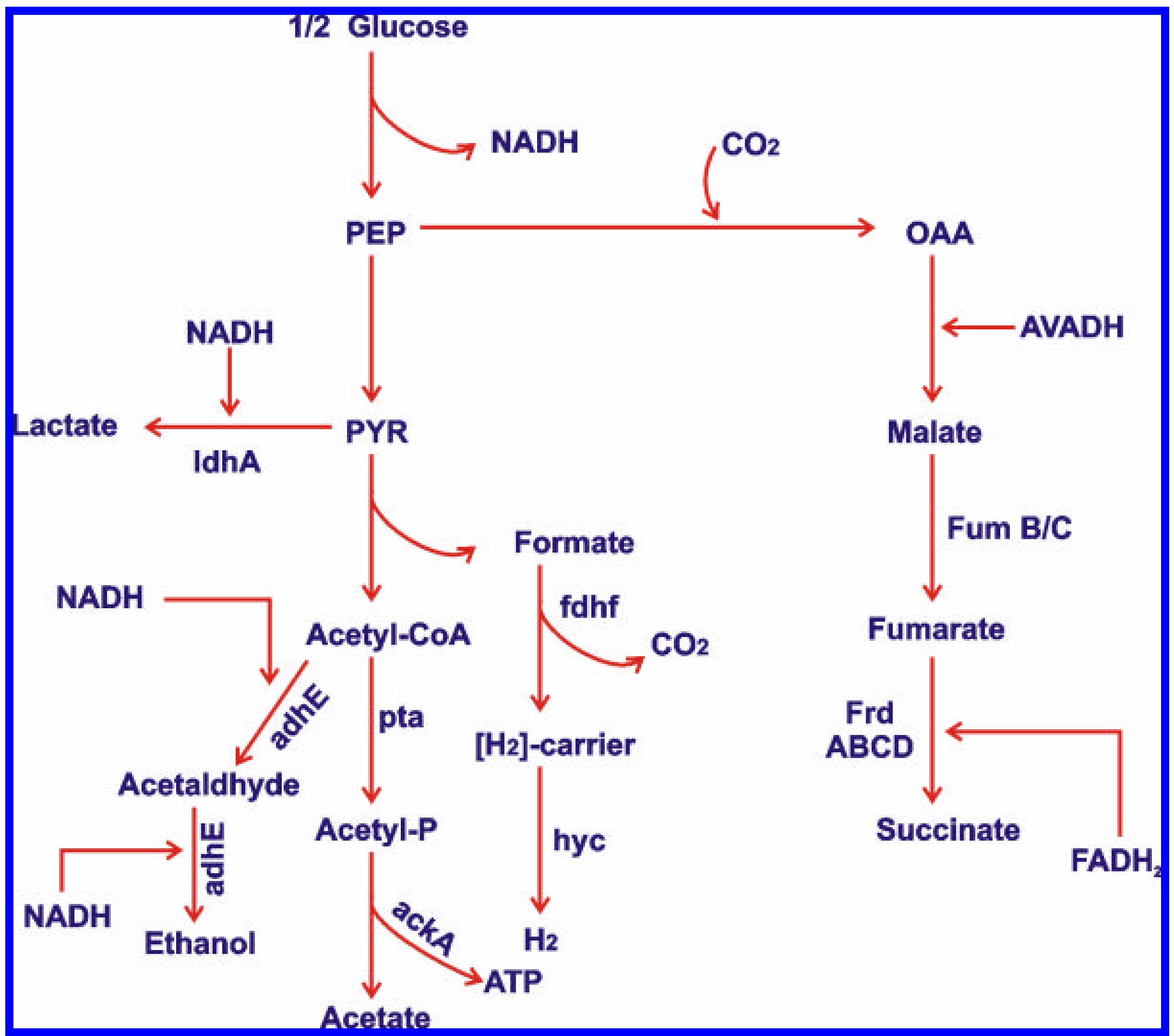


Figure VI.3

Common to the metabolism of various energy sources is the generation of electrochemical gradients that provides an electron donor for metabolism and allows for maintenance of a membrane potential and proton motive force. In microbial metabolism, the energy produced from the driving force of electrons is directly proportional to the ? E_0' value between the initial electron donor (the dehydrogenating reaction) and the final electron acceptor (final hydrogenating reaction) **(Thaur et al., 1977**).

