Microbial decolorization of textile dye effluent

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CERTIFICATE

Certified that the work included in the thesis entitled, **"Microbial decolorization of textile dye effluent",** submitted to the Department of Biotechnology & Environmental Sciences, Thapar University, Patiala, for the degree of Doctor of Philosophy, was carried out by Ms. Bella Devassy at the Department of Biotechnology & Environmental Sciences, Thapar University, Patiala, under our supervision. This is an original work and has not been submitted in part or full for any other degree/diploma at Thapar University or any other University/Institute.

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I hereby declare that the work which is being presented in this thesis **"Microbial decolorization of textile dye effluent"** submitted by me for the award of the degree of Doctor of Philosophy in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervision of Dr. Sunil Khanna and Dr. Dinesh Goyal. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree.

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LIST OF ABBREVIATIONS

cm	Centimeter
°C	Degree Centigrade
g	Gram
g/L	Gram per liter
HRT	Hydraulic Retention Time
L	Liter
mg	Milligram
mg/L	Milligram per liter
min	Minutes
ml	Milliliter
ml/h	Milliliter per hour
mg/ml	Milligram per milliliter
mmoles	Millimole
hrs.	Hours
BHB	Bushnell Haas Broth Medium
µl	Microlitre
N	Normal
nm	Nanometer
OD	Optical Density
%	Percent
rpm	Revolutions per minute
UV	Ultra-violet radiations
v/v	Volume by volume
w/v	Weight by volume
λmax	Wavelength of maximum absorbance
PCR	polymerase chain reaction
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
NaOH	sodium hydroxide
BOD	Biochemical Oxygen Demand
COD	Chemical Oxygen Demand

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PUBLICATIONS:

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SUMMARY

1. Textile industry discharges large quantities of wastewater effluents containing residual dyes in the ecosystem which deteriorates the water quality, is a source of esthetic pollution, eutrophication, perturbations in aquatic life and a serious threat to public health. Additionally, most of the dyes and their metabolic intermediates are mutagenic and carcinogenic. Synthetic dyes are xenobiotic as they are not synthesized in the biosphere and they resist mineralization in the conventional biological waste water system. World wide production of these dyes is currently estimated at nearly 450,000 tons, with 50,000 tons being lost in effluents during application and manufacture.

2. Azo dyes constitute the largest and most important class of commercial dyes and make up 60–70% of all textile dyes produced. Physico-chemical methods used for treating wastewater from dyeing operations have shortcomings like excess amount of chemical usage and sludge generation with obvious disposal problems. Bioremediation for removal of dyes is gaining interest as it is cost effective, environmentally friendly, and produces less sludge. Thus, for the successful implementation of biological treatment process for removal of azo dyes from wastewater, an efficient microbial inocula having wide versatility to work on azo dyes decolorization was developed in this research.

3. Three aerobic mixed bacterial cultures SKB-I, SKB-II and Con3 were developed and screened for consistent decolorization efficiency using six selected azo dyes *viz*: Direct Red 28 (499 nm), Direct Red 7 (510 nm), Acid Blue 113 (611 nm), Direct Blue 53 (564nm), Reactive Orange 107 (410 nm) & Reactive Red 120 (535 nm) at a concentration of 10 mg/L.

4. Among the three cultures, SKB-II was the most efficient decolorizer as it decolorized four out of the six selected azo dyes. Dyes: Direct Red 28, Direct Red 7, Acid Blue 113 and Direct Blue 53 were decolorized efficiently and the remaining two dyes Reactive Red 120 & Reactive Orange 107 were resistant to decolorization. Therefore, further work was carried out using mixed culture SKB-II with four dyes: Direct Red 28, Direct Red 7, Acid Blue 113 and Direct Blue 53 respectively.

5. Effect of various concentrations of medium components (starch: 0.65 and 1.3 g/L along with NaCl: 4, 2 and 0%) on the efficiency of mixed culture SKB-II to decolorize was studied and it was observed that best decolorization was achieved with 1.3 g/L starch and 4% NaCl.

6. The fifftythree pure bacterial strains isolated from mixed culture were screened for their ability to decolorize four individual azo dyes (Direct Red 28, Direct Red 7, Acid Blue 113 and Direct Blue 53) at a concentration of 10mg/L. Among the fifftythree, only Direct Red 28 dye was efficiently decolorized by five distinct bacterial strains, identified by 16 srDNA analysis as *B. pumilus* (GenBank accession no. FJ386542), *B. megaterium* (GenBank accession no. FJ386540), *B. cereus* (GenBank accession no. FJ386543), *B. vallismortis* (GenBank accession no. FJ386541) and *B. subtilis* (GenBank accession no. FJ386544).

7. Efficiency of mixed culture was studied under batch conditions (37^{o}C and 120 rpm for 120 hrs) to decolorize four azo dyes (Direct Red 28, Direct Red 7, Direct Blue 53 & Acid Blue 113 at a higher concentration from 10-100 mg/L individually and as a mixture (two and four dyes) and also to decolorize real raw untreated effluent collected from various dyeing industries and local dyeing houses. In both cases, it was observed that decolorization efficiency and growth (as protein) of the mixed culture SKB-II was observed to be decreasing with increasing dye concentration and high effluent concentration.

8. After flask scale, mixed culture SKB-II was used as seed inoculum for development of immobilized cell bioreactor studies. Optimization of parameters was carried out in glass column bioreactor with plastic clips and marble chips as immobilization support material. Various parameters studied on decolorization efficiency and biofilm stability in bioreactors with two different immobilization supports (plastic clips and marble chips) were: hydraulic retention time (11, 7.3 and 5.5 hrs for plastic clips and 8.16, 5.4 and 4 hrs for marble chips) across two aeration rates (0.4 and 0.6 mmoles/min.).

9. By comparing the initial and final absorbance, values of the dye (Direct Red 28 at a concentration 10mg/L in BHB medium) after treatment through the bioreactor maximum decolorization was observed with marble chips as matrix at an aeration rate of 0.6 mmoles/min. and hydraulic retention time of 8.16hrs. And therefore, the real raw untreated effluent (5%) was also treated at the same parameter in the bioreactor.

1. INTRODUCTION

The history of dyes goes back over a hundred years. William Henry Perkin synthesized the first dye, mauve, by accident in 1856 from chemicals derived from coal and later commercialized his innovation and developed the production processes for this new dye. For a colored compound to act as a dye, presence of a chromophore and an auxochrome are the prime requisites. On the basis of the chemical structure of the chromophore group, dyes are classified as azo dyes, triphenylmethane dyes, anthraquinone dyes, phthalocyanine dyes, etc. Azo dyes are by far the largest and most important group of dyes (Carliell *et al* 1998) mainly due to the simple synthesis. The fundament of the production of azo dyes was laid in 1858 when P. Gries discovered the reaction mechanism, diazotization, for the production of azo compounds (Zollinger 1987). Due to their chemical structure azo dyes absorb light in the visible spectrum. Their chemical structure is characterized by one or more azo groups (-N=N-). The azo group is substituted with benzene or naphthalene groups, which can contain many different substituents such as chloro (-Cl), methyl ($-CH_3$), nitro ($-NO_2$), amino ($-NH_2$), hydroxyl (-OH) and carboxyl (-COOH). The azo compound class accounts for 60-70% of all dyes. The general formula for making an azo dye requires two organic compounds, a coupling component and a diazo component.

While textile mills predominantly use them, azo dyes can also be found in the food, pharmaceutical, paper and printing, leather, and cosmetics industries. They are considered to be easy to use, relatively cheap and to provide clear, strong colors. There are more than 10,000 dyes available commercially and approximately 2000 azo dyes on the market and more than 7×10^5 tons of dyestuffs are produced annually (Zollinger 1987). The huge growth in the textile dyeing and dyestuff manufacturing industries has resulted in an increase in the volume and complexity of the wastewater discharged to the environment. During textile processing, inefficiencies in dyeing result in large amount of dyestuff being directly lost in the wastewater, which ultimately finds its way into the environment. During manufacturing and usage of azo dyes an estimated amount of 10-15% is released into the environment (Tan *et al* 2000) while in the case of reactive dyes, as much as 50% of the initial dye load is present in the dye bath effluent (Easton 1995). The wastewater characteristics from a dye house are highly variable from day to day, depending on the type of dye, the type of fabric and the concentration of the agents added. Textile industries consume substantial volumes of starch for the stiffening of cloth and NaCl for fixation of dye to the cloth besides using water for the processing of textiles. Volume of wastewater varies considerably from mill to mill and lies in the range of 35 to 520 litre/kg of cloth. The dye wastewater generally has high BOD, COD, suspended solids and high alkalinity with fairly high temperature. The COD of wastewater from textile industry varies between 2000-5000 mg/litre whereas it should be around 100 mg/litre to meet the regulatory standards.

The presence of very small amounts of dyes in water (less than1 ppm for some dyes) is highly visible and affects the aesthetic merit, water transparency and gas solubility in lakes, rivers and other water bodies. Apart from the aesthetic problems, the greatest environmental concern with the dyes is their absorption and reflection of sunlight entering the water. This interferes with the growth of bacteria and plants, causing a disturbance of the ecology of the receiving water. Thus, loss of dyes to the environment has become an environmental hazard. This is because of the low biodegradability and the toxic nature of the dyes. Some dyes, dye precursors, and their biotransformation products such as aromatic amines have been found to be toxic, mutagenic, and carcinogenic in nature, apart from having the potential of bioaccumulation in the food chain (Lourenco *et al* 2000, Sani *et al* 1999a). Treatment of such wastewaters is therefore, essential but difficult.

Past years, efforts have been focused on various chemical and physical techniques for treating azo dye wastes. All these physico-chemical methods possess significant differences in color removal results, volume capability, operating speeds and capital costs (Easton 1995). Among other shortcomings are excess amount of chemical usage and sludge generation with obvious disposal problems; costly plant requirements or operating expenses; lack of effective color reduction, particularly for sulfonated azo dyes and sensitivity to a variable wastewater input and formation of hazardous by-products (Slokar *et al* 1998, Verma *et al* 2003, Zhang *et al* 2004).

The cost of restoring the burgeoning global inventory of contaminated ecosystems is virtually incalculable. As a result, government, industry and public have recognized the immediate need for more cost effective alternatives to physical and chemical methods of contaminant remediation. The new color removal technologies include the use of bioremediation systems.

Bioremediation provides enormous scope for increasing the range and extent of biodegradation procedures that can be used to reduce problems of the unpleasant and hazardous waste of dyes. For the treatment of effluent economically Co-metabolism is the best suitable method. This phenomenon is of importance in utilization of dyes as a

C-source under artificial conditions in the presence of a main C-source like starch, glucose etc. Another new approach that is gaining importance to get rid of recalcitrant is of consortia or communities. It is dangerous always to focus attention on biodegradation by cultures of single pure strains. In natural ecosystems and in various types of waste treatment procedures, mixtures of organisms may well be the norm. Consortium consists of different species of microorganisms that exist together and jointly carry out reactions that could not be achieved by any of the single component species.

Increasingly stringent legislation demands that discharged effluents achieve high standards including the new concept of “water white” discharges. This places the industries in the position of having to decide the most cost effective route to pursue and achieve compliance within the near future.

2. REVIEW OF LITERATURE

Ever since the beginning of humankind, people have been using colorants for painting and dyeing of their surroundings, their skins and their clothes. Until the middle of the 19th century, all colorants applied were from natural origin. Inorganic pigments such as soot, manganese oxide, hematite and ochre have been utilized within living memory. Earlier dyes originated usually from plants (e.g. the red dye alizarin from madder and indigo from wood) also from insects (e.g. the scarlet dye kermes from the shield-louse *Kermes vermilio*), fungi and lichens. Synthetic dye manufacturing started in 1856, when the English chemist W.H. Perkin, in an attempt to synthesize quinine, obtained instead a bluish substance with excellent dyeing properties that later became known as Aniline purple, Tyrian purple or Mauve.

There are two different types of dyes:

Natural dyes: occur in nature e.g. Indigo (a blue dye), alizarin (a red dye).

Synthetic dyes: are man-made dyes e.g. azo dye, triphenylmethane dyes, nitroso dyes, disperse dyes, solvent dyes etc.

Dyes consist of two essential components:

a) Chromophore

These are the groups responsible for producing color to a dye because they are capable of absorbing light in the near ultraviolet region. The absorption and reflection of visible and UV irradiation is ultimately responsible for the observed color of the dye (Zollinger, 1991). Some important chomophores are: $-N=O$, $-N=N-$, $-C=N$, $-NO_2$, $-C=O$, $-C=S$, $(CH=CH)n$. The compounds bearing chomophores are known as chromogens.

b) Auxochrome

Dyes should be attached to the fibers by means of stable chemical bonds. These chemical bonds are formed by some groups which may be either acidic or basic in nature. Some examples are: $-OH$, $-COOH$, $-SO_3H$ (acidic), $-NH_2$, $-NHR$, $-NR_2$ (basic). A chromogen without auxochrome can never act as a dye.

2.1. Classification of dyes

Dyes can be classified according to either by the chemical structure or by their usage or application method. The most appropriate system for the classification of dyes is by chemical structure as it readily identifies dyes as belonging to a group that has characteristic properties and there are manageable numbers of chemical groups.

Dyes can be grouped under different classes:

Acridine
Anthraquinone
Arylmethane
Azo
Diazonium
Nitro
Phthalocyanine
Quinone amine
Tetrazolium
Thiazole
Triphenylmethane
Xanthene

Among the various dyes about 60-70 % of all known dyes produced are azo dyes, making them the largest group of synthetic colorants (Carliell *et al* 1998) mainly due to their simple synthesis, followed by the anthraquinone type (Vandevivere *et al* 1998). The fundamental of the production of azo dyes was laid in 1858 when P. Gries discovered the reaction mechanism, diazotization, for the production of azo compounds (Zollinger 1987). Synthesis of most azo dyes involves diazotization of a primary aromatic amine, followed by coupling with one or more nucleophiles. Amino and hydroxy groups are commonly used coupling components (Zollinger, 1991). Because of the diversity of dye components available for synthesis, a large number of structurally different azo dyes exist and are used in industry (McCurdy, 1992).

2.2. Azo Dyes Toxicity

The azo dyes, their precursors and degradation metabolites have been reported to be potentially toxic to the environment and humans. 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 of azo dyes and their intermediates/products. Azo dye microbial toxicity is thought to be caused by the intercalation of dye compounds between DNA base pairs (Ogawa *et al* 1988) thus; diffusion of dye molecules through cell membranes and the insertion between base pairs is needed. Most textile effluents contain heavy metals that are complexed in the azo dyes. High concentrations of salt are often used to force the dyes out of solution and onto substrates (Zollinger 1991). These compounds can cause high electrolyte and conductivity concentrations in the dye wastewater, leading to acute and chronic toxicity problems. Degradation of the azo dyes leads to the production of metabolites such as 1, 4 phenylenediamine, 1-amino-2-naphthol, benzidine and substituted benzidines, like otolidine (Rosenkranz and Klopman 1990) which are toxic in nature. The complex structure of some azo dyes makes it more probable that aromatic amines formed during azo dye reduction could be responsible for inhibitory effects, because these intermediates can pass through membrane cells more easily and affect DNA structure (Carliell *et al* 1995). Chung and Cerniglia (1992) reported that mutagenicity of azo dyes was related to chemical structure of dye molecule. It was further reported that mutagenic moiety of most mutagenic dyes may be attributed to presence of *p*-phenylenediamine and benzidine moieties. Mutagenicity of azo dyes may therefore, be predicted by their structure-activity relationships (Chung and Cerniglia 1992). Greene and Baughman (1996) studied the toxic effects of 46 commercial dyes on population growth of freshwater green algae *Selenastrum capricornutum* and reported that the cationic basic dyes are more toxic dyes for algae In mammals, metabolic activation (*i.e.* reduction) of azo dyes is mainly due to bacterial activity in the anaerobic parts of the lower gastrointestinal tract. After azo dye reduction in the intestinal tract, the released aromatic amines are absorbed by the intestine and excreted in the urine. The acute toxic hazard of aromatic amines is carcinogenesis, especially bladder cancer.

In 1996, Ministry of environment and forests, Government of India, issued a notification (S.O. 292CE) dated March 29, 1996 (Prohibition of handling of azo dyes in the Gazette of India Extraordinary Part-II, Section-3, subsection-II dated April 8, 1996) banning the use of 74 dyes including dyes based on Benzidine and naphthylamine amines due to the reported carcinogenicity of these compounds.