Thus, electron transfer processes drive the energetic of living systems. Electrons are funneled from a source that becomes oxidized to final acceptor that becomes reduced.

The advantages for a bacterial cell survival of sensing several environmental signals (oxygen, light, redox potential, and energy levels) have been well recognized. Many microorganisms are adapted for living within a certain range of oxygen concentrations. There is a increasing evidence that depletion of cellular energy levels is first seen in a decreased electron transport or proton motive force that precede an observable change in **ATP** level. ***E.coli*** senses intracellular redox changes and migrates to a microenvironment with a preferred redox potential **(Bespalov et al., 1996)**. The metabolic effects of oxygen, proton motive force and redox potential are interrelated on the level of the flow of reducing equivalents through the electron transport system **(Table VI.1, Figure VI.4, VI.5, VI.6)**.

Table VI.1 Electron flow during aerobic and anaerobic respiratory conditions (Park and Gunsalus)

Condition	e^- Donors	Quinone	e^- Acceptors
Aerobic	NADH Succinate Glycerol Lactate Fatty acids	→ Q	1 → 2 → Oxygen
Anaerobic	NADH Glycerol Formate Hydrogen	→ MK	3 → Nitrate 4 → Nitrite 5 → DMSO/TMAO 6 → TMAO 7 → Furmarate