2.3. Environmental Concern

The azo dyes have a negative aesthetic effect on the wastewater. Understanding the basic composition of azo dyes is necessary to envision how these molecules can be destroyed. To ensure the safety of effluents, proper technologies need to be used by treatment facilities when degrading azo dyes. Textile mill effluent (TME) is a complex mixture, having high concentrations of suspended solids, metals, chemical surfactants and dyes. Normally colors are noticeable at a dye concentration of more than 1 mg/L and an average concentration of 300 mg/L have been reported in effluents from textile manufacturing processes (O'Neill *et al* 1999, GonCalves *et al* 2000). The discharge of large volumes of colored untreated textile mill effluent into the environment is aesthetically displeasing, impedes light penetration, damages the quality of the receiving streams and is toxic to flora and fauna. In order to meet the criteria necessary for industrial applications, these dyes present a diversity of colors, molecular structures and resistance to fading upon exposure to light, water and many chemical compounds (Correia *et al* 1994). The complex aromatic substituted structures of the dyes make conjugated system and are responsible for intense color, high water solubility and resistance to degradation of azo dyes under natural conditions (Rajaguru *et al* 2000).These required criteria thus yield compounds that cause serious environmental pollution problems. Interest in the pollution potential of textile dyes has been primarily prompted by concern over their possible toxicity and carcinogenicity. Furthermore, dyes can be detrimental to the microbial population present in such treatment works and may lead to decreased efficiency or treatment failure in such plants (Ogawa *et al* 1988). As a result of the environmental legislation, industries are being forced to treat dye contaminated effluent (Robinson *et al* 2001). Since many textile plants have rural locations and municipal treatment costs are increasing, both industries and scientists are compelled to search for innovative novel treatment technologies directed particularly towards the decolorization of dyes in effluents. Dyes usually have a very low rate of removal ratio for BOD to COD (BOD/COD less than 0.1) (De Angelis and Rodriguez 1987). Biological methods, being cheap and simple to use, have been the main focus of recent studies on dye degradation and decolorization.

2.4. Textile Dyes Treatment

The increasing pressure due to strict pollution control regulations compelled the dye manufacturing and textile processing industries to search for innovative and novel treatments/technologies that are directed particularly towards the decolorization of dyes in effluents (Banat *et al* 1996). Previous research efforts primarily depended on using physical or chemical-treatment processes, occasionally in conjunction with biological treatment. The physical and chemical techniques were numerous and included flocculation combined with flotation, electrofloatation, flocculation with Fe (II)/$Ca(OH)_2$, membrane-filtration, electrokinetics coagulation, electrochemical destruction, ion-exchange, electrochemical destruction, irradiation, precipitation, ozonation, adsorption and Katox treatment method involving the use of activated carbon and air mixtures (Pandey *et al* 2007). All the methods possess significant differences in color removal results, volume capability, operating speeds and capital costs (Easton 1995). Some of these techniques have been shown to be effective (Wamik *et al* 1998), although they have shortcomings like, excess amount of chemical usage and sludge generation with obvious disposal problems; costly plant requirements or operating expenses; lack of effective color reduction, particularly for sulfonated azo dyes and sensitivity to a variable wastewater input and formation of hazardous by-products (Zhang *et al* 2004).

Advantages and disadvantages of physical and chemical methods available for the treatment of dye waste water (Robinson *et al* 2001) are mentioned in Table 1. Many reviews are available on the physicochemical for decolorization of azo dyes (Pandey *et al* 2007).

2.5. Bioremediation

Bioremediation is gaining importance as it is cost effective, environment friendly, and produces less sludge (Banat *et al* 1996; Robinson *et al* 2001) and also due to the genetic diversity and metabolic versatility of microorganisms. Lie *et al* (1998) reported that microorganisms are known to play a crucial role in the mineralization of biopolymers and xenobiotic compounds, like azo dyes. The treatment of dye wastewater currently focuses on the biological methods which include possible mineralization of dyes to harmless inorganic compounds like carbon dioxide and water, and formation of a lesser quantity of relatively harmless sludge and when used properly has a lower operating cost than other remediation processes.

Among the various bioremediation technologies, decolorization using microbial cells has been widely used. A newer approach on the use of microbial enzyme holds promises for effective decolorization of industrial wastewater from dyeing industries as well as degradation of ecosystems contaminated with dyes.

Table1: Advantages and Disadvantages of Physiochemical Methods Of Treatments.

Physical & Chemical methods	**Advantages**	**Disadvantages**
Fenton’s reagent	Effective decolorization of both soluble and insoluble dyes	Sludge generation
Ozonation	Applied in gaseous state; no alteration of volume	Short half life (20 mins)
Photochemical	No sludge generation	Formation of by-products
NaOCl	Initiates and accelerates azo-bond cleavage	Release of aromatic amine
Activated Carbon	Good removal of wide variety of dyes	Very expensive
Peat	Good adsorbent due to cellular structure	Specific surface areas are lower than Carbon
Membrane filtration	Removes all dye types	Concentrated sludge generation and very expensive
Ion exchange	Regeneration; no adsorbent loss	Not effective for all dye types
Irradiation	Effective oxidation at lab scale	Requires a lot of Oxygen
Silica gel	Effective for basic dye removal	Side reactions prevent commercial application
Electro-kinetic coagulation	Economically feasible	High sludge production
Wood chips	Good sorption capacity for acid dyes	Requires long retention time
Electrochemical destruction	Breakdown compounds are non-hazardous	High cost of electricity

2.5.1. Enzymatic Approach

Recent studies indicate that an enzymatic approach has attracted much interest as they can act on a broad range of substrates and can also catalyze the degradation or removal of organic pollutants present in very low concentration at the contaminated sites. In view of the potential of these enzymes, several microbial and plant peroxidases and polyphenol oxidases have been considered for the treatment of dyes but none of them has been exploited at the large scale due to low enzymatic activity in biological materials and high cost of purification (Bhunia *et al* 2001; Verma and Madamwar, 2002). The major reason that enzymatic treatments have not yet been applied on an industrial scale is the huge volume of polluted wastewater demanding remediation. Soluble enzymes suffer from certain drawbacks such as thermal instability, susceptibility to attack by proteases, activity inhibition, etc. (Husain and Jan 2000). An important disadvantage of using soluble enzymes in the detoxification of hazardous aromatic pollutants is that the free enzyme cannot be used in continuous processes. To overcome all these limitations enzyme immobilization is the best alternative to exploit the enzymes at the industrial level.

Because this research explores the use of microbial sludge for the treatment of a textile effluent, the focus of this literature review will be on biological techniques used for degrading wastewaters containing azo dyes.

2.5.2. Bacterial Decolorization

Decolorization of the dye solution by bacteria could be due to adsorption to microbial cells or due to biodegradation. Dye adsorption would result in cell mats which are deeply colored because of adsorbed dyes, whereas those retaining their original color are accompanied by the occurrence of biodegradation (Knapp *et al* 1995, Chen *et al* 1999). Bacterial degradation of azo dyes is often initiated by an enzymatic biotransformation that involves reductive cleavage of azo bonds with the aid of azoreductase by utilizing both NADH and NADPH as electron donors (Zimmerman et al 1982). The resulting aromatic amines are further degraded by multiple step bioconversion occurring aerobically or anaerobically (Sheshadri *et al* 1994, Flores *et al* 1997).

2.5.2.1. Aerobic Decolorization

Reports have demonstrated that some bacterial strains can mineralize various dyes under aerobic conditions (Blumel *et al* 1998, Coughlin *et al* 1997; 1999, Nigam *et al* 1996 a) (Fig.1.). Bacterial isolates of *Bacillus subtilis & Bacillus cereus* (Horitsu *et al* 1977, Wuhrmann *et al* 1980), *Aeromonas hydrophila* (Idaka and Ogawa, 1978), and *Pseudomonas cepacia (*Idaka and Ogawa, 1987*)* were reported to be capable of degrading azo dyes by azoreductase. Urushigawa and Yonezawa (1977) had reported that azo compounds with amino or hydroxyl groups in the 2nd or 4th position of the aromatic ring aerobically degraded better than compounds with methyl, carboxyl or sulfonyl groups in these positions. They also observed that compounds with carboxyl or sulfonyl groups degraded poorly due to their higher solubility. Some specialized strains of aerobic bacteria have developed the ability to reduce the azo group by special oxygen-tolerant azo reductases, but have limited substrate range (Zimmermann *et al* 1982, Ghosh *et al* 1993).

Ever since, efforts to isolate aerobic microorganisms capable of transforming dyes and dye related compounds have continued. *Pseudomonas luteola,* isolated from an activated sludge system for treatment of dye-bearing wastewater was capable of efficiently expressing azoreductase activity to decolorize myriads of azo dyes via co-metabolism initiated by azo bond reduction (Bailey 1986). Hu *et al* (1994) also showed the capacity of bacterium *Pseudomonas luteola*, isolated from the sludge of an “activated sludge treatment” system treating dyeing wastewater, to decolorize four reactive azo dyes: Red G, RBP, RP_2B, and V_2RP by 37, 93, 93, and 88% respectively when incubated under shaking conditions for 48 hrs. followed by static incubation for another 48 hrs. Studies on the biodegradation of methyl violet into three unknown intermediate metabolites by *Pseudomonas mendocina* MCM B-402 was reported by Sarnaik and Kanekar (1999) while Nachiyar and Rajkumar (2003) showed that *P. aeruginosa* decolorized a commercial tannery and textile dye, Navitan Fast blue S5R, in the presence of glucose under aerobic conditions. The unidentified metabolites were further degraded to phenol by a phenol-utilizing *Pseudomonas sp.*

A consortia comprising of *Pseudomonas sps*, *Bacillus sps Halomonas sps, Orthrobacter sps,* and *Micrococci sps* degraded commercial azo dyes: Red RB, Remazol Red, Remazol Blue, Remazol Violet, Remazol Yellow, Golden Yellow, Remazol Orange, Remazol Black efficiently in the presence of various co-substrates, such as glucose, starch, lactose, sewage and whey water. Among the various co-substrates starch was the best source of carbon for decolorization of reactive azo dyes. In presence of 250 mg/L of starch, all the reactive dyes were decolorized within 24 hrs with the reduction in COD in the range of 75.15–95.9 %. The COD reduction was the maximum in Remazol Orange and Red RB followed by Remazol Yellow, Remazol Violet, Remazol Black, Remazol Red, Golden Yellow and Remazol Blue (Padmavathy *et al* 2003). An aerobic bacterial consortium consisting of two isolated strains (BF1 and BF2) and a strain of *Pseudomonas putida* (MTCC1194) was developed by Senan and Abraham (2004) for the decolorization of seven commercially available dyes (Direct Yellow 86, Basic orange 2 C.I.11270, Reactive green 19, Direct Blue 54 C.I.27960, Reactive Blue 171, Reactive Red 141, Acid Red 260) as individual and mixture under pH (9–10.5) and salinity (0.9–3.68 g/l) at 28 ± 2°C. Laccase was detected in the culture supernatant. The analysis of degradation products showed that dyes are converted to low molecular weight compounds and amines were absent after microbial treatment of the dyes. Hsueh & Chen (2007) showed that variation of molecular structures and functional groups present among the azo dyes i.e., Congo red, Eriochrome black T, methyl orange, and methyl red affected biodecolorization capability of *Pseudomonas luteola*. The optimal cellular age for the most effective decolorization was 7 days after static incubation in dye-free cultures at pH 7-9. Methyl red was not decolorized in contrast to methyl orange, Congo red, and Eriochrome black T. The presence of electron-withdrawing groups as the substituents on azo dyes enhanced decolorization capability for biodegradability. In addition, Monod kinetic model provided better predictions to all dye decolorization at initial short periods of time due to negligible intermediate formed at initial short time duration.

Zissi *et al* (1997) showed the biological oxidation of p-aminoazobenzene (pAAB) by *Bacillus subtilis* in the presence of glucose, and ammonium chloride, by breaking the N=N double bond and producing aniline and p-phenylenediamine. *Bacillus sp*. strain PS decolorized and degraded Terasil black dye in effluents under aerobic condition in the presence of glucose or starch (1 %) and the decolorization was faster in the presence of 0.5% yeast extract (Pourbabee *et al* (2005). They also reported that the strain was able to complete decolorization of high concentration of Congo red in 48 hrs. under aerobic conditions with 96% removal at 30-40°C and no noticeable effects of different pH values (5.5-8.0) on decolorization were observed. A 16S rDNA analysis revealed an isolate from textile effluent contaminated soil as *Bacillus sp. VUS* (Dawkar *et al* 2008) which degraded textile disperse dye Brown 3 REL by 100% within 8 hrs under static anoxic condition at 40 degrees C and pH 6.5-12.0. It also decolorized (80%) textile dye effluent within 12 hrs.

A strictly aerobic bacterium, *Flavobacterium* reduced 4, 4’-dicarboxy-azobenzene to two molecules of amino-benzoic acid under aerated conditions (Kulla 1984). Among the two bacteria *Klebsiella pneumoniae RS-13* and *Acetobacter liquefacien*s S-1, *K. pneumoniae RS-13* completely decolorized and degraded 100 mg/L toxic azo dye Methyl Red (MR) in the culture medium (Wong and Yuen, 1996). In addition, MR degradation by *K. pneumoniae* required less nutrients and was less sensitive to the environmental change than that of *A. liquefaciens.*

Blumel *et al* (1998) reported that bacterial strain S5, derived from *Hydrogenophaga palleronii S1* used sulfonated azo compound 4-carboxy- 4′-sulfoazobenzene (CSAB) as sole carbon and energy source. Completely reduction of CSAB (0.35mM) under aerobic conditions was achieved in 40 min., and its dye reduction products: 4-aminobenzenesulfonic acid (4-ABS) and 4-aminobenzoic acid were mineralized by previously established degradative pathways. *Sphingomonas sp* strain 1CX isolated from a wastewater treatment plant was capable of aerobically degrading a suite of azo dyes by reductive cleavage of the azo bond (Orange II, Acid Orange 8, Acid Orange 10, Acid Red 4, and Acid Red 88), by using them as a sole carbon and nitrogen source. The addition of glucose or inorganic nitrogen inhibited growth and decolorization of the azo dyes by strain 1CX (Coughlin *et al* 1999).