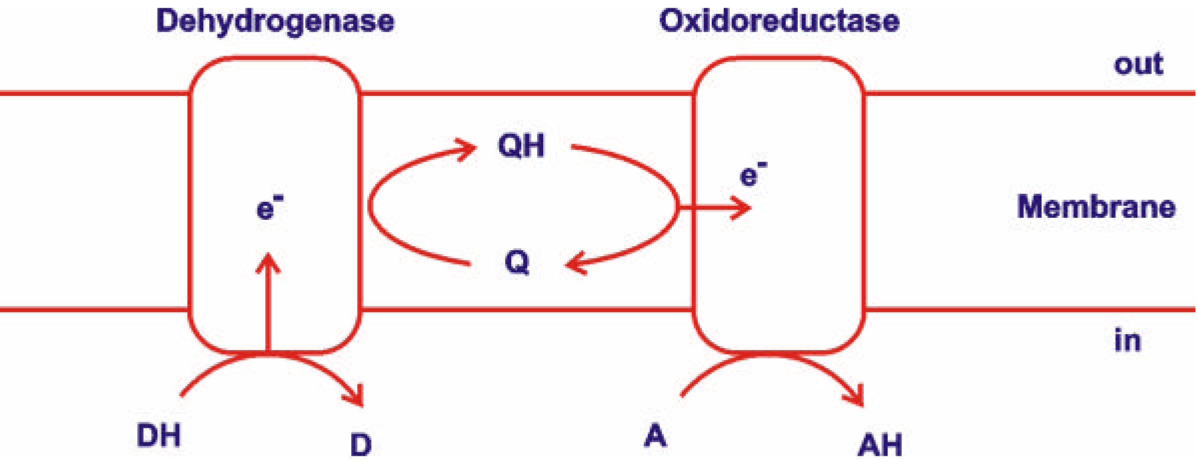


Figure VI.4

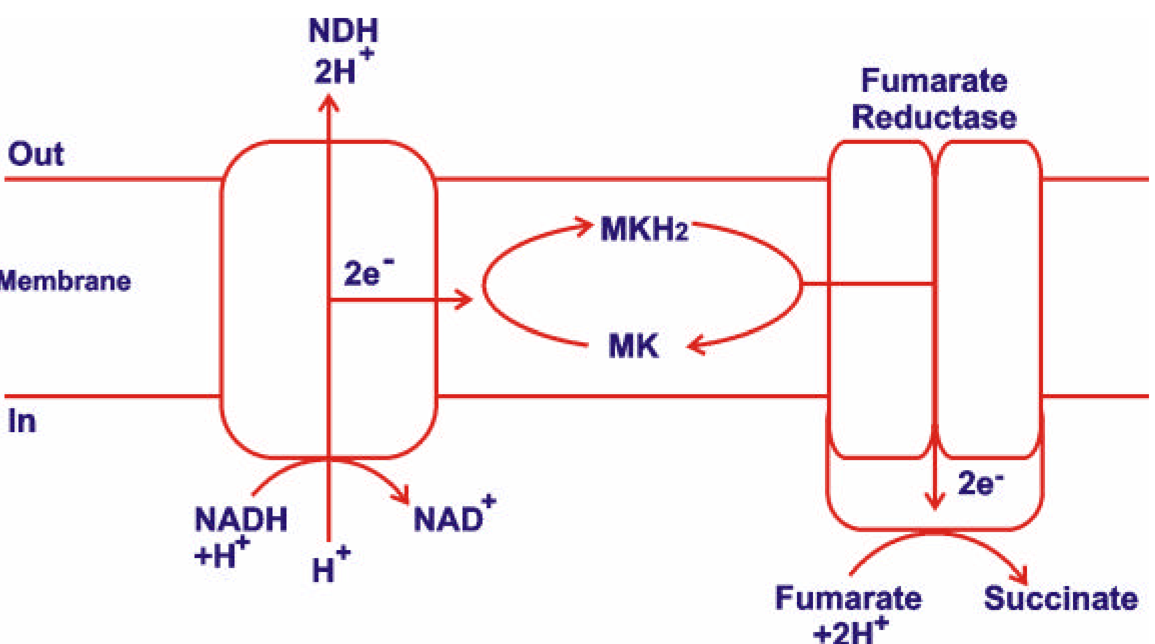


Figure VI.5

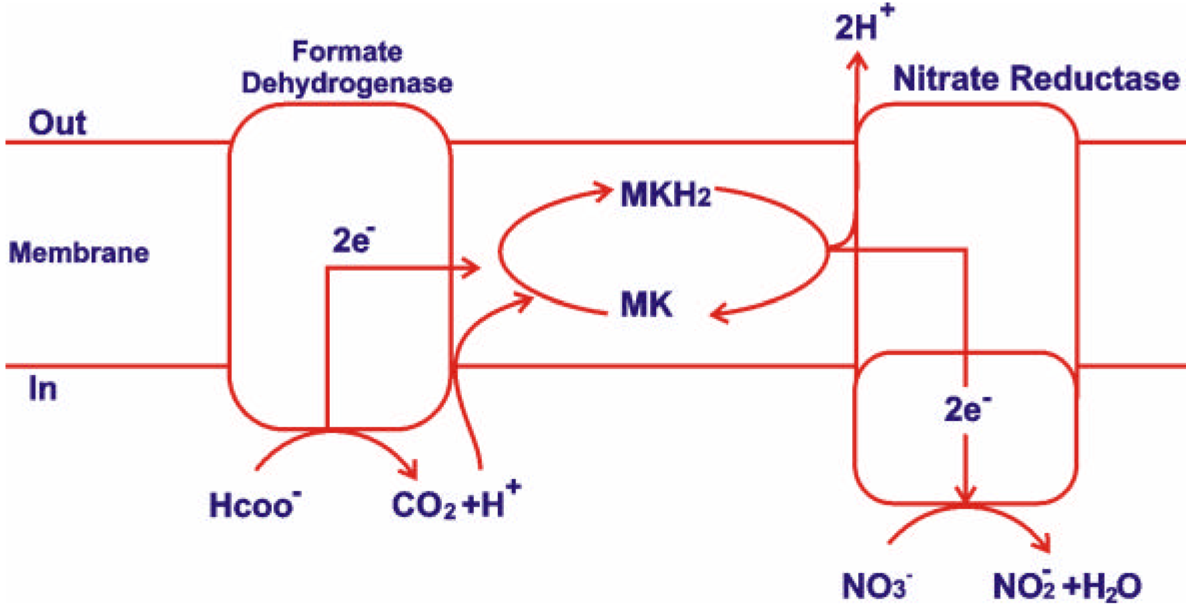


Figure VI.6

NADH serves as the major electron donor during growth on glucose: other electron donors couple electron flow to the quinone pool via their specific dehydrogenises. Ubiquinone (Q) is the intermediate electron carrier during respiration with O_2 or NO_2, whereas menaquinone (MK) functions under anaerobic conditions with any of the anaerobic terminal oxidorudutase enzymes. The levels of these two lipophilic carriers in the cytoplasmic membrane also vary depending on the availability of oxygen. If neither oxygen nor any of the alternative anaerobic electron acceptors are present, the facultative organisms must resort to a fermentative mode of carbon catabolism, also called mixed acid fermentation.