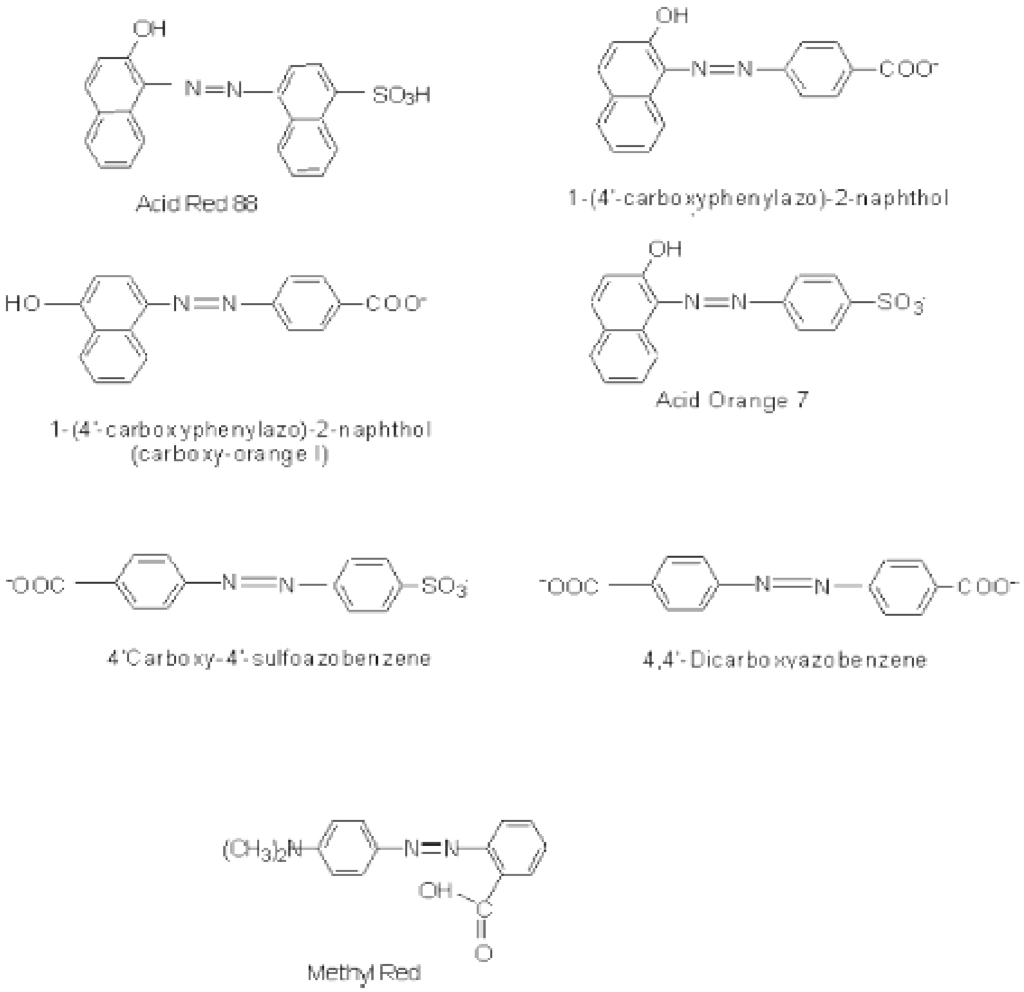
A bacterial isolate C7 was capable of oxygen-insensitive azo bond cleavage of dyes such as Acid orange 7 and acid red 151 during aerobic growth in glucose-enriched minimal medium (Tabor 1991). Govindaswami *et al* (1993) using Eubacterial consensus oligonucleotide primers amplified by polymerase chain reaction the nearly full-length 16S rRNA gene of isolate C7, a gram-negative rod capable of aerobic degradation of azo dyes and by phylogenetic analysis based upon this DNA sequence placed isolate C7 closely to *Caulobacter subvibrioides*. This was the first phylogenetic classification of a potentially useful aerobic azo dye-degrading microorganism

Heiss *et al* (1992) cloned the azoreductase gene of *Rhodococcus* sp.,capable of effectively decolorizing two sulfonated azo dyes, Orange II and Amido black, into a mutant which had lost decolorization capability, thereby conferring sulfonated azo-dye decolorization ability. Aerobic decolorization of azo dyes has been confirmed by a number of investigators but in most cases the toxicity of the dye degradation products has not been assessed. Adedayo *et al* (2004) reported complete decolorization within 6 hrs. at 5 mg/L methyl red concentration, and 16 hrs. at 20–30 mg/L, using a mixed culture out of which two were identified as *Vibrio logei* and *Pseudomonas nitroreducens* at pH 6, 30°C. Remarkable improvement in color removal of azo dye Direct Fast Scarlet 4 BS by the microbial consortium in the presence of oxygen was shown by He Fang *et al* (2004) wherein optimal decolorization activity was observed between pH 4 and 9, and between 20 to 40°C. Kodam *et al* (2005) reported a 100% decolorization of the sulfonated azo dyes, Reactive Red 2, Reactive Red 141, Reactive Orange 4, Reactive Orange 7 and Reactive Violet 5 by an unidentified bacterium, KMK 48. High effectiveness was attained within 36 hrs. of incubation at room temperature and neutral pH.

Snyder *et al* (2006), developed three aerobic mixed microbial cultures from the aeration basin of the wastewater treatment plant and acclimated it in shake flasks utilizing various combinations of 2, 4, 6-Trichlorophenol (25 to100 mg/L), phenol (300 mg/L) and glycerol (2.5 mg/L) as substrates. The phenol acclimated mixed microbial culture consisting of primarily Gram positive and negative rods was capable of degrading 2, 4, 6 Trichlorophenol completely. Asgher *et al* (2006) developed four different aerobic mixed consortia were collected from basins of wastewater streams coming out of dying plants of Crescent Textile (CT), Sitara Textile (ST), Chenab Fabrics (CF) and Noor Fatima Textile (NF), Pakistan for the decolorization of Drimarene Orange K-GL, Drimarene Brilliant Red K-4BL, Foron Yellow SE4G and Foron BlueRDGLN. CT consortia showed the best decolorization potential where all dyes were decolorized within 3–5 days followed by ST consortia which showed effective decolorization of Foron Yellow SE4G and Drimarene Brilliant Red K-4BL within 4 and 7 days, respectively. NF consortia took comparatively longer time periods (5–7 days) and showed 100% decolorization on Foron Yellow SE4G and Foron Blue RDGLN where as, the NF consortia had decolorized only 40% and 50% of Drimarene orange and red, after 10 days. CF consortia completely decolorized Foron Blue RDGLN and Drimarene Brilliant Red K-4BL after 4 and 8 days, respectively but it showed poor performance on the other two dyes.

Ren *et al* (2006) reported an aerobic broad-spectrum dye-decolorizing bacterium strain DN322, identified as *Aeromonas hydrophila* which decolorized azo, triphenylmethane and anthraquinone dyes, the most suitable pH and temperature were pH 5.0–10.0 and 25–37 °C, respectively. *Citrobacter sp.* strain KCTC 18061P was found to be able to decolorize textile plant effluent containing different types of reactive dyes

(Jang *et al* 2007). The observed changes in the visible spectra indicated color removal by absorption of dye to cells. This strain showed higher decolorization ability under aerobic than static culture conditions, with 1% glucose, and removed 70% of effluent color within 5 days. The potential of isolated bacteria *Paenibacillus larvae* for the effective decolorization of Indigo carmine was evaluated by Ramya *et al* (2007). Maximum decolorization was observed at 30°C (100%) followed by 40°C (92%) in 8 hrs. of incubation when the medium was incorporated with 10 g/L of yeast extract and peptone.



Acid Red 88

1-(4'-carboxyphenylazo)-2-naphthol

1-(4'-carboxyphenylazo)-2-naphthol
(carboxy-orange I)

Acid Orange 7

4'Carboxy-4'-sulfoazobenzene

4,4'-Dicarboxyazobenzene

Methyl Red

Fig.1. Structure of Azo dyes degraded under aerobic conditions.

Besides the degradative studies of azo dyes reported by many researchers, Brown and Laboureur (1983) studied the aerobic biodegradability of the possible by-products of azo dyes: aniline, o-toludine, p-anisidine, p-phenetidine, o-dianisidine, and 3, 3'-dichlorobenzidine and concluded that aniline, p-anisidine, p-phenetidine and o-toludine were readily biodegradable by aerobes, while o-dianisidine and 3, 3'-dichlorobenzidine were inherently biodegradable. They suggested that these compounds could be stabilized if released into the environment or directly from a dyehouse into a conventional wastewater treatment plant.

2.5.2.2 Anaerobic Decolorization

As early as 1987, Brown *et al* reported that aromatic amine residues formed after anaerobic decolorization resisted further anaerobic degradation and these aromatic amine residues were reported as mutagens by Haug *et al* (1991) which could be degradaded only aerobically. The anaerobic step is responsible for color removal but it does not remove the dye-related hazards from the wastewater since the anaerobic degradation of aromatic amines formed due to anaerobic degradation has not been reported, except for a few hydroxyl and carboxyl substituted amines. The aromatic amines formed are toxic and carcinogenic (Carliell *et al* 1995, Field *et al* 1995). The azo bond reduction of dyes involves the electron transport linked reduction in the extracellular environment.

Thus, the electron transport components must be localized in the bacterial membrane, where they can make direct contact with either the azo dye substrate or a redox mediator at the cell surface (Myers and Myers 1992). Brown and De Vito (1993) reported that a wide variety of faecal anaerobes have the ability to produce aromatic amines from azo dyes such as tartrazine, and may do so in the human gut, producing carcinogenic amines such as benzidine and 4-aminoanilin. Nigam *et al* (1995) demonstrated that a mixture of dyes was decolorized by anaerobic microbial bacterial consortium PDW in 24-30 hrs., using free growing cells or in the form of biofilms on various support materials. Nigam *et al* (1996 b) further showed that decolorization was dependent upon the presence of a carbon and energy source in addition to the textile dyes and decolorization of dyes took place when utilizing cheap and readily available carbon sources such lactose, starch and distillery waste. Overall, PDW removed 76% of color from textile plant effluent after 3 days.

In 1996, Zissi and Lyberatos reported that pure culture of *Bacillus subtilis* degraded p-amino-azobenzene under anoxic conditions, producing aniline and p-phenylenediamine as main decomposition products. Under anaerobic conditions, Mordant Orange 1 and Azodisalicylate were reduced and decolorized using methanogenic granular sludge by Razo-Flores *et al* (1997). An azo dye-degrading bacterium, *Caulobacter subvibrioides* strain C7-D, reduced the azo bond of the dyes Acid Orange (AO) 6, AO7, AO8, AO12, Acid Red (AR) 88, AR151, and Methyl Red (MR) and concluded that this activity was oxygen insensitive and among all the dyes tested, AO7 was the best inducer and the most rapidly reduced substrate suggesting that dye AO7 most closely mimics the natural physiological substrate for this enzyme (Mazumdar *et al* 1999). Yoo *et al* (2000) reported that sulfate reducing bacteria *Desulfovibrio desulfuricans* decolorized Reactive Orange 96 and Reactive Red 120 under anaerobic conditions.

The nonspecific ability of anaerobic sludge bacteria obtained from cattle dung slurry was investigated for 17 different dyes in a batch assay system. Among the 17 dyes Reactive Violet 5 (RV 5) was decolorized effectively up to 1000 mg/L with a decolorization efficiency of above 75% during 48 hrs. of incubation by sludge bacteria. The optimum inoculum size was 9.0 g /L at 37 °C. Of sixteen other dyes tested, nine (Reactive Black 5, Reactive Blue 31, Reactive Blue 28, Reactive Red HE8B, Reactive Yellow, Reactive Golden Yellow, Mordant Orange, Novatic Olive R S/D & Navilan Yellow GL) were decolorized with more than 88% efficiency; three (Orange II, Navy Blue HER & Novatic Blue BC S/D) were decolorized with about 50–65% efficiency, whereas other three dyes (Procion Orange H2R, Procion Brilliant Blue HGR & Novatic Blue BC S/D) were decolorized with less than 40% efficiency (Keharia *et al* 2004).

Anaerobic degradation of Acid Orange 7 was shown to be influenced by chemical reduction by sulphide in the presence of glucose as carbon source (Carliell *et al* 1996) while tapioca starch was found to be more effective for efficient decolorization of synthetic blue waste water by Chinwekitvanich *et al* (2000). Among the various isolates from sludge samples and mud lakes, *Aeromonas hydrophila* exhibited the greatest color removal from various dyes (Acid Blue 74, Acid Orange 7, Acid Red 106, Direct Yellow 4, Direct Yellow 12, Acid Black 172, Acid Blue 264, Acid Yellow 42, Direct Black 22, Direct Orange 39, Direct Red 224, Direct Red 243, Direct Yellow 86, Reactive Black NR, Reactive Black 5, Reactive Blue 160, Reactive Blue 171, Reactive Blue 198, Reactive Blue 222, Reactive Green 19, Reactive Red 120, Reactive Red 141, Reactive Red 198 and Reactive Yellow 84) under anoxic or anaerobic conditions. Dye RED RBN (3000 mg/L) was reduced to >90% within 8 days and the decolorization efficiency enhanced strongly in presences of yeast extract or peptone while glucose inhibited decolorization activity because the consumed glucose was converted to organic acids that decreased the pH of the culture medium, thus inhibiting the cell growth and decolorization activity

(Chen *et al* 2003a). Soil samples collected from contaminated sites of Vatva, India were screened for isolation of organisms capable of decolorizing textile dyes by Moosvi *et al* (2005). Bacterial consortium RVM11.1 was selected on the basis of rapid dye decolorization of Reactive Violet 5 (RV 5). Decolorization of 94% was achieved within 37 hrs. under a wide pH range from 6.5 to 8.5 and at 25 to 40 °C under static conditions in the presence of glucose and yeast extract. Bacterial consortium RVM11.1 also showed an ability to decolorize in the presence of starch instead of glucose. Moosvi *et al* (2007) developed dye decolorizing microbial consortium JW-2 comprising of three isolates: *Paenibacillus polymyxa, Micrococcus luteus* and *Micrococcus sp.* from soil samples from dye contaminated sites of Jetpur, Gujarat and showed that the concerted metabolic activity of these isolates led to complete decolorization of Reactive Violet 5R (100 ppm) within 36 hrs. whereas individual isolates could not show decolorization even on extended incubation also the consortium showed complete decolorization utilizing low amount of co-substrates like glucose (0.1% w/v) and yeast extract (0.05% w/v) and could also utilize a cheaper carbon source like starch in place of glucose as an alternative co-substrate under anaerobic conditions.

Cetin & Donmez in 2006 studied mixed cultures isolated from textile dye effluent to study decolorization activity in a batch system under anaerobic conditions. Dye decolorization of 94.9% for Reactive Red RB, followed by 91.0% for Reactive Black B and 63.6% for Remazol Blue was observed. They concluded that pretreatment of effluent with anaerobic bacteria will provide color removal, but anaerobic cleavage of azo dyes leads to formation of hazardous aromatic amines.

Xu *et al* (2006) reported a new anaerobic species genus *Shewanella*, *S. decolorationis*, which had a high capacity for rapid decolorization of anthraquinone dye, Reactive Brilliant Blue K-GR. Liu *et al* (2006) showed that *Rhodopseudomonas palustris* AS1.2352 possessing azoreductase activity decolorized azo dyes only under anaerobic conditions at pH 8 and 30–35°C, respectively. Kalme *et al* (2007b) reported decolorization of Red HE7B (100 mg/L), a sulfonated azo dye, under static condition by *Pseudomonas desmolyticum* NCIM 2112.

The decolorization of the azo dye Reactive Red 2 (RR2) under anoxic conditions was investigated by Beydilli & Pavlostathis in 2007 using a mesophilic (35°C) halotolerant enrichment culture capable of growth at 100 g/L sodium chloride (NaCl). The maximum biomass-normalized RR2 decolorization rate by the halotolerant enrichment culture under batch, anoxic incubation conditions was 26.8 mg dye mg^{-1}

VSS. Guo *et al* (2008) reported that the newly isolated anaerobic bacteria *Halomonas sp* strain GTW (identified by 16S rDNA sequence analysis from coastal sediments contaminated by chemical wastewater) could decolorize different azo dyes (Reactive brilliant red X-3B, Acid black 10B, Acid scarlet GR, Acid red B, Acid Red G, Reactive brilliant red K-2BP) under high salt concentration conditions, and the decolorization rate of five tested azo dyes was above 90% in 24 hrs. Joe *et al* (2008) identified isolate SL186 as *Clostridium bifermentans* (by phenotypic characterization and 16S rDNA sequence comparison) which under anaerobic conditions decolorized Reactive Red 3B-A, Reactive Black 5, and Reactive Yellow 3G-P dyes by 90% within 36 hrs. The bacterium retained decolorizing activity over a wide range of pH (6–12), with peak activity at pH 10. Additionally, SL186 decolorized a relatively high concentration of Reactive Red 3B-A dye (1000 ppm) by 80% and raw industrial effluent effectively.

Under anaerobic conditions, amines are not degraded and get accumulated. Some aerobic bacteria can degrade many aromatic amines. For this reason, anaerobic treatment in combination with aerobic treatment, sequentially or simultaneously to achieve complete mineralization of dyes has been suggested.

2.5.2.3 Decolorization by single bacterial strains

Kulla *et al* (1983) reported that *Pseudomonas* sp. achieved 90% decolorization of azo dye Orange II but could decolorize only 35% of another azo dye Orange I. The difference in the azoreductase specificity towards the hydroxy group of Orange I and Orange II dyes was responsible for these variations in decolorization rate. Similarly, cell-free extract of *Pseudomonas stutzeri* was capable of 80-90% color removal from aqueous solutions of 9 different azo dyes having amino-benzene and naphthalene-azobenzene moieties (Yatome *et al* 1990). Yatome *et al* (1991) reported that the permeability to the dye and presence of azoreductase affected the reduction of azo dye by pure culture of *P.stutzeri, B.subtilis* and *P.cepacia*, under aerobic condition and variation in the rate of decolorization of individual dyes could be attributed to their structural difference. Hu (1994) used *Pseudomonas luteola* for achieving 37-93 % decolorization of four reactive azo dyes viz. Red G, RBB, RP_2B and V_2RP after 42 hrs. incubation under static conditions. Dyes Amaranth, Orange II and Tartrazine were decolorized in 8 hrs. upto 80, 50 and 20% respectively by *Bacteroides fragilis* under anaerobic conditions. The variation in decolorization rate was correlated to the redox potential of the dyes.

Chen *et al* (1999) isolated *Proteus mirabilis* from acclimated sludge of a dye wastewater treatment plant which decolorized more than 95% of azo dye Red RBN (1.0 g/L) within 20 hrs. under anoxic static incubation and out of which, 13-17% of dye was adsorbed on inactivated microbial cells. In yet another study, *Aeromonas* sp. B-5 completely decolorized 100 mg/L of Bordeaux S dye under static conditions in 24 hrs. Though, the decolorization was suppressed under shaking conditions, but rapid decolorization was observed when the culture was incubated under static conditions after cultivation under shaking conditions (Hayase *et al* 2000) while Meehan *et al* (2001) reported 98% decolorization of Remazol Black B within 24 hrs., at a dye concentration of 100 mgdm^3 using *Paenibacillus azoreducens* sp. nov. Isik and Sponza (2003) studied the effect of aerobic, anaerobic and microaerophilic conditions on decolorization efficiency of *Escherichia coli* and *Pseudomonas* sp. using two azo dyes: Congo Red (CR) and Direct Black 38 (DB-38). Under anaerobic incubation the dyes were removed up to 98 and 72 % respectively by *E. coli*, while under microaerophilic conditions, up to 39 and 75% decolorization of CR and DB-38 was achieved. With *Pseudomonas* sp. under anaerobic incubation, color of CR and DB-38 dyes was removed up to 100 and 83%, while under microaerophilic conditions, 76 and 74% color removal efficiencies were observed whereas, no decolorization occurred under aerobic incubation. Kalme *et al* (2007a) observed that *Pseudomonas desmolyticum* NCIM 2112 degraded diazo dye Direct Blue-6 (100 mg/L) completely within 72hrs. with 88.9% reduction in COD under static anoxic condition. Handayani *et al* (2007) investigated the decolorization efficiency of *Enterococcus faecalis* on azo dyes: Acid Red 27 and Reactive Red 2 as well as growth characteristics of *E. faecalis* on these dyes. High decolorization efficiency (95–100%) was achieved within 3 hrs. of incubation for Acid Red 27, and 12 hrs. for Reactive Red 2, at room temperature, neutral pH, static and non-aerated condition.

Kalyani *et al* (2008) isolated a novel bacterial strain *Pseudomonas sp.* SUK1 from contaminated sites of textile industry which decolorized reactive textile dye Red BLI (50 mg/L) to 99.28% within 1 hr. under static anoxic condition at pH range from 6.5 to 7.0 and 30°C. Khalid *et al* (2008), screened 288 strains of azo-dye degrading bacteria using a mixture of four structurally different dyes (Acid Red 88, Reactive Black 5, Direct Red 81, and Disperse Orange 3) as sole source of Carbon and Nitrogen and found that among all isolates *Shewanella putrefaciens* was the most efficient strain which required 4 hrs. for complete decolorization of AR-88 and DR-81 dyes (100 mg/L) under static conditions while complete decolorization of RB-5 and DO 3 was observed within 6 and 8 hrs., respectively. Similarly, Joe *et al* (2008) studied decolorization of textile reactive azo dyes by bacterial strain *Clostridium bifermentans*; isolated from a contaminated site. Under anaerobic conditions, *C. bifermentans* decolorized 90% of Reactive Red 3B-A, Reactive Black 5, and Reactive Yellow 3G-P after 36 hrs. The bacterium decolorized a 1,000 ppm of Reactive Red 3B-A by 80% and raw industrial effluent effectively. The addition of glucose increased the decolorization rate a little.

2.5.2.4. Decolorization by mixed bacterial culture

A facultatively anaerobic culture, PDW: mixed culture of *Alcaligenes faecalis* & *Commamonas acidovorans*, decolorized textile dyes (Cibacron Red C-2G, Cibacron Orange CG (both reactive dyes); Remazol Navy Blue GG, Remazol Red RB, Remazol Blue B, Remazol Black B (all diazo dyes); Remazol Golden Yellow RNL (azo dye); Disperse Navy D2GR (Disperse dye); Remazol Turquoise Blue133, (phthalocyanine dye) under anaerobic conditions. Neither of these isolates, however, were able to decolorize textile dyes to the same extent independently as the original PDW culture (Nigam *et al* 1996). A mixed bacterial culture developed from activated sludge of effluent and sewage treatment plant was used by Bromley-Challenor *et al* (2000) for achieving 30 mg/L/hrs. decolorization rate of azo dye Reactive Red 3.1 (40 mg/L) under anaerobic conditions. Bras *et al* (2001) showed the decolorization of commercial azo dye C.I. Acid Orange 7 by methanogenic and mixed cultures, and it was concluded that dye degradation occurred for both groups of bacteria when a co-substrate was present and addition of electron donors (sucrose/glucose and acetate) stimulated the reductive cleavage of azo bonds.

A microbial consortium isolated from sludge of petrochemical wastewater treatment plant and immobilized on polyvinyl alcohol gel beads, decolorized 75% of Red RBN dye (500 mg/L) within 12 hrs. in flask culture. Color removal efficiency of 90% was obtained in continuous bioreactor treatment operated at an average hydraulic retention time higher than 10 hrs. (Chen *et al* 2003 b). Mathew & Madamwar (2003) concluded that consortium, SV5, formed from combining three isolates exhibited maximum decolorization of Ranocid Fast Blue with a 0.1% (w/v) concentration of both starch and yeast extract supplemented in Bushnell Hass Medium under static conditions at 37°C in less than 24 hrs. at a concentration of 100 ppm and the authors suggested that a mixed microbial community is required to mineralize azo dyes completely through a combined metabolism.

Vijaya & Sandhya (2003) isolated mixed bacterial culture from a domestic wastewater treatment plant which degraded 700 mg/L of methyl red efficiently in the presence of 200 mg/L of glucose within 18 hrs., whereas in the absence of glucose it could degrade only 100 mg/L of methyl red. The mixed microbial cultures could degrade methyl red efficiently through three cycles but further degradation was not possible as the pH of the medium decreased to 3.5.

Various soil and sludge samples were collected by Sharma *et al* (2004 d) isolated twenty five bacterial cultures from the vicinity of textile dyeing industries and waste disposal sites using triphenylmethane dye Acid Violet-17 (10 mg/L) as the inducer. When these twenty five bacterial isolates were used in various combinations together, the rate of decolorization of Acid Violet-17 was 82% within 48 hrs. of incubation whereas 86% decolorization was achieved in 72 hrs., whereas the individual isolates normally showed around 50% decolorization. Among these twenty five bacterial cultures *Bacillus* sp., *Alcaligenes* sp. and *Aeromonas* were the most efficient and decolorized other triphenylmethane dyes such as Malachite green and Brilliant green (80%) within 24 hrs. of incubation, whereas 82% of crystal violet was decolorized within 48 hrs.

Khehra *et al* (2005) enriched and isolated bacterial strains *Bacillus cereus (BN-7), Pseudomonas putida (BN-4), Pseudomonas fluorescence (BN-5) and Stenotrophomoas acidaminiphila (BN-3)* collected from waste disposal sites of local textile industries and designated the developed consortium HM-4 which completely decolorized azo dye Acid Red 88 (AR-88). The concerted metabolic activity of these isolates led to complete decolorization of AR-88 (20 mg/L) in 24 hrs., whereas individual cultures took more than 60 hrs. to achieve complete decolorization of the added dye. In addition to AR-88, consortium HM-4 decolorized 78% of Acid Red 88, 99% of Acid Red 119, 94% of Acid Red 97, 99% of Acid Blue 113 and 82% of Reactive Red 120 dyes at an initial concentration of 60 mg/L in 24 hrs.

The abilities of two bacterial strains of opposite tinctorial type, the Gram-negative *Alcaligenes faecalis* and the Gram-positive *Rhodococcus erythropolis*, to decolorize reaction medium containing initially 10, 50, 100, 200 and 500 mg/L of the monoazo dye Acid Orange 7 was studied by Mutafov *et al* (2006). The experimental data revealed relative independence of the decolorization dynamics on the dye-binding properties of the cell, which could be regarded as an indirect confirmation of the known extracellular redox-mediator- dependent mechanism of azo group reduction. Junnarkar *et al* (2006) studied a novel bacterial consortium on the basis of 90% decolorization of textile dye

Direct Red 81 within 35 hrs. The bacterial consortium exhibited maximum decolorization at 0.6 g/L starch and 0.9 g/L casein supplement in the medium along with pH 7.0 and 37 °C and dye concentration of 200 mg/L. The bacterial consortium had an ability to decolorize nine other structurally different azo dyes: Reactive Black B, Reactive Blue 172, Reactive Violet 5R, Reactive Red 5B, Reactive Black RL, Ponceau 4R, Raspberry Red, Tartrazine and Sunset Yellow FCF.

Dafale *et al* (2008a) in their studies reported that the bacterial consortium responsible for effective decolorization of the azo dye Remazol black-B (RB-B) comprised of strains *Pseudomonas aeruginosa*, *Rhodobacter sphaeroides*, *Proteus mirabilis*, *Bacillus circulance*, *NAD 1* and *NAD 6*.

2.5.2.5. Integrated anaerobic/aerobic Treatment

The biotransformation products generated by anaerobic decolorization are not further susceptible to anaerobic attack, but are readily biodegraded under aerobic conditions (Stolz *et al* 1992). Anaerobic decolorization followed by aerobic post treatment was recommended for treating colored wastewater from textile and dyestuff manufacturing industries (Brown 1987, Field *et al* 1995). This condition can be implemented either by spatial separation of the anaerobic and aerobic sludge using a sequential anaerobic–aerobic reactor system (Zitomer *et al* 1993) or within one reactor, commonly termed an integrated anaerobic–aerobic reactor system (Field *et al* 1995). The basis of these systems lies in the fact that anaerobic and aerobic microorganisms can co- exist beneficially in a single biofilm (Knackmss 1997, Zitomer and Shrout 1998).

Study conducted by Tan *et al* (1999), demonstrated that two azo dyes, 4-phenylazophenol (4-PAP) and mordant yellow 10 (MY10), were appreciably degraded in an integrated anaerobic–aerobic treatment system by exposing anaerobic granular sludge to oxygen. Anaerobic degradation led to the temporary accumulation of aromatic amines, that is, 4-aminophenol (4-AP) and aniline from 4-PAP, and 5-aminosalicylic acid (5-ASA) and sulfanilic acid (SA) from MY10, which were subsequently mineralized either by facultative aerobic bacteria. Lodato *et al* (2007) studied the biodegradation of the monoazo-sulphonic dye Acid Orange 7 by *Pseudomonas* sp. OX1 which grew during the aerobic phase while the dye conversion occurred only during the anaerobic phase.

Azo dye biodegradation by combination of two main steps, the reductive cleavage of the azo bond under anaerobic conditions and the subsequent aerobic mineralization of the produced aromatic amines have been reported. The combination have used various technologies for both anaerobic and aerobic such as UASB+UBAF (Lacalle *et al* 2001), SBR (Shaw *et al* 2002), Anoxic + Anaerobic/Aerobic SBR (Panswad et al (2001), Aerobic-anaerobic packed-bed reactors (Lin & Liu; 1994), Aerobic-anaerobic fluidized-bed reactors (Seshadri *et al* 1994), Aerobic-anaerobic sequential batch or Continuous-flow reactors (Oxspring *et al* 1996).

2.5.3. Decolorization Capability of the Fungi

Fungal degradation is shown to be mediated by lignin modifying enzymes, laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP) (Fu and Viraraghavan, 2001). Biodecolorization of lignin-containing pulp and paper wastewater, as measured by the decrease in color absorption, using two white-rot basidiomycete fungi: *Phanerochaete chrysosporium* and *Tinctoporia sp.*, was reported as early as 1980 (Eaton *et al* 1980 and Fukuzumi 1980). *Phanerochaete chrysosporium* has been shown to decolorization various dyes: Red HE-8B, Malachite Green, Navy Blue HE-2R, Magenta, Crystal Violet and an industrial effluent of dying unit to varying extent (20-100%) in shake and static culture (Sani *et al* **,**1998) with Red HE-8B being decolorized most rapidly. Decolorization rates for all the dyes in static condition were found to be less than the shake culture and also dependent on biomass concentration. They further reported the decolorization of Acid Green 20 at 30°C by *P. chrysosporium* in a low-cost medium where the dye was initially converted to an unknown product, showing λmax at 522 nm and then transformed into another colorless compound (Sani and Banerjee *et al* 1999 b).

Meilgo *et al* (2001) studied the continuous decolorization of azo dye orange II using a packed bed reactor with *Phanerochaete chrysosporium* for periods longer than 30 days. Nearly complete decolorization (>95%) was achieved when working at a high dye load rate of 0.2 g/L/d at 37 °C, with hydraulic retention time of 24 hrs. and applying oxygen gas in a pulsed flow. These conditions allowed Manganese peroxidase (MnP) production and subsequent Orange II decolorization and also caused the breakdown of the chromophoric group as well as the cleavage of the aromatic ring.

Radha *et al* (2005) investigated the effect of various operational parameters, dye concentration (20–400 mg/L), pH (2–7), temperature (20–45 C°) and inoculum size (0.25–4 ml) on the maximum percentage decolorization by *Phanerochaete chrysosporium* which decolorized several synthetic dyes of varying structures namely, Methyl violet, Congo red, Acid orange, Acid red 114, Vat magenta, Methylene blue and Acid green. They reported that 39% of the decolorization was due to the physical adsorption on the mycelia. Therefore, both the mycelium (biosorption) and the extra cellular fungal enzyme (biodegradation) are involved in dye decolorization.

Besides *Phanerochaete chrysosporium*, other fungal strain *Neurospora crassa* and *Schizophyllum commune* have been reported to decolorize diazo dyes (Corso *et al* 1981and Belsare and Prasad,1988) Other white-rot fungi, also capable of decolorizing dyes, include *Funalia trogii* (Yesilada *et al* 1995), *Umbelopsis isabellina, Aspergillus foetidus, Rhizopus oryzae* (Polman and Breckenridge 1996), *Coriolus versicolor* (Knapp and Newby 1999), *Trametes versicolor* (Wong and Yu, 1999), *Phlebia tremellosa* (Kirby *et al* 2000), *Irpex lacteus, Pleurotus ostreaus* (Novotny *et al* 2001) and *Trametes modesta* (Nyanhongo *et al* 2002), *Penicillium geastrivous* (Yang *et al* 2003). Some other strains of fungi like *Kurthia sp., Cyathus bulleri, Coriolus versicolor, Funalia trogii, Laetiporous sulphureus, and Streptomyces sp.* have also been reported to decolorize some triphenylmethane and azo dyes (Yesilada *et al* 1995). Young and Yu (1997) studied the decolorization of eight synthetic dyes: azo, anthraquinone, metal complex and indigo by *T. versicolor* and also reported that MnP did not decolorize these dyes while ligninase-catalyzed oxidation removed over 80% of the dyes because *T. versicolor* releases laccase as its major extracellular enzyme, its major mechanism in decolorizing anthraquinone, azo and indigo dyes was laccase activity (Wong and Yu, 1999).

Selvam *et al* (2003) reported decolorization of azo dyes such as Orange G (50 µM), Congo red (50 µM), and Amido black 10B (25 µM) by a white rot fungus *Thelephora sp*. Orange G was decolorized to 33.3%, Congo red (97.1%) and amido black 10B (98.8%). Further they showed that 19% orange G was removed by laccase at 15 U/ml whereas lignin peroxidase (LiP) and manganese dependent peroxidase (MnP) at the same concentration decolorized 13.5% and 10.8% of Orange G, respectively. A maximum decolorization of 12.0% and 15.0% for Congo red and Amido black 10B, respectively, was recorded with laccase. They suggested batch mode of treatment using *Thelephora sp* to be more effective than the continuous mode for color removal from dye industry effluents.

Ambrosio and Campos-Takaki (2004) evaluated the decolorization of dyes with different molecular structures by *Cunninghamella elegans UCP542.* In the presence of sucrose and peptone, using 72 hrs. old mycelium decolorization of 83% after 96 hrs. was observed with Orange II but not with Reactive black or Reactive red or a mixture of the three dyes. Conversely, in the presence of peptone alone decolorization was not satisfactory, the most significant decolorization (48% after 72 hrs.) being achieved for the Reactive red. Biological decolorization of textile dye Basic Blue 22 (C.I. 61512), a phthalocyanine type reactive dyestuff, by *Phanerochaete sordida* ATCC90872 was studied in a rotating biological contactor by Yang Ge *et al* (2004). The effects of different operating parameters including disc type, rotational speed, glucose and dyestuff concentration on the decolorization performance were investigated and the system was operated in repeated-batch mode with 48 hrs. hydraulic retention time. Highest decolorization efficiency was obtained with a rotational speed of 40 rpm. Minimum glucose concentration for 78% decolorization efficiency was 5 g/l. TOC removal efficiency was around 80% for 50–200 mg/L initial dyestuff concentrations and decreased to 52% for 400 mg/L.