The feed-forward motif cluster comprising the **"AEROBIC-ANAEROBIC SWITCH MODULE"** contains the coding genes for the central aerobic regulatory transcriptional factors, **FNR** (Fumarate – Nitrate – Reductase regulator), which globally regulates the gene expression in response to oxygen deprivation (oxygen tension) in ***E.coli*** and several other bacteria. The transcriptional regulation in response to oxygen is effected by two transcriptional regulators **arcAB** (aerobic respiratory control) and fnr. These two global regulators are the major controlling factors of catabolic gene expression and in most cases operate coordinately to fine – tune the catabolism in response to oxygen. The **FNR**, along with other sets of genes and/or

operons comprise a distinct model in ***E.coli***. **FNR** controls transcription of many genes whose functions facilitate adaptation of a cell to grow under oxygen limiting conditions. Thus, responding to the changes in the state of the growth medium, the **FNR** protein functions as the main regulator at the time of aerobic to anaerobic switch during the growth of ***E.coli*** (**Kiley and Beinert, 2003; Sawers, 1993**).

Low oxygen tension activates **FNR** protein, which is expressed constitutively and is inactive under aerobic conditions. The results of chemostat measurements and theoretical prediction in a report have shown that the genes for aerobic respiration (the **sdh gene** encoding succinate dehydrogenase) are expressed efficiently only at oxygen tensions above **5 m bar** (˜ **5 µm O_2**), genes for micro-aerobic or anaerobic respiration are expressed between **1 – 5 m bar**, while genes for fermentation are expressed or function at O_2 tensions below 1 m bar **(Unden, 1998)**. In a recent study by **Gunsalus** group **(Salmon et al., 2003)**, DNA microarray technology was used to identify genes involved in the regulatory networks that facilitate the transition of ***E. coli*** **K – 12** cells from an aerobic to an anaerobic growth states. They have identified several genes regulated by **FNR** and demonstrated that the one-third of the genes expressed during growth under aerobic conditions are altered when ***E. coli*** cells transition to an anaerobic growth state. They also showed that the expression of **49%** of these genes is either directly or indirectly modulated by **FNR**.

In another recent study by the same group **(Wang and Gunsalus, 2003)**, the role of **NarL** and **NarP** genes in regulation of genes of formate dehydrogenase operon (**fdhGHI** and **fdh F**) in response to nitrate, nitrite and formate in anaerobic chemostat cultures has also been shown. Another major part of this motif cluster (two component **arcA** / **arcB** system) is the major control system for the regulation of expression of genes encoding enzymes involved in both aerobic and catabolic pathways. **ArcA** is a cytoplasmic regulatory protein and **ArcB** is a transmembrane protein, which senses unfavorable respiratory conditions.

VI.1.4 Reduction of fumarate and nitrate by facultative microorganisms

The amount of energy that can be potentially be generated by a given respiratory pathways depends on the redox potentials of the electron donor (DH) the electron acceptor (A)

Redox couple Ox/Red	E_0' (mv)	Redox couple Ox/Red	E_0' (mv)
SO_4^{-2} / HSO_3^-	- 516	HSO_3^- / HS^-	- 116
CO_2/ formate	- 432	Monoquinone Ox / Red	- 74
H+ / H2	- 414	APS / AMP + HSO_3^-	- 60
$S_2O_3^{-2}$ / HS^- + HSO_3^-	- 402	Fumarate / Succinate	+ 33
NAD^+ /NADH + H^+	- 320	UQ Ox / Red	+ 113
CO_2 / Acetate	- 290	HSO_3^-/ NO	+ 350
S^0 / HS^-	- 270	NO_3^- / NO_2^-	+ 433
CO_2 / CH_4	- 244	Fe^{3+} / Fe^{2+}	+ 772
Acetaldehyde / ethanol	- 197	O_2 / H_2O	+ 818
Pyruvate / Lactate	- 190	NO / N_2O	+ 1175
DHAP / Glycerol-p	- 190	N_2O / N_2	+ 1355
HSO_3^- / $S_3O_6^{2-}$	- 173		

VI.2 The present studies

In the present study, the focus of the research was to isolate and screen bacteria capable to decolorize azo dyes and experiments were programmed to explore and optimize physiological condition and attempted to analyses the important metabolic situation(s) that compulsively lead to the dye decolorization.