Kasinath *et al* (2003) studied the immobilization of white rot fungus *Irpex lacteus* on polyurethane foam and pine wood to study decolorization of Remazol Brilliant Blue R and concluded that reusability and regenerative capacity of the immobilized culture, is important for application to water bioremediation. Alberto & Susana (2005) showed that laccase produced by *Trametes hirsuta* and immobilized into alginate beads in an airlift bioreactor decolorized 96% for indigo carmine and 69% for phenol red in 24 hrs., indicating the suitability of this process for application to synthetic dye decolorization. The rate of decoloration of dyes depended on the composition of medium and on the dye-microorganism pair. It was assumed that various extracellular peroxidases (lignin peroxidase and Mn-depen-dent peroxidase) or laccase are involved in the decolorization process. The decolorization of two different textile dyes, Reactive Red 2 and Reactive Blue 4, was studied by Axelsson *et al* (2006) in batch (individual as well as in a mixture) and continuous experiments using *Bjerkandera* sp. Strain BOL 13. The results showed that the fungus decolorized both dyes at different concentrations in a continuous rotating biological contactor. Decrease in absorbance at 538 nm was 96% at the two lower dye concentrations while it was 81% at the highest concentration and the corresponding figures at 595 nm were 94 and 80%. Zeroual *et al* (2007) used biomass of *Geotrichum sp.*, immobilized in calcium alginate and polyacrylamide gels for decolorization of azo dyes Orange G, Trypan blue, Azorubine, and Methyl red in fluidized bed bioreactor. The highest specific decolorization rate was obtained when the fungal biomass was entrapped in calcium alginate beads. Shedbalkar *et al* (2008) reported that *Penicillium ochrochloron* decolorized cotton blue a triphenylmethane dye (50 mg/L) within 2.5hrs. under static condition at pH 6.5 and 25°C.

El-Rahim *et al* (2003) conducted studies wherein the tested dyes were used as carbon and nitrogen sources for isolation of soil and/or water microorganisms capable of removing textile dyes wastes from factories effluent. Six fungal isolates were obtained from two genera, *Aspergillus* and *Trichoderma*. Results revealed the potential capacity of these fungi to decolorize 34 to 100% decolorization of dyes Direct Yellow, Direct Brown, Erio Red and Maxilon Yellow in comparatively short time (2-24 hrs.) indicating strong efficiency of dye bioremediation by the fungal isolates. They concluded that since the process involved is mostly fast interaction between the fungal mycelium and the dye in the media, the possible mechanism could be based on a biosorption of such chemicals on the intact fungal biomass, rather than direct biodegradation of the compounds. Actinomycetes *Nocardia corallina* IAM 12121 degraded Crystal Violet (BV3) at a low concentration, below 5 µmol dm^{-3} in 24 hrs. but was inhibited at a dye concentration of 7 µmol dm^{-3}. The degradation product of BV3 was identified as 4,4'-bis (dimethylamino) benzophenone (Michler's ketone; MK) (Yatome *et al* 1993).

2.5.4. Decolorization by Yeast

Kluyveromyces marxianus IMB3 decolorized Remazol Black-B dye under aerobic conditions and 98%, color removal was achieved at 37^{o} C in the pH range of 3.0-5.5. Also little or no color removal was detected when *K. marxianus* IMB3 was incubated under anaerobic conditions. Further investigation, in which decolorization was monitored under extreme temperatures and low pH (to inhibit growth) and using ten fold dense inoculum, revealed that decolorization was due to biosorption to the yeast cells and not due to a metabolic reaction (Meehan *et al* 2000). *Candida zeylanoides* degraded a number of simple azo dyes (Azo dyes derived from 2-naphtholaminoazobenzene and p-N,N-dimethylaminoazobenzene) in liquid aerated batch cultures with glucose as a carbon and energy source. The extent of color removal ranged from 44 to 90%, after 7 days, for 5 out of 6 dyes studied in shake cultures, without pH control, and from 46 to 67%, after 22 hrs., for 6 out of 8 dyes in batch experiments, at controlled pH (Martins *et al* 1999)

Among the 44 yeast strains tested for their ability to degrade textile dyes *Pseudozyma rugulosa* Y-48 and *Candida krusei* G-1, exhibited excellent color removal of 99% of reactive azo dye, Reactive Brilliant Red K-2BP (200 mg/L), in 24 hrs. *P. rugulosa* Y-48 could also remove 22–98% of (50 mg/L) and *C. krusei* G-1 62–94% (Yu & Wen 2005). *Candida oleophila* decolorized up to 200 mg/L of Reactive Black 5 dye under aerobic conditions in 24 hrs. of incubation at 26°C in the presence of 5 g/L glucose and without visible signs of dye adsorption to yeast cells (Lucas *et al* 2006).

2.5.6. Decolorization by Algal and Cyanobacterial Cultures

The potential of *Spirogyra* species was investigated by Mohan *et al* (2002) as viable biomaterial for biological treatment of simulated synthetic azo dye (Reactive Yellow 22) effluents and the results obtained showed that decolorization was dependent both on the dye concentration and algal biomass. Maximum dye color removal was observed on the third day for all the system conditions. Based upon the results, the dye–algal treatment mechanism was attributed to biosorption (sorption of dye molecules over the surface of algal cells), bioconversion (diffusion of dye molecules into the algal cells and subsequent conversion) and biocoagulation.

Acclimatized *Chlorella vulgaris* showed COD removal efficiencies of 69, 66 and 63% for initial Tectilon Yellow 2G (TY2G) concentrations of 50, 200 and 400 mg/L respectively, whereas, acclimatized cells of *C. vulgaris* resulted in increased COD removal efficiencies of 88, 87 and 88 % for same concentrations of dye respectively (Acuner and Dilek 2004). The main mechanism of the removal was proposed as partial biotransformation in case of un-acclimated algae and complete mineralization in case of acclimated algae. The equilibrium and kinetic analysis of the biosorption of two acidic dyes AB 290 and AB 324 on *S. rhizopus* has been investigated by Ozer *et al* 2006. *S. rhizopus* appeared to be effective for the removal of these acidic dyes from aqueous solutions. The optimum biosorption conditions were determined at pH 2.0 and 30°C for AB 290; and pH 3.0 and 25 °C for AB 324 at dye concentration 100 mg/L, biosorbent concentration 0.5 g/L.

Cyanobacterial cultures isolated from sites polluted by industrial textile effluents were screened for their ability to decolorize cyclic azo dyes. *Gloeocapsa pleurocapsoides* and *Phormidium ceylanicum* decolorized Acid Red 97 and FF Sky Blue dyes by more than 80% after 26 days. *Chroococcus minutus* was the only culture which decolorized Amido Black 10B (55%). Chlorophyll *a* synthesis in all cultures was strongly inhibited by the dyes (Parikh and Madamwar 2005). Sadettein and Donmez (2006) studied thermophilic cyanobacterial strains: *Synechococcus sp.* and *Phormidium sp.,* isolated from hot springs and their reactive dye bioaccumulation for Remazol Blue, Reactive Black B and Reactive Red RB dye in a batch system. The optimum pH at

25 mg/L initial reactive dye concentration was 8.5 for Remazol Blue and Reactive Black B dyes and 9.5 for Reactive Red RB dye. At the optimum pH value of each dye, dye bioaccumulation was between 10 mg/L and 78 mg/L initial dye concentrations at 45 °C and *Phormidium* sp. showed high dye bioaccumulation than the *Synechococcus* sp., with maximum uptake yields ranging from 13% to 97%. At all tested dye concentrations *Synechococcus* sp., showed the maximum uptake yields from 8% to 66% for all dye concentrations. Highest uptake yields at high dye concentrations for *Synechococcus* sp. was measured as 23.0% for 78.3 mg/L Reactive Red RB, 39.9% for 72.4 mg/L Remazol Blue and 13.7% for 62.0 mg/L Reactive Black B at 40 °C. At the same temperature, the uptake yield of *Phormidium* sp varied between 38.5% and 40.7% at 75 mg/L initial dye concentration.

2.6. Biological techniques for treatment of azo dye wastewater

Immobilization of microbial cells has received increasing interest in the field of wastewater treatment (Yang *et al* 1995, Oxspring *et al* 1996, Meilgo *et al* 2001, Frounda *et al* 2001, Kasinath *et al* 2003, Yang Ge *et al* 2004, Sharma *et al* 2004 a; b; c, Moutaouakkil *et al* 2004, Jane-Yii 2005, Chen *et al* 2005, Khehra *et al* 2006, Chen 2007). Studies on transport and biodegradation of azo dyes in aerobic biofilm was conducted by Zhang *et al* (1995) using three lab-scale rotating drum biofilm reactors (RDBRs) for culturing the biofilm. Synthetic, municipal-type wastewater, was spiked with several azo dyes, and continuously fed into these RDBRs. The study revealed that Acid Orange 7 (AO7) and Acid Orange 8 (AO8) degraded aerobically in an efficient and effective manner however the other dyes were non-degradable in an aerobic reactor. The results showed that biofilm were non-homogeneous and highly stratified and characterized by aerobic and anaerobic zones, with the anaerobic part used to break the azo bond and the aerobic surface layer to achieve the mineralization of anaerobically recalcitrant intermediates. Kanekar and Sarnaik (1995) reportedly used a fixed film bioreactor to treat waste effluent coming from dye industry manufacturing methyl violet rhodamine B, nigrosine and chrysoidine by *Pseudomonas alcaligenes*, isolated from cattle dung (which was adaptive to the waste effluent) and immobilized on rock media packed in a rectangular steel tank. The waste effluent was treated by recycling it for 24 hrs. in the form of a shower through rock media at 17 day hydraulic retention time at an influent loading rate of 0.055 m super (3)/m super (2) stone bed area per day. The microbial treatment resulted in the removal of 51% COD, 82% BOD, 74% TOC (Total

Organic Carbon), 76% phenol, 67% acetic acid and 60% color in terms of methyl violet. The pH of the waste effluent remained stable at 8.2.

Donlon *et al* (1997) reported partial mineralization and detoxification of azo dye Mordant Orange I using a continuous up-flow anaerobic sludge blanket reactor seeded with methanogenic granular sludge from a full-scale UASB reactor treating petrochemical industry effluent. Another sequential anaerobic-aerobic treatment process based on mixed culture of bacteria isolated from textile dye-effluent contaminated soil was developed by Rajaguru *et al* (2000) wherein biodegradation of azo dyes Orange G, Amido Black 10B, Direct Red 4BS and Congo red at the maximum degradation rate of 61, 571, 112 and 135 mg/L per day was achieved. Chang and Lin (2000) reported decolorization of azo dye Reactive Red 22 by *Pseudomonas luteola* strain in a two-stage fed-batch process consisting of an aerobic cell growth stage and an anaerobic fed-batch decolorization stage. They reported that dissolved oxygen reportedly inhibited the azo reduction activity strongly but slight agitation was found favorable and with periodical feeding strategy, the specific decolorization rate and overall decolorization efficiency increased with increase in concentration of dye in feed. However, feeding the reactor with dye concentration (>600 mg/L) lead to significant reduction of decolorization efficiency due to substrate inhibition. Chinwetkitvanich *et al* (2000) investigated the effect of addition of Tapioca starch as a co-substrate on the color removal efficiency of a two-stage up-flow anaerobic sludge blanket (UASB) system treating effluent of a reactive dye-bath. Supplement of Tapioca upto 500 mg/L gave a better color removal performance but the excessively high concentration of Tapioca did not enhance the process capability in terms of color removal efficiency. O'Neill *et al* (2000) studied effect of varied ratios of starch and azo dye in the combined anaerobic-aerobic treatment of simulated textile industry wastewater containing azo dye Procion Red H-E7B. The treatment system achieved a maximum of 77 % decolorization of dye at concentration of 0.15 g/L in feed with addition of 3.8 g/L starch. An anaerobic/aerobic sequential batch reactor system was designed by Panswad and Luangdilok (2000) for treatment of synthetic dye wastewater consisting of four different reactive dyes; C.I. Reactive Black 5 (Remazol Black B), C.I. Reactive Blue 19 (Remazol Black R), C.I. Reactive Blue 5 (Cibacron Blue CR) and C.I. Reactive Blue 198 (Procion Blue H-EGN). It was concluded from this study that different chemical structures of dyes affected the color removal rate of the system and nitrate could interfere with the color removal of azo dyes because of its better electron accepting potential than dye.

Talarposhti *et al* (2001) used two phase anaerobic packed bed reactor for color removal from a simulated dye wastewater containing mixture of dyes (Basic Yellow 28, Basic Yellow 21, Basic Red 18.1, Basic Violet Red 16, Basic Red 46, Basic Blue 16 and Basic Blue 14). The reactor removed 90% of color from mixed cationic dye wastewater at 1000 mg/L dye concentration however the color removal decreased with increase in influent dye concentration, but increased with increase in hydraulic retention time and organic loading. Lourenco *et al* (2001) used a sequential batch reactor with cyclic combination of anaerobic and aerobic periods to decolorize simulated textile effluents containing azo reactive dyes; Remazol Brilliant Violet 5R and Remazol Black B which were decolorized by 90 and 75% respectively. Kapdan *et al* (2003) showed the effect of hydraulic retention time (12-72 hrs.) and initial dyestuff concentration (50-400 mg/L) on COD removal and decolorization of textile dye Reactive Red 195, in a sequential anaerobic-aerobic treatment system consisting of facultative anaerobic bacterial consortium comprising of *Alcaligenes faecalis* and *Commomonas acidourans.* This system could be operated at hydraulic retention time of 18 hrs. with an initial COD load of 3000 mg/L and 100 mg/L dyestuff concentration to obtain over 85% decolorization efficiency in anaerobic unit and upto 90% COD load removal in aerobic unit of the system.

Semi-continuous reactors with hydraulic retention time of 10 days for degradation of dyes: Reactive Red 141, Reactive Blue 113 and hydraulic retention time five days for Vat Blue 1 operating under anaerobic conditions for treatment of simulated dye wastewaters were used to achieve 95 to 99% decolorization by Manu and Chaudhari (2003). In a fluidized bed reactor seeded with anaerobic cultures obtained from anaerobic sludge digesters of a wastewater treatment plant Sen and Demirer (2003) achieved upto 82, 94 and 59% removal of COD, BOD and color respectively by anaerobic treatment of real textile wastewater supplemented with glucose (2 g/L). Sponza and Isik (2004) reported 84% COD, 86% color and 52% total aromatic amines removal at an hydraulic retention time of 2.9 days, organic loading rate of 1.17 kg COD m^{-3} per day and a maximum dye loading rate of 46.4 gdyem-3hrs.$^{-1}$ respectively by the system treating 3200 mg/L Direct Black 38 dye.

Microorganisms isolated from activated sludge from a municipal wastewater treatment plant were immobilized on a porous volcanic rock decolorized 99% azo dye Acid Red 151 and showed 73% transformation of initial azo dye to CO_2 at an initial concentration of 50 mg/L in a sequential batch biofilter reactor (Buitron *et al* 2004)

Similarly a bacterial consortium enriched from samples collected from effluent disposal sites were able to degrade/decolorize Acid Violet-17 (AV 17) in an up-flow immobilized cell bioreactor using seashells for support (Sharma *et al* 2004 a). The bioreactor, at a flow rate of 6 ml/hrs., resulted in 91% decolorization of 30 mg/L of AV-17 with 94 and 95.7% removal of BOD and COD of the feed. Sharma *et al* (2004 b) in another study decolorized/degraded triphenylmethane dye Acid blue-15, in an up-flow immobilized cell bioreactor using refractory brick pieces as support material for biofilm formation by the consortium comprising of isolates *Bacillus sp*., *Alcaligenes sp*. and *Aeromonas sp*. The dye was degraded to simple metabolic intermediates with 94% decolorization at a flow rate of 4 ml/hrs. Sharma *et al* (2004 c) developed an up-flow immobilized cell bioreactor using a microbial consortium, comprising of *Bacillus sp., Alcaligenes sp. and Aeromonas sp* and immobilized on refractory brick pieces. The dye Malachite green was decolorized by >93% within 48 hrs. (dye concentration 30 mg/L; flow rate 6 ml/hrs.).

Moutaouakkil *et al* (2004) used immobilized cells of *Enterobacter agglomerans,* in fluidized bed reactor to decolorized 100 mg/L of azo dyes methyl red (MR) after only 6 hrs. of aerobic incubation. Chen *et al* (2005) using fixed-bed bioreactors with gel-entrapped cells of *Pseudomonas luteola* decolorized azo dye Reactive Red 22 in continuous mode with a constant feeding dye concentration of 50 mg/L, the beds with CA-immobilized cells had an optimal volumetric decolorization rate of 30.6 mg/hrs./l and a specific decolorization rate of 2.61 mg/g cell/hrs. when HRT and dye loading rate was 1.12 hrs. and 2.25 mg/hrs., respectively. Under aerobic conditions azo degrading bacteria entrapped into a phosphorylated PVA gel were used to degrade azo dye, Red RBN in a fluidized bed reactor (Jane Yii *et al* 2005) with an efficiency of 90% at initial dye concentration of <2200 mg/L under a continuous-flow condition, but the removal efficiency dropped dramatically after the dye concentration was increased over 2200 mg/L.

Khehra *et al* (2006) used a sequential anoxic-aerobic bioreactor to decolorize and degrade azo dye Acid Red 88 using polyurethane foam as immobilization support and a consortium comprising of *Stenotrophomonas sp*., *Pseudomonas sp.* and *Bacillus sp.*, isolated from waste disposal sites of textile processing industries as inoculum. The reactor was operated at flow rate of 7 ml/hrs. with hydraulic retention time of 12 hrs. and the treatment of synthetic dye wastewater feed having 100 mg/L of Acid Red 88 dye resulted in the 98% color and 95% COD removal. Vijayaraghavan *et al* (2007) developed an up-flow packed column loaded with polysulfone-immobilized with *Corynebacterium* *glutamicum* biomass performed well in the continuous treatment of Remazol effluent. Samples collected after 14hrs. of column operation revealed almost zero color and TOC. Column regeneration experiments revealed that the biosorbent was able to continuously treat Remazol effluent over ten cycles, with more than 90.6% decolorization efficiency. In a two stage anoxic–oxic reactor system, effective decolorization/degradation of azo dye Remazol black-B (RB-B) was achieved by bacterial consortium (comprising of *Pseudomonas aeruginosa*, *Rhodobacter sphaeroides*, *Proteus mirabilis*, *Bacillus circulance*, NAD 1 and NAD 6) where, at a concentration of 25 and 50 ppm, RB-B was decolorized within 12 and 20 hrs. and further at 75 and 100 ppm, about >90% color was removed in 24 hrs. while it increased to 96% at 35 hrs hydraulic retention time wherein COD removal was optimum at hydraulic retention time of 24 hrs. About 70% COD was removed during aerobic condition while 26% COD was removed under anoxic condition concentration of RB-B and Dafale *et al* (2008a).

2.7. Gene Manipulation

Azoreductase gene has been widely implicated in the decolorization of azo dyes. Cloning of the azoreductase gene in *E coli* could lead to the use of *E coli* for the process of decolorization. The cloning and expression in *E. coli* of an 'azoreductase' gene from *Clostridium perfringens* (Rafii and Coleman 1999), from *Bacillus sp.* (Suzuki *et al* 2001), from *Pseudomonas luteola* (Hu 1994) have been reported (Table 2). Furthermore, the feasibility of the use of a recombinant *E. coli* strain, harboring azo-dye-decolorizing determinants from *Rhodococcus sp.* (Chang and Lin 2001), and recombinant *Sphingomonas sp.* (Russ *et al* 2000) for the decolorization of dye wastewater has been demonstrated. The exoenzymes of white-rot fungi have also been objects of genetic engineering. The laccase of various filamentous fungi was successfully transmitted into yeast. These manipulations enhanced the capacity of microorganisms to decolorize synthetic dyes. The expression of oxidases from higher plants augmented the catabolic potential of microbes (Haudenschild *et al* 2000; Morawski *et al* 2001) and in turn microbial genes straightened the tolerance of higher plant to Poly R-487 (Iimura *et al* 2002).

Table 2: Improvement of Decolorization Activity Of Organisms By Interspecific Transfer Of Genetic Elements.

Donor	Acceptor	Function	References
Clostridium perfringens	*E.coli*	Azo reductase	Raffi and Coleman(1999)
Bacillus sp	*E.coli*	Azo reductase	Suzuki *et al* (2001)
Rhodococcus sp	*E.coli*	Azo reductase	Chang and Lin (2001)
Caulobacter subvibrioides	*E.coli*	Azo reductase	Govind *et al* (1993)
Xenophilus azovorans	*E.coli*	Azo reductase	Blumel *et al* (2002)
Pseudomonas leutola	*E.coli*	Azo reductase	Chang *et al* (2000)

The textile industries based in the various cities of Punjab state (India) extensively use azo dyes for dyeing cotton, polyester and woolen fabrics and the discharge of partially / untreated effluents into the water bodies and disposal of sludge are causing extensive pollution in the catchments areas. In view of these facts this research was focused on the decolorization of selected azo dyes using microbial consortium developed from the effluent treatment plant of Nahar Group of Textile & Dyeing Industry, Punjab, India and also using aerobic immobilized bioreactors.

OBJECTIVES:

- Development of microbial systems for the decolorization of dyes in wastewater from the textile printing and dyeing industry
- Techno economic evaluation of nutritional and process parameters for the decolorization of the wastewater from the textile printing and dyeing industry using packed bed column
- Characterization of wastewater to quantify the reduction in the color as well as the total COD and BOD load of the wastewater

3. MATERIALS & METHODS

3.1. Biological Material

3.1.1. Microbial Culture

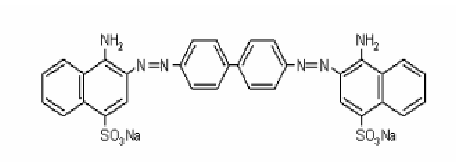
Three bacterial consortiums SKB-I, SKB-II and Con3 were used to study the efficient decolorization of various dyes. The bacterial consortiums: SKB-I and SKB-II were developed from the effluent treatment plant of Nahar group of textile & dyeing industries, Punjab, India while consortium Con3 was developed from a site contaminated with organic compounds but not dyes in Kalyan, Patiala District, Punjab, India.

The bacterial cultures were preserved at –80°C in 50% glycerol throughout this study and subsequently sub-cultured in Luria broth.

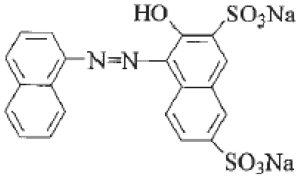
3.2. Chemicals

Chemicals of pure analytical grade like Bushnell Haas Broth, Bushnell Haas Agar, Luria broth, Luria agar and Starch soluble were obtained from the following sources: Merck (India), Himedia (India). Sodium chloride, silicon high vacuum grease and silicon tubes were obtained from the following sources: Qualigens (India), Ranbaxy (India), SD-fine (India).

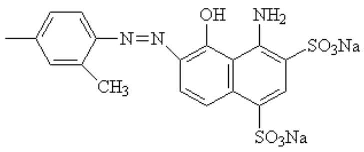
Textile Dye: Direct Red 28 (499λ max.), Direct Red 7 (510 λ max.) Acid Blue 113 (564 λ max.), Direct Blue 53 (611 λ max.) Reactive Orange 107 (410 λ max.), Reactive Red 120 (535 λ max.) were generously provided by Nahar Group of Textile Dyeing Industry, Punjab, India.



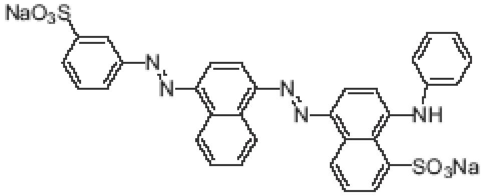
C.I. name Direct Red 28



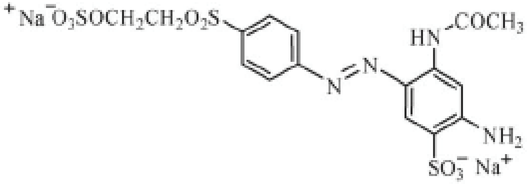
C.I. name Direct Red 7



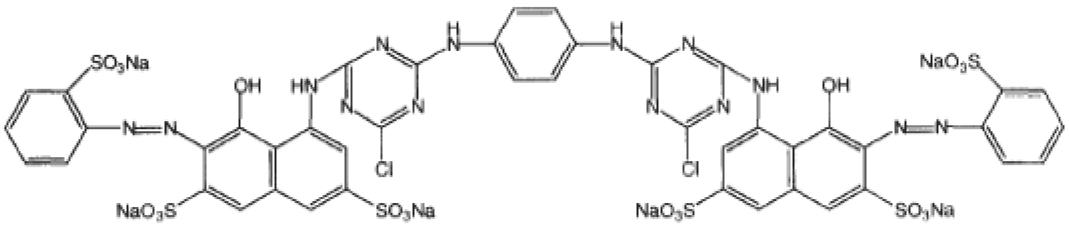
C.I. name Direct Blue 53



(C.I. name Acid Blue 113)



(C.I. name Reactive Orange 107)



(C.I. name Reactive Red 120)

Fig.2. Chemical structures of the azo dyes

3.2.1. Preparation of dye stock solutions

Stock solutions of the dyes were prepared by dissolving one gram of the dye powder in hundred milliliters of distilled water and added to the medium after filter sterilization (Millipore filter 0.22 µm, Sharma *et al* 2004a). Wavelength maxima of the dye were spectrophotometrically measured by wave length scan, against water as blank.

3.2.2. Preparation of media

The medium used for growth unless otherwise mentioned was Bushnell Hass Broth and referred as 'Basal Medium'. Bushnell Hass Broth (BHB) contained per liter: $MgSO_4$ (0.20 gms), $CaCl_2$ (0.02 gms), KH_2PO_4 (1.00 gm), K_2HPO_4 (1.00 gm). Starch 0.13 %(w/v) as carbon and energy source and 4% (w/v) NaCl was supplemented to provide salinity conditions (since textile dye effluents have a high salt concentration as their constituent (Mathew and Madamwar 2003) for fixing of the dye on the cloth. pH was adjusted to 7. The dye solutions were added from the filter sterilized stock solutions.

3.3. Development of dye decolorizing consortia

Effluent samples collected at different time periods from sludge samples (aerated tank) of effluent treatment plant of a Nahar Group of Textile & Dyeing Industry, Punjab, India were used for the development of the bacterial consortium capable of decolorizing dye. After 10 transfers on the basal medium containing the particular dye, the stabilized consortiums were used for further studies.

3.4. Growth conditions, Subculturing & Culture preservation

The growth of either the consortiums or the pure bacterial culture were done in basal medium and incubated at 37˚C at 120 rpm on a reciprocating shaker. The overnight grown culture (consortiums or the pure bacterial culture) was checked for absorbance colorimetrically by Biuret method using spectrophotometr (Itzhaki and Gill 1964). Upon attaining an Optical density of 0.800 ≥, the culture were preserved in 50% glycerol stock solution and stored at -80Cº and was subsequently sub-cultured on basal medium.

3.5. Screening of dye decolorizing consortia

Screening of three consortiums SKB-I, SKB-II and Con 3, for their decolorization ability was conducted in batch culture along with two controls, abiotic control (dye medium, without inoculum) and biotic control (dye free medium, with inoculum) also designed to perform under same condition. Different dyes used for screening were Direct Red 28 (499λ max.), Direct Red 7 (510 λ max.) Acid Blue 113 (564 λ max.), Direct Blue 53 (611 λ max.) Reactive Orange 107 (410 λ max.), Reactive Red 120 (535 λ max.). The flasks were incubated at 37˚C on a rotatory shaker (120 rpm). All observations are a mean of two replicates. At specific time interval, aliquots from each flask were withdrawn aseptically and total dye decolorization was observed spectrophotometrically at the given wavelength of the respective dye and the process continued up to 120 hours. Growth was monitored by estimation of the protein spectrophotometrically. The cell pellet obtained upon centrifugation (10,000x g for 15 min.) of 3 ml aliquot, was re-suspended in 3 ml distilled water and its protein was determined by Biuret Method at 340 nm (Itzhaki and Gill, 1964).

3.5.1. Varying concentrations of single dyes

The three pre-grown bacterial consortiums (4%) Con3, SKB-I and SKB-II were inoculated in a separate 100 ml basal broth medium containing single dye. The varying concentrations of dyes used for screening were 10, 20, 30, 50, 75 and 100 mg/L.

3.5.2. Varying concentrations of mixture of dyes

The textile industry uses many dyes at a time for dyeing of the cloth. Between 20-50 percent of the reactive dye used by the textile industry is lost in exhaust and wash water (Lewis 1999). Pre-grown bacterial consortiums (4%) were inoculated in a separate 100ml basal broth medium containing mixture of dyes at varying concentrations of 10, 20, 30, 50, 75 and 100 mg/L.

3.5.3. Varying concentrations of starch

The textile industry uses Starch for stiffening of the fabric; excess of this starch comes out in the effluent. To observe the decolorization efficiency of the consortium SKB-II to decolorize dyes, pre-grown bacterial consortium SKB-II (4%) was inoculated in 100ml basal broth medium supplemented with different Starch concentrations of 1.3 g/L, 0.65 g/ L and 0 g/L. Filter sterilized single dye Direct Red 28; Direct Red 7; Acid Blue 113 and Direct Blue 53 was also added in the basal medium.

3.5.4. Varying concentrations of NaCl

The textile industry uses NaCl for fixing the dyes onto the fabric; excess of this salt comes out in the effluent. Pre-grown bacterial consortium SKB-II (4%) was inoculated in 100 ml basal medium supplemented with different concentrations of NaCl (4 g/L, 2 g/L and 0 g/L). Filter sterilized single dye Direct Red 28; Direct Red 7; Acid Blue 113 and Direct Blue 53 at concentration of 10 mg/L was also added in the basal medium.

3.5.5. Varying concentrations of effluents

The composition of effluent from textile industry varies depending upon the dyeing schedules and the types of dyes used in the dyeing processes. In this study, the capability of mixed microbial consortium SKB-II, to decolorize effluents containing diverse dyes was evaluated.

Pre-grown bacterial consortium SKB-II (4%) was inoculated in sterile basal media. Different concentrations of 1-10% real raw untreated effluent (procured from local dyeing house and textile mill) was added to the basal medium. pH was set at 7 using 1N HCl.

3.8. Pure Cultures

3.8.1. Isolation and Screening of dye decolorizing bacterial isolates from consortium SKB-II

An aliquot of 1ml of pre-grown culture (in BHB medium, without dye) with an optical density of 0.800 ≥ was added to 10 ml sterile water blank and shaken well by vortexing. This microbial suspension was serially diluted up to 10^{-12} dilution. From each dilution aliquot of 100µl inoculum was taken and plated on Bushnell Hass Agar medium plates (BHA) for isolation of bacterial isolates. The inoculated plates were incubated at 37ºC for 24 hours and observations recorded. The individual pure isolates were stored in 50% glycerol at -80 ºC and picked and patched further to study the efficiency of the bacterial isolates to decolorize different dyes.

Aliquots (6 ml) of pre-grown isolates with an optical density of 0.800 ≥ was inoculated separately in 100 ml basal media containing 10 mg/L respective dye in 250 ml Erlenmeyer flask and incubated at 37ºC on rotatory shaker (120 rpm).

Spectrophotometer absorbance was recorded after every 24 hours at respective wavelength of the dye and at 550 nm for growth of the isolate.
For calculating dye decolorization activities see section 3.11.1.

3.9. Molecular identification

The chromosomal DNA of the strains ST 6, 33, 43, 47 and 53 were isolated according to the procedure described by Rainey et al, (1996). The 16S rRNA gene was amplified with primers 8-27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1500r (AGAAAGGAGGTGATCCAGGC-3'). The amplified DNA fragment was separated on 1% agarose gel, eluted from the gel and purified using Qiaquick gel extraction kit (Qiagen, Germany). The purified PCR product was sequenced with four forward and three reverse primers namely 8-27f (5'AGAGTTTGATCCTGGCTCAG-3'), 357f (5'-CTCCTACGGGAGGCAGCAG-'), 704f (5'-TAGCGGTGAAATGCGTAGA-3'), 1114f (5'-GCAACGAGCGCAACC-3'), 685r (5'-TCTACGCATTTCACCGCTAC-3'), 1110r (5'-GGGTTGCGCTCGTTG-3') and 1500r (5'-GAAAGGAGGTGATCCAGGC-3'), respectively (*Escherichia coli* numbering system). The rDNA sequence was determined by the dideoxy chain-termination method using the Big - Dye terminator kit using ABI 310 Genetic Analyzer (Applied Biosystems, USA).