VI.2.1 Screening of suitable isolates

Several pure cultures **(33 isolates, Table V.2)** isolated and screened showing decolorization activity against several dyes. As shown in **Table V.2 and V.6**, four bacterial isolates (named as **Isolate – I** to **Isolate – IV**) and five azo dyes were employed in the present work. Attempts were made to categorization has been considered tentative.

1. From the results described, it was quite evident that all the 33 isolates have been observed to decolorize the dye ranging from 50% to 100%. Isolate I to IV are the most efficient to perform the process within 24 h (**Table V.2**).

2. Experimental results **(Table V.6, V.7, V.8 and V.9)** obtained led to infer that:
   - Both the broths (N-broth and SM6) are supporting the favorable condition to biomass increase.
   - It can also be noticed that normal growth stages have been in progress.
   - The defined medium SM6 has been considered as the suitable medium in this study.
   - It is also noticeable that $(NH_4)_2SO_4$ has been the favorable nitrogen source indicating that the inorganic nitrogen was utilized by all the four isolates. Thus, the organisms are capable of assimilating the inorganic nitrogen for the synthesis of nitrogenous organic molecules.
   - The four isolates were exposed to 13 different azo dyes **(Table V.6)**. The isolates could completely decolorize all the azo dyes and the biomass yielded almost 1.0 OD under the experimental stationary incubation conditions.

VI.2.2 Profile of the cultural medium property during the course of incubation

The question is that "How does the development of biomass (increase in cell population) influence the characteristic of the medium?"

The medium (SM6) composition is well-defined containing all the essential nutrients C, N, P, S macro and microelements. Glucose has been selected to act as a source of carbon. Two salts of phosphate, KH_2PO_4 and Na_2HPO_4 were included in the medium to serve as a source of phosphate and also acting as buffer(s) against change in pH.

Over the course of study, the Isolate-I was used as the "suitable organism". Albeit, whenever comparison to be made, two all four isolates were considered.

Combination of several physical and chemical parameters, were selected to determine their influence on the process of biodecolorization.

Physical conditions:

- Shake batch culture system
- Stationary batch culture system

Chemical parameters:

- Influence of complex and chemically defined medium
- Selection of carbon sources
- Selection of optimum concentration of carbon source
- Influence of different azo dyes
- Change in pH of the medium over the course of incubation
- Formation of biomass under different conditions

Physiologically all the four isolates were chemoorganotrophic that able to grow and multiply under both aerobic and facultative (may be referred as partially anoxic or oxygen limited) conditions. However, the results obtained indicating that oxygen limited conditions did not encourage the process of the cell multiplication (i.e. either late exponential or stationary growth phase).

The fact of matter is that the cultures did not remove the dye color in the assay medium when grown aerobically (i.e. under shaking conditions) to significant level, while the opposite stationary batch conditions did show lightly significant changes of the dye color in the assay mixtures.

From the findings, it is confirmed that none of the isolates was capable to carry out the process of decolorization in aerobic conditions. Further, the initial conditions (i.e. when the isolate achieve culture was inoculated) in which sufficient level of oxygen available for the organisms consequently consumed O_2 and glucose during their exponential phase (i.e. 8 to 14 hours). When the culture entered in to late exponential to stationary phases, the assay medium reached to oxygen limited (depleted) conditions. However, glucose level was not limiting the growth but the oxygen limited conditions led to a physiological condition, which might be either anaerobic or fermentative.

The findings clearly demonstrated that the process of dye decolorization initiated in all the experiments after six hours of incubation time and maximum (or complete) reduction (removal of color) of azo bonds could be observed, in most cases, within 24 hours **(Figure V.3, V.4, V.6)**.

Thus, as the biomass increased the oxygen level in the medium was assured to decrease, but the amount of carbon source-glucose, was enough to support fermentative way of growth. The result also support that the maximum biomass yielded during aerobic growth condition was not significantly reduced even after incubation for 48 h. This also indicates that net growth rates were near to zero.

The most essential steps during the incubation were (i) the availability of O_2 in the initial hours of incubation (0.0 to 24 hours) which favoured the increase in biomass and (ii) the oxygen depletion would occur in the medium to support the reducing conditions when the flow of reducting equivalents would search for alternative electron sink(s).

Thus, it was very much necessary to build up anaerobic conditions in the assay medium which again be favoured by the catabolic generation of enough CO_2 that usually persists in the headspace of the cotton-plugged assay flasks (100 ml medium in 250 ml conical flask) that do not permit oxygen to diffuse in to the assay medium (high partial pressure of CO_2). It is worthwhile to mention that the pH of the assay medium contains to fall down (i.e. becoming acidic) as course of incubation time increases) were inoculated that on incubation reached to 4.25 within 32 h.

VI.3 Optimization of nutrient requirements

Though the complex medium, such as N-broth showed favourable conditions for biomass and process of dye decolorization, the synthetic medium (chemically defined) was employed because of its case to manipulate each ingredient as required in designing experiments.

It is also quite evident from the results **(Figure V.2)** that the maximum biomass could be obtained (four isolates) within 24 hours of incubation under stationery as well as shaking condition. In this experiment, it has been assumed that the shaking conditions could supply oxygen to the cells and favoured faster growth which can be accounted to almost complete conservation of carbon source in to CO_2 + H_2O + biomass. Under the stationary experimental conditions, biomass could be achieved at maximum

level(s) (i.e. up to 1.30 OD) which was lesser than the shake cultures, and also the growth rates were comparatively slower than the shake cultures.

Comparison of the biomass development of four cultures under stationary and shake batch systems

All the four isolates were studied to determine their biomass development when grown in SM6 medium and incubated at 30^{0C} under stationary and shake (aerobic) conditions. Figure clearly demonstrates that the biomass developed much faster when incubated under shake conditions (100 RPM). Initial OD of the medium (SM6, 100 ml) was adjusted to almost of similar values (0.038 to 0.04 OD) without dye and riboflavin. Measurements of OD were read at 4 hours intervals for 24 h.

At each interval the shake cultures showed almost double the growth then the stationary cultures of the four isolates (up to 8 to 12 h incubation).

The starting point of the work was the observation that dye could be decolorized when the inoculated bacterium did allow growing under stationary batch system in which the carbon source remained unlimited but the organism consumed the available oxygen by the time it entered in the late exponential growth stage.

The anoxic or near anoxic environment did favor the process of color removal by reducing the azo bonds of the dye(s). The four isolates, three being **gram negative (Isolate I, II, IV)** and one **(Isolate III)** a gram-positive bacillus species, were enabled to grow aerobically and facultatively (i.e. either anaerobic respiration or fermentative) in **N-broth** as well as **SM6** broth. All the isolates showed inability to reduced azo bonds, even after prolonged incubation under shake cultures. These observations led us to assume that the process of dye decolorization may be referred as biodecolorization, i.e. azo bond reduction.

To test the above stated primary observation, several experiments have been performed. To address the possibility of involvement of electron mediator, RF was added in the medium and its influence on the dye decolorization process was determined.

Facultative bacteria switch off the **TCA cycle**, if the alternative electron acceptor(s) besides oxygen, is not available and have to depend on fermentative metabolism. Thus, depriving the bacteria of the necessary energy to continue exponential and/or stationary phase and may enter into decline stage. From the medium conditions, after **12 h**, contained enough carbon source glucose (**1.0%** or **55 mM** glucose in **SM6** medium) that allowed to support energy and carbon to the cells in medium event during stationary growth stage (glucose was never limiting throughout incubation course in all the experiments).

Throughout growth course (**0.0 h** to **24** or **48 h** or prolonged time) the cells of the four isolates in the medium were not substantially depleted of carbon source The biomass development in the course of incubation consistently progressing (depending on the initially added amount of inoculum), attaining the exponential phase at slower or higher rates and reached to late exponential stage between **8** to **14 h**.

All the four strains were tested shake and stationary batch cultures have been reported to change pH of medium **(Table V.9)**. The observations were made under both the conditions, clearly elucidated that the noticeable decrease in pH values started from **12h (6.5, 6.2, 6.1, 6.2)** to **32 h (5.3, 5.8, 5.1, 5.4)**, becomes acidic conditions. Contrast to stationary conditions shake batch system did not show any significant in medium pH.

Further the values of the biomass formation from **0.0** h to **32 h** under both the conditions were more in cases of shake cultures for all the four isolates than stationary system **(Table V.9)**. However, the process of the dye decolorization found significant high in stationary compare to shake cultures (**100%**, within **24 h**; **18%**, within **32 h** respectively).