The 16S rDNA sequence of the strains generated in this work were aligned with the 16S rDNA sequence of other closely related Bacillus species retrieved from the GenBank data base. A sequence similarity search was done using GenBank BLASTN (Altschul *et al* 1997). Sequences of closely related taxa were retrieved, aligned using Clustal X programme (Thompson *et al* 1997) and the alignment was manually corrected. For the neighbour-joining analysis (Saito and Nei, 1987), the distances between the sequences were calculated using Kimura's two-parameter model (Kimura, 1980). Bootstrap analysis was performed to assess the confidence limits of the branching (Felsenstein, 1985).

3.10. Bioreactor

The packed bed bioreactor was built from a borosilicate glass column (44.5 cms x 6 cms, volume 1260ml). For support material various cheap and sturdy matrices like thermocol, foam, plastic clips (7-8 mm) and marble chips (2-3 mm) procured from local stores, were screened for bacterial immobilization. The support material (matrix) were washed with tap water to remove adhering dust and dirt particles and then rinsed thoroughly with distilled water. Further the matrix was treated in dye water for maximum dye adsorption. The reactor was packed with the dye treated matrix and sealed with silicon grease. Silicon tubing was attached at the openings (inlet and outlet) of the reactor. The whole system was autoclaved for 30 min. at 15 psi. For adsorption of the dye onto the matrix sterile basal media containing Direct Red 28 dye was pumped in upward direction using an Electrolab PP-106 peristaltic pump. After maximum adsorption of the dye onto the matrix, sterile basal media without Direct Red 28 dye was pumped through the reactor to remove loosely bound color.

Biofilm development

Mixed culture was pre-cultured for 18 hrs at 37°C and upon attaining an optical density of 0.800 ≥, the culture (32ml) was inoculated in the bioreactor at an aeration rate of 0.4 & 0.6 mmoles/min (which was provided using air pumps and controlled with Bubble flowmeter) and hydraulic retention time (HRT) of 11, 7.3 and 5.5 hrs for plastic clips and 8.16, 5.4 and 4 hrs for marble chips. The total working volume of the reactor with plastic clips as matrix was 600 ml and with marble chips as matrix was 790 ml respectively. The basal medium was pumped into the bioreactor using peristaltic pump. This was followed by incubation period of 7 days at 37°C during which bacterial consortium forms a biofilm by attaching itself over the surface of the matrix. After incubation period, sterile basal media without the dye was introduced into the bioreactor at the respective HRT to check the biofilm formation by collecting washouts at regular time intervals from the outlet of the bioreactor and checking the optical density of the washout spectrophotometrically by Biuret method. Once stabilization period (stable value of washout) was attained, the feeding stream was continuously pumped with dye in BHB through bottom of the reactor. The bioreactor was operated under these conditions for 40- 45 days. Another bioreactor was operated under same conditions as an abiotic control to compare the total decolorization of the dye in the biotic reactor.

3.10.1. Decolorization studies of Direct Red 28 dye treated through the bioreactor

At aeration rates of 0.4 & 0.6 mmoles/min. along with hydraulic retention time (11, 7.33 and 5.5 hrs for plastic clips and 8.16, 5.4, 4.08 hrs for marble chips) basal medium (Bushnell and Hass broth (BHB) containing the following in g/L: $MgSO_4$, 0.2; $CaCl_2$, 0.02; KH_2PO_4, 1.0; K_2HPO_4, 1.0; $(NH_4)\ _2NO_3$, 1.0; $FeCl_3$, 0.05 supplemented with Starch 1.3 g/L as carbon and energy source and 4% NaCl along with Direct red 28 dye solution of 10 mg/L was run through the bioreactor (packed with respective matrix) at 37

°C. Pre-cultured Consortium SKB-II (optical density of 0.800≥) was inoculated in the bioreactor.

3.10.2. Decolorization studies of untreated textile effluent (procured from local dyeing houses) treated through the bioreactor

At aeration rates of 0.6 mmoles/min., and hydraulic retention time of 8.6 hrs, textile effluent (5%) in Bushnell and Hass broth medium (BHB) containing the following in g/L: $MgSO_4$, 0.2; $CaCl_2$, 0.02; KH_2PO_4, 1.0; K_2HPO_4, 1.0; $(NH_4)_2NO_3$, 1.0; $FeCl_3$, 0.05 supplemented with Starch 1.3 g/L as carbon and energy source and 4% NaCl was run through the bioreactor (packed with marble chips as matrix) at 37 °C. pH was set using 1N HCl. Pre-cultured consortium SKB-II (optical density of 0.800≥) was inoculated in the bioreactor.

3.11. General Techniques

3.11.1. Decolorization Assay

To measure the decolorization capacity of the culture, an aliquot was separated aseptically at regular time interval and centrifuged at 10,000 x *g* for 15 min. (Khehra *et al* 2005). Decolorization percentage was calculated by measuring the decrease in color of the supernatant by taking optical density in glass cuvettes at the respective wavelength maxima of the dyes spectrophotometrically. The un-inoculated sterile medium with dye was used as blank.

Decolorization percentage was calculated as follows:

$$\text{Decolorization (\%)} = \frac{(I - F) \times 100}{I} \qquad \text{(Khehra } et \text{ al 2005)}$$

Where, I = initial absorbance and F = Final Absorbance of decolorized medium

Growth (as protein) of the culture was observed by Biuret method (Itzhaki and Gill 1964) by using the pellet separated from the supernatant during centrifugation. The inoculated dye free medium was used as blank.

3.12. Gram staining

Gram staining of all the 53 dye decolorizing isolates was done and the strains were analyzed microscopically. Preparation of a fixed bacterial smear: The bacterial smear placed on a slide with the help of an inoculation needle was dried and heat-fixed. On a staining rack the slide was flooded with crystal violet for about 1 min. The stain was washed gently with iodine solution and stained with fresh iodine solution for 1 min followed by washing in tap water or by dipping in a beaker containing water. Few drops of decolorizer were added and continued until color ceased to come out of the preparation. Gentle washing was repeatedly given with water. The slide was counter-stained with dilute Safranin for 10-30 seconds and again the slide was washed with water and left for drying by evaporation. The stained dry slide was examined under the microscope directly without a cover slip first under low power and then under oil immersion at higher magnification.

3.13. Scanning Electron Microscopy (SEM)

The support particles for scanning electron micrographs were collected from the bioreactor and were fixed with 4% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 1 h and subsequently washed with 0.1 M phosphate buffer for 10 min. The fixed samples were then dehydrated through a graded series of ethanol solutions (30, 50, 70, 90 and 100 % ethanol) for 30 min each. The samples were then dried in critical point drier and coated with gold (Sharma et al 2004). The un-inoculated support material sample was also treated as per the described procedure. Scanning electron micrographs were taken on a JSM-6100, JEOL (Japan) scanning electron microscope.

3.14. Waste water analysis

Waste water was collected from the effluent treatment plant (ETP) of Nahar Spinning Mills, Punjab, India. Samples from raw (untreated tank) were collected in jerry cans and were then stored at 4 C^o for further characterization in terms of pH, total COD, total BOD, total solids, total dissolved solids, total suspended solids and alkalinity.

3.14.1. Physical analysis

3.14.1.2. Determination of pH

pH was determined electrometrically .The pH meter was standardized by immersing the electrode in buffer solution of known pH 4 and 9.2. And then the electrode was immersed in the sample till a stable reading was obtained.

3.14.1.3. Total Solids

Sample (250 ml) was taken in separate dried and weighed beaker and evaporated to dryness in an oven at 103°C-105°C to get a constant weight. The beaker was cooled in a dessicator then weighed to determine total solids

$$\text{Total residue [mg/l]} = \frac{\text{[wt.of beaker with residue – wt.of empty beaker] mg} \times 100}{\text{250 ml of sample}}$$

3.14.1.4. Total Dissolved Solids

Samples were filtered through Whatman filter paper no. 44 and100 ml of the filtrate was taken in a weighed beaker. It was allowed to evaporate to dryness in an oven at 103° C-105° C to a constant weight. Beaker was cooled in a dessicator then weighed.

$$\text{Dissolved residue [mg/l]} = \frac{\text{[wt.of crucible with residue-wt.of empty crucible] mg} \times 100}{\text{100 ml of sample}}$$

3.14.1.5. Total Suspended Solids

Total suspended solids = Total solids – Total dissolved solids

3.14.2. Chemical analysis

3.14.2.1. Measurement of COD

Two tubes of each sample were prepared with one set as blank. One ml $K_2Cr_2O_7$, 2 ml sample in each tube and 3 ml of H_2SO_2 and Ag_2SO_4 was added in the COD tubes. These tubes were digested for 2 hours at 150ºC so that $K_2Cr_2O_7$ effectively digested the organic matter in the COD digester. The whole content was transferred to titration flask by rinsing the tube with water. The samples were titrated with FAS, using

Ferroin as indicator and end point was green to wine red. Volume of FAS used was noted.

$$\text{COD mg/l} = \frac{(A-B)\ C \times 8 \times 1000}{\text{ml sample}}$$

Where,

A=ml $FeSO_4\ (NH_4)_2\ SO_4$ used for blank

B=ml $FeSO_4\ (NH_4)_2\ SO_4$ used for sample.

C=Normality of $FeSO_4\ (NH_4)_2\ SO_4$ solution determined above.

3.14.2.2. Measurement of BOD

The required volume of distill water was aerated in a container by bubbling compressed air for 24 hrs. to attain DO saturation. After 24 hrs. of aeration, 1 ml of each phosphate buffer, magnesium sulphate, calcium chloride and ferric chloride solution was added for each litre of dilution of water and mixed well. Bacterial seed (2 ml) was added to 1000ml of dilution water because wastewater does not have sufficient bacterial population.

Dilution of Sample:

As pH of the sample was not highly alkaline so neutralization of the sample was not done. Different dilutions of the sample were made and the total volume was made up to 1000 ml by using dilution water. The prepared diluted sample were siphoned in three labeled BOD bottles and stoppered immediately. Out of the three BOD bottles, one bottle was used for determining the initial DO and the other two bottles were incubated at 20ºC for 5 days. Abiotic control in duplicate was kept by siphoning plain dilution water (without seed) to measure the oxygen consumption in dilution water. Initial DO of the sample and the abiotic control was estimated the same day and final DO of the sample and abiotic control was determined after 5 days of incubation.

RESULTS

4.1. Screening of dye decolorizing bacterial consortiums

Aerobic consortiums SKB-I and SKB-II were developed from the aeration tank of the effluent treatment plant of textile dyeing industry and consortium Con-3 was developed from a site contaminated with organic compounds but not dyes. This industry uses variety of dyes namely: azo, triphenylmethane, nitroso, disperse and solvent dyes during their dying process. After 10 transfers in basal medium with starch (1.3 g/L) and NaCl (4%) and the dyes, the consortia's were tested for consistent decolorization using azo dyes *viz* Direct Red 28, Direct Red 7, Acid Blue 113, Direct Blue 53, Reactive Red 120 and Reactive Orange 107.

Consortium Con 3 decolorized the selected azo dyes very poorly and after 120 hrs only 6.3, 2.8, 9.7, 2.9, 5.4 and 2.8 % of 10 mg/L of Direct Red 28, Direct Red 7, Acid Blue 113, Direct Blue 53, Reactive Red 120 and Reactive Orange 107 had been decolorized (Fig 3).

Consortium SKB-I showed gradual and higher decolorization of the azo dyes Direct Red 28 (78%), Direct Red 7 (69%), Acid Blue 113(70%) & Direct Blue 53(74%) but only 7 and 8% with Reactive Orange 107 & Reactive Red 120 after 120 hrs of incubation at 37 °C (Fig 4).

Consortium SKB-II showed maximum and best decolorization of four out of six dyes and after 72 hrs 70% decolorization was achieved with Direct Red 28 which increased to 81 and 96% decolorization at 96 and 120 hrs. Dyes Direct Red 7, Acid Blue 113 and Direct Blue 53 showed maximum decolorization of 90, 82 and 82% at the end of 120 hrs. Least decolorization of 9.8 & 11% was observed with Reactive Red 120 & Reactive Orange 107 respectively (Fig.5)

Among the three consortiums SKB-II was the most efficient decolorizer wherein 10 mg/L of four out of six dyes i.e. Direct Red 28, Direct 7, Acid Blue 113 and Direct Blue 53 were decolorized by 96, 90, 82 and 83% respectively (Fig.6). With consortium SKB-I maximum decolorization observed was 78, 69, 70 and 74% with Direct Red 28, Direct Red 7, Acid Blue 113 and Direct Blue 53 respectively while con3 showed poorest decolorization of only 6% (Direct Red 28), 3% (Direct Red 7), 10% (Acid Blue 113) and 3% (Direct Blue 53).

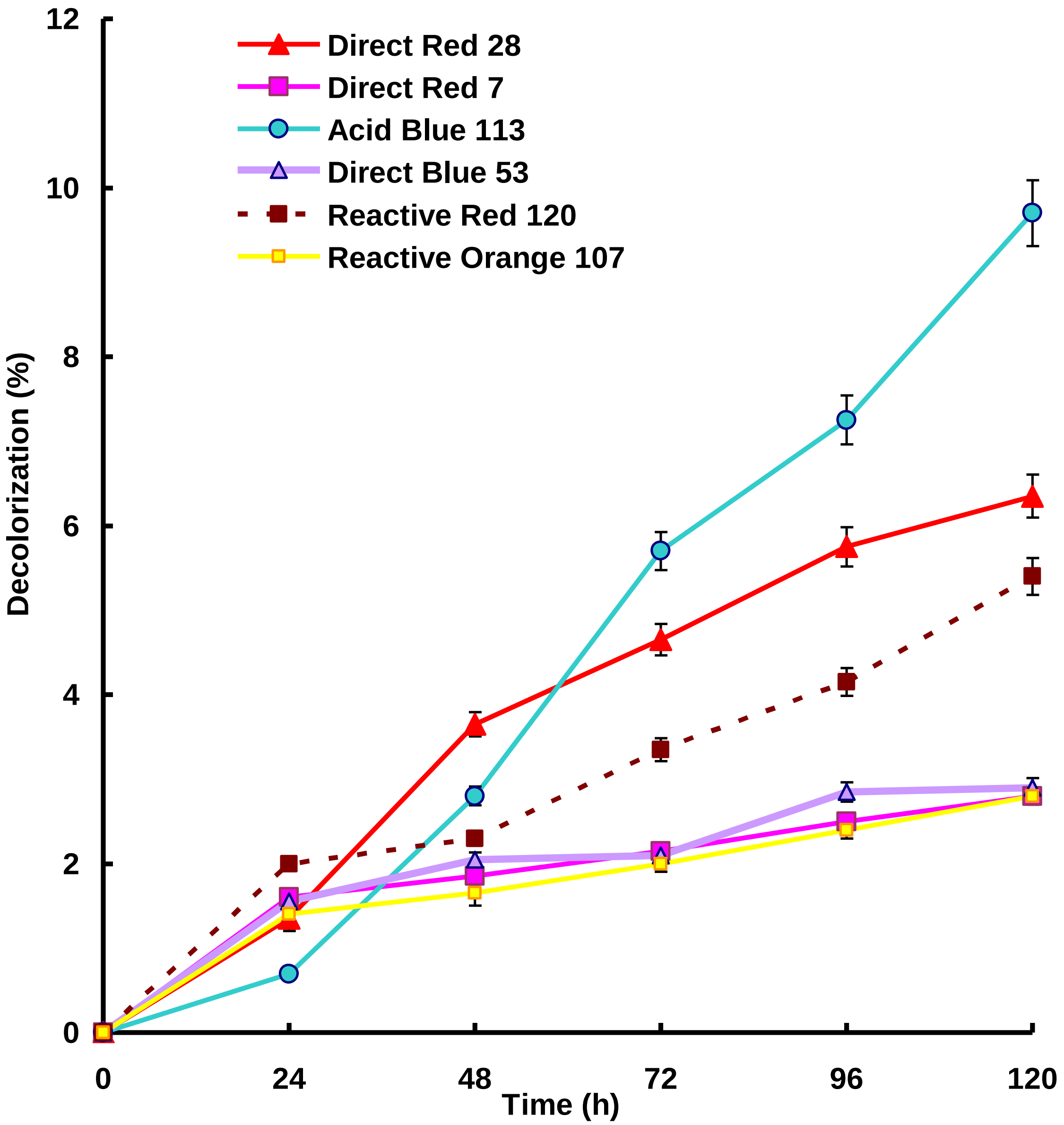


Fig.3. Decolorization (%) of azo dyes (10 mg/L) by consortium Con3

Consortium Con 3 was grown at 37 °C on Bushnell Haas Broth (BHB) medium with starch (1.3 g/L) and NaCl (4%) at pH 7. Decolorization was estimated at regular time intervals in the supernatant (as described in section 3.11.1.)