In view of the process of decolorization the successive events occurring in the reaction assay mixture have been analyzed. The events occurred reported had led to further investigate weather the process of decolorization was influenced by other parameters, beside the most important, the stationary batch cultures.

The data obtained from the experiments for optimizing the natural conditions as well as other parameters; (i) different mineral combinations, (ii) the most suitable nitrogen

source, (iii) various carbon sources, (iv) glucose was considered to be a carbon source at 1.0% (55 mM) throughout the study, (v) the effect of NO_3^- and RF, separately and in combination (vi) the UV-Visible scanted profile of control and treated samples, (vii) confirmation of further degradation of decolorized products (primary amines).

VI.4 Analysis of UV-Visible profile

The azo dyes, Biebrich Scarlet (BS) and Sunzol Violet 5R (SV 5R) have $?_{max}$ at 500nm and 560nm **(Figure V.8 and V.9)**. Comparisons of the absorbance values at these wavelengths (C-1, C-2 and C-3) the peaks were completely disappeared. The loss of peaks:

$?_{max}$ nm	A at 0.0 h	A at 42 h
500	1.076	0.00
560	0.46	0.00

The new peaks appeared after 24 h incubation, at 250 nm (for BS 2.101) and at 270 nm (for SV 5R 1.347) clearly demonstrate that the new peaks belong to the product(s) after reductive decolorization of azo bonds.

VI.5 Involvement of redox mediator

The concept of redox mediators compounds that accelerate the process of electron transfer from a primary reduced donor to terminal acceptor, which may increase the reaction rates by one to several orders of magnitude **(Cervantes, 2002)**. Redox mediators have shown to effective not only for reductive decolorization, but also reductive transformation of iron **(Loveley et al., 1998)**. **Keck et al., (2002)** found that quinoid redox mediators were produced by *Sphingomonas xenophoga*, which mediated the reduction of azo dye under anaerobic conditions. **They (1997)** also suggested that non-specific enzyme mediator reduction in two distinct steps.

In the present investigation the effect of RF as redox mediator and presence of NO_3^- on electron transfer and subsequent color removal was assessed, by the isolates.

Time	Isolate - I		Isolate - II		Isolate - III		Isolate - IV	
	In presence of RF							
	*St	#Sh	St	Sh	St	Sh	St	Sh
12	0.493	0.892	0.582	0.932	0.564	0.941	0.586	0.893
24	1.241	1.321	1.102	1.424	1.105	1.568	1.234	1.528
	Without RF							
12	0.731	0.981	0.840	1.132	0.720	1.081	0.832	1.182
24	0.981	1.342	0.998	1.411	0.910	1.481	1.231	1.482

*St: Stationary condition #Sh: Shake condition

The values given in the above table indicates that the presence of RF in the medium did not enhance any significance biomass for 24 h incubation. The differences in the biomass at 12 h interval was due to two distinct cultural conditions, i.e. shake cultures where there was adequate level of available oxygen while under stationary conditions, the depletion in oxygen tension in to the medium had slower growth. However, the 24 h incubation showed less difference. Thus, it shows that RF does not affect the biomass.

Further, similar results on biomass were found under stationary conditions, without RF by the isolates. The analyzed data also support that in the RF less medium, the process of dye decolorization was not at all affected. There was 100% color removal within 24 hours. The data also supports that the oxic condition does not allow dye decolorization process to occur at significant level, i.e. compared to stationary cultures, it was negligible (refer the original **Table V.7** and **V.8** and **Figure V.3**).

Secondly, the UV-Visible spectra measured **(Figure V.4)** also strongly support the completely removal of azo bond(s) present in the dyes. The evidence of shifting of peak(s) in the UV range indicates formation of new product(s), mostly aromatic amines.

A possible explanation for the dye to decolorize, without supplemented RF in the medium, that either the bacterial cells produced any mediator(s) or the reducing equivalents from reduced biological molecules **(NADH/FADH$_2$)** might have

transferred to quinone/monoquinone. The later are lipophilic in nature that remained mobile in the membrane directly shuttle the electron to azo dye, since there was no other electron acceptors available.

It has been mentioned that the initial level of glucose was never a limiting (at least for 24 -30 h incubation) that sustained the biomass and thus the flux reducing equivalents were never stopped.

The choice of alternative electron acceptor was further studied under stationary batch culture by adding NO_3^- in the medium. When the medium SM6 containing NO_3^- as a sole source of nitrogen or in combination with $(NH_4)_2SO_4$, there was pronounced effect of NO_3^- on the process of azo bond reduction (Figure V.6). In absence of NO_3^-, there was 100% of decolorization, where as in the assay medium (5 mg/10 mg per ml NO_3^-) the color removal was inhibited significantly less (i.e. 35%) than assay medium (NO_3^- + $(NH_4)_2SO_4$) in which total amount of dye decolorized was only 40%.

The results clearly demonstrate that the addition NO_3^- species in the medium significant inhibit the dye decolorization, indicating again that NO_3^- ions compete with azo dye for reducing equivalents. Further, it is also important to note that NO_3^- is always a better choice to transfer electron in the anoxic oxygen depletion condition, i.e. favour more energy capturing ability than any other acceptor except oxygen. The experimental study noticeably showed that azo bond reduction process was considerably higher in absence of NO_3^- and combination of NO_3^-/RF did indicate the same results.

In an effort to establish a relationship between biomass and change in pH of the assay mixture, the data showed that under stationary conditions, a pronounced decrease in pH (from 7.0 to 5.0 pH) when incubated for prolonged period (32 h) by all four isolates. This finding shows that the cells have switched on to fermentative metabolism during early stage of stationary phase. However, the shifting in pH (acidic) did not affect the decolorization process.

The subsequent experiments performed and data evaluated show that azo dye could not be decolorized in presence of adequate oxygen level. Results expressed in **Tables**

V.14, V.15 and **Figure V.8** and **V.9**, further confirmed that the process of decolorization essentially require the presence of suitable organism(s) that carry out biologically mediated physico-chemical transformation and reduction of azo bonds. Though the reduction reaction may not be enzymatic or biological, but occurred only in the medium containing sufficient level of biomass, oxygen limiting conditions, supply of glucose (or carbon source) that consequently lead to flux of electrons from the metabolism of the cells in assay mixture.

While today there is fairly enough evidence to suggest that under anaerobic conditions, several bacteria and yeast are capable to reduce azo bonds, but we know much less about the small molecules that participate in extracellular shutting of electron transport. However, the shutting of the reducing equivalents from the intracellular donor molecules to extracellular acceptor(s) having required redox potential gradient that enable to generate proton-motive force across the membrane to sustain the metabolic energy requirements.

Indeed the cells growing in such settings (the assay mixtures) are likely to benefit from this type of extracellular electron transfer circuit, in context of the azo dye(s) occurring either in the present undertaken laboratory study or in the natural environment.

The fundamental molecular events involved in such reactions remain unanswered, despite the fact that more than two decades have passed since appreciate the importance of the transfer of electron to such molecules.

Although several workers have claimed to demonstrate the azo bond reducing specific enzymes azoreductases in vitro conditions, the fact that none of the claims provided only further conclusion. The results obtained and evaluated in the current investigation, clearly show that the isolates employed (Isolate I to IV) did not show any likely role of involvement of enzyme or protein to shuttle the electrons, to reduce the azo bonds. The reaction proceeds when the conditions became anaerobic in the presence of the bacterial population in the given broths. The conditions of the medium appeared to switch on to fermentative metabolism with the generation of **NADH** (or

$FADH_2$) molecules that essentially required to reoxidize and passed on to the reactions to generate **NADH** again.

Thus, in the absence of oxygen and other alternative terminal acceptors, the grown cells have to opt for any other acceptor(s) to transfer electron flux to extracellular molecules having sufficient redox potential. The resulting decolorized product(s), the aromatic amines, may be further mineralized (ultimate biodegradation) aerobically **(Table V.15 Figure V.8 and V.9)** when the same culture was employed to sequential anaerobic – aerobic batch culture systems. The spectra of the reaction corresponded with disappearance of the maximum absorption peak and appearance of new peaks in UV region.

The reduction mechanism(s) observed and reported in the current study, on azo dye decolorization, seems to occur only when growing or grown cell culture of facultative bacteria, which are the source of reducing equivalents, transferring them to the azo bond, without involvement of any enzyme(s). It is also proposed that any attempts to search for gene(s) of such bacterial strains would be futile.

CHAPTER VII: REFERENCES

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