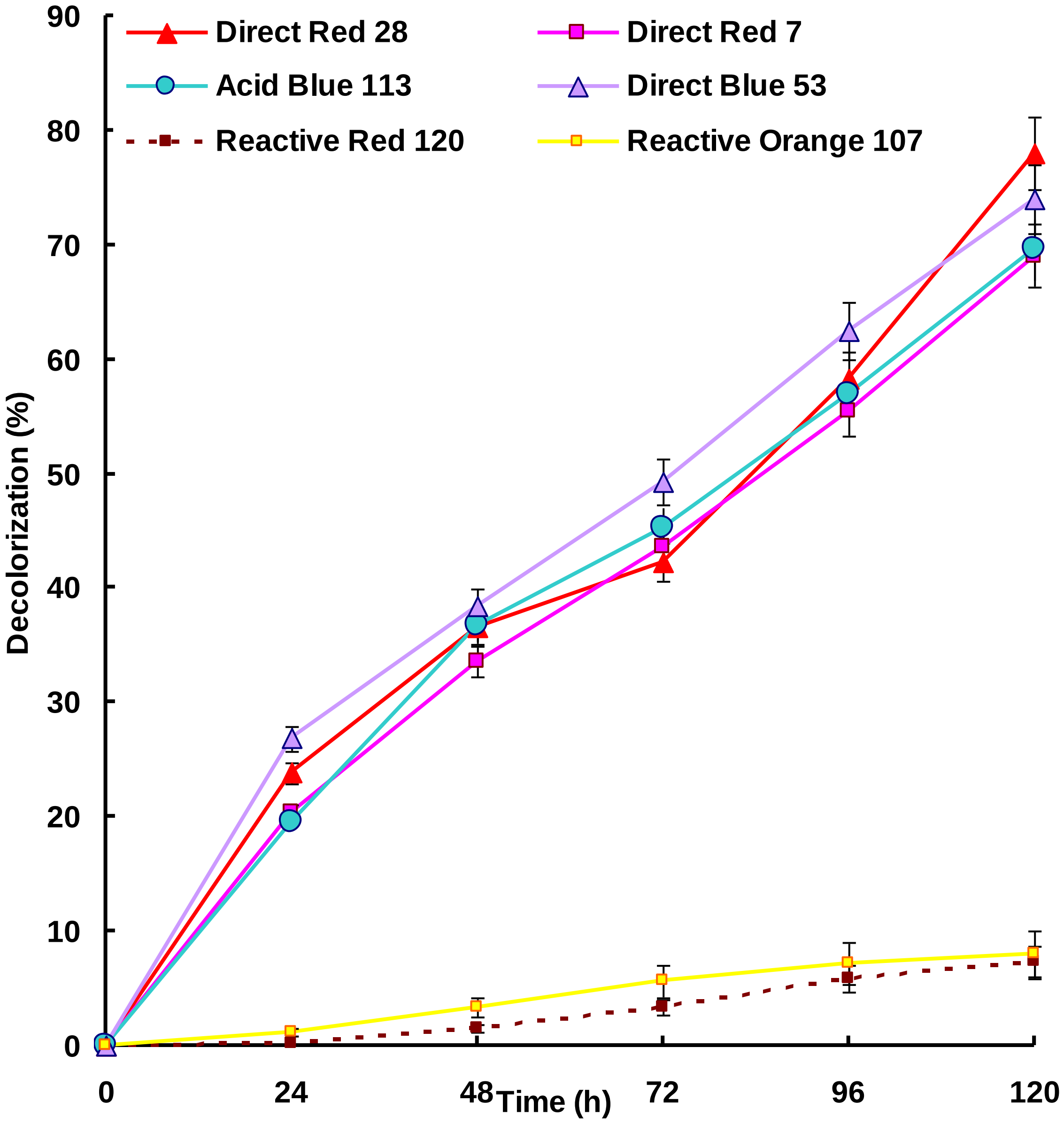


Fig.4. Decolorization (%) of azo dyes (10 mg/L) by consortium SKB-I

Consortium SKB-I was grown at 37 °C on Bushnell Haas Broth (BHB) medium with starch (1.3 g/L) and NaCl (4%) at pH 7. Decolorization was estimated at regular time intervals in the supernatant (as described in section 3.11.1.)

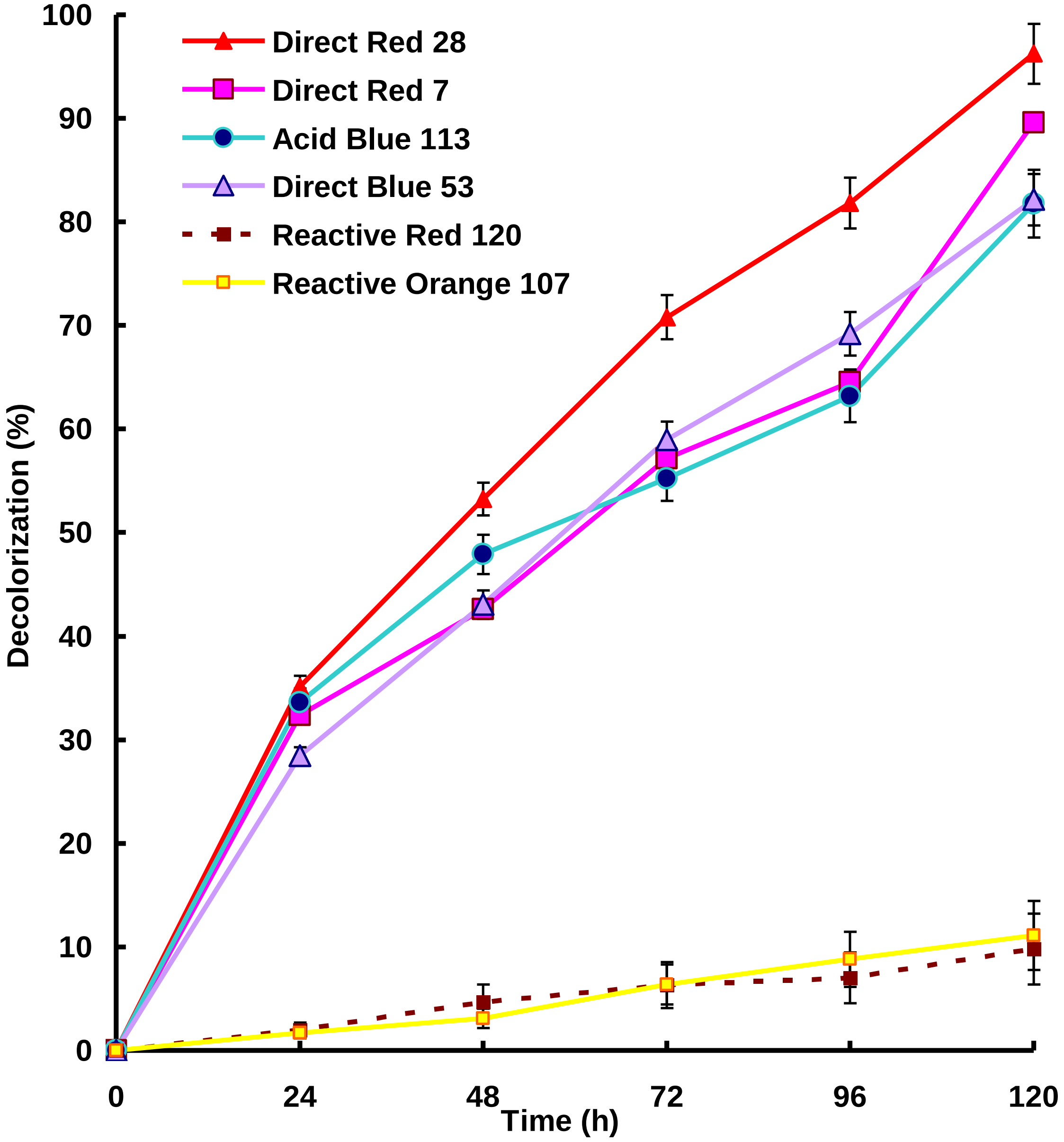


Fig.5. Decolorization (%) of azo dyes (10 mg/L) by consortium SKB-II

Consortium SKB-II was grown at 37 °C on Bushnell Haas Medium (BHB) with starch (1.3 g/L) and NaCl (4%) at pH 7. Decolorization was estimated at regular time intervals in the supernatant (as described in section 3.11.1.)

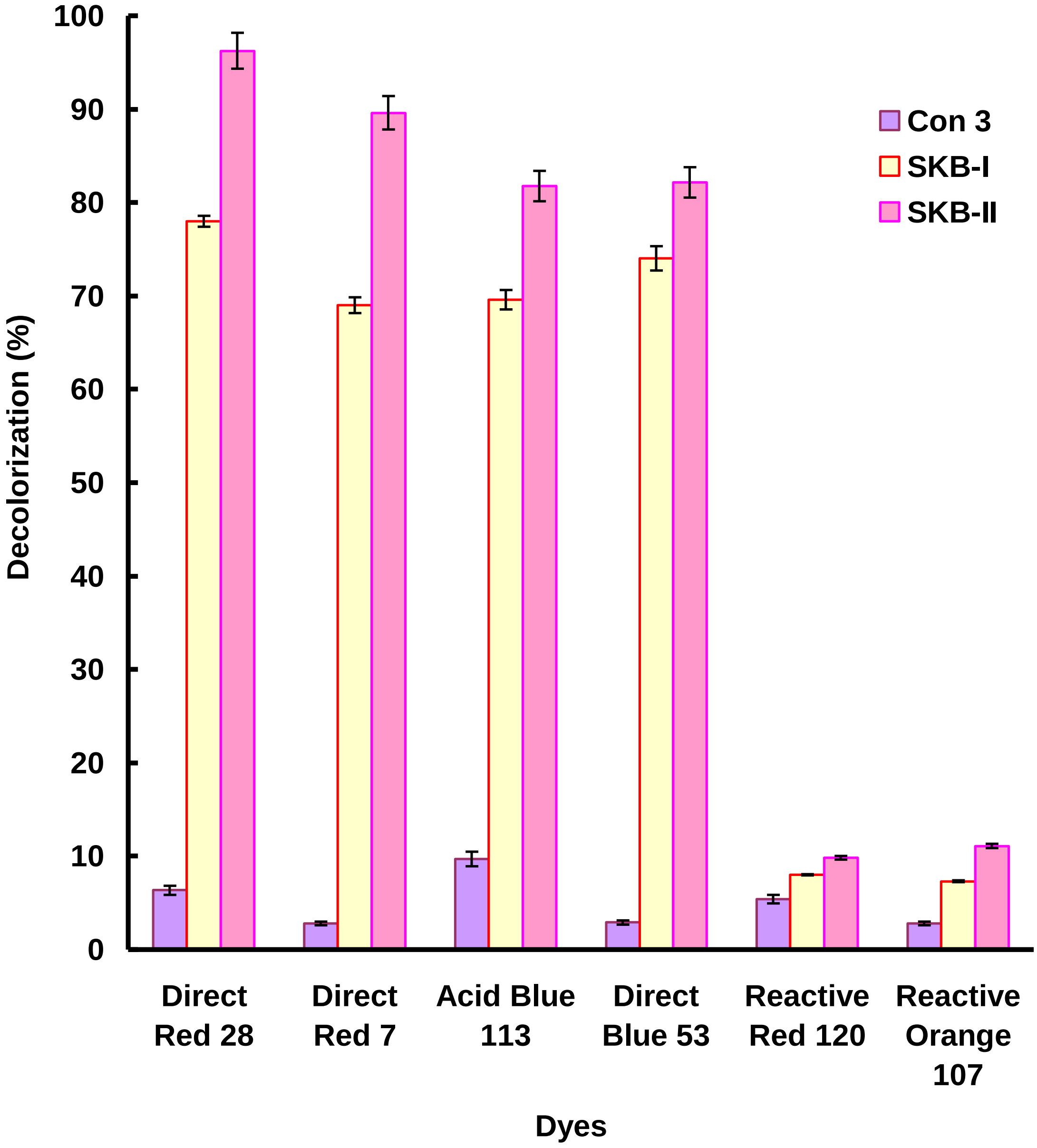


Fig.6. Decolorization (%) of azo dyes (10 mg/L) consortium Con3, SKB-I and SKB-II

All three consortiums were grown at 37 °C on Bushnell Haas Medium (BHB) with starch (1.3 g/L) and NaCl (4%) at pH 7. Decolorization was estimated at 120 hrs. in the supernatant (as described in section 3.11.1.)

Azo dyes Reactive Red 120 and Reactive Orange 107 were quite resistant to decolorization, as none of the three consortiums were able to decolorize the two dyes beyond a maximum of 12%. Among the three, consortium SKB-II being the most efficient decolorizer was henceforth selected for further studies with only four azo dyes viz. Direct Red 28, Direct 7, Acid Blue 113 and Direct Blue 53.

4.2. Optimization of medium components

Starch, is most commonly used as dye additive in textile finishing and a part of this comes into the effluent. The effect of different concentrations (0, 0.65, 1.3 g/L w/v) of starch on the decolorization efficiency of consortium SKB-II to decolorize selected dyes was studied (Table 3). The results indicate that additional carbon source is essential for decolorization, as least decolorization was observed with all dyes in absence of starch. With 0.65 g/L starch concentration decolorization of 69% of Direct Red 28, 67% Direct Red 7, 66% Direct Blue 53 followed by 40% Acid Blue 113 and 6% each of Reactive Red 120 and Reactive Orange 107 was observed after 120 hrs (Table 3). Upon increasing the starch concentration to 1.3 g/L sudden increase in decolorization (39 to 67%) of Direct Red 28 was observed from 24 to 48 hrs which gradually increased to 96.5% after 120 hrs. Higher decolorization was also observed with rest of the dyes *viz*: Direct Red 7, Direct Blue 53, Acid Blue 113, Reactive Red 120 & Reactive Orange 107 (87, 84, 81, 12 and 10% respectively) after 96 hrs but further not much variation in decolorization was observed after 120 hrs. Hence, 1.3 g/L starch concentration was the best suited and preferred throughout this study.

In the process of fastening color applied to textiles, NaCl is added during the application of the dyes. The wastewater contains salt and it generally ranges between 2-4% (w/v). Therefore the effect of NaCl concentration on the growth and decolorization ability of the consortium SKB-II was studied. Growth (as protein) of the consortium SKB-II without NaCl was observed to be 0.71mg/ml with Direct Red 28 dye, and 42% (protein=1.23 mg/ml) higher with 2% NaCl concentration compared to no salt. Increasing the concentration to 4% NaCl resulted in 48% increase in growth (protein=1.38 mg/ml) compared to no salt in presence of Direct Red 28 dye after 120 hrs. Other dyes *viz*. Direct Red 7, Acid Blue 113 and Direct Blue 53 showed growth (as protein) of 0.56 to 0.69 mg/ml without NaCl and protein of 1.14 to 1.23 mg/ml with 2% NaCl and highest growth (as protein) of 1.28 to 1.31 mg/ml with 4% NaCl after 120 hrs.

Table.3. Decolorization (%) by consortium SKB-II at varying starch concentrations

Consortium SKB-II was grown in Bushnell Haas broth (BHB) medium with varying concentration of starch (0, 0. 65 g/L, 1.3 mg/L), NaCl (4%) and azo dyes (10 mg/L) at 37ºC and 120 rpm. Decolorization was estimated at regular time intervals in the supernatant (as described in section 3.11.1.)

Dyes/Hrs.	**24**	**48**	**72**	**96**	**120**	**24**	**48**	**72**	**96**	**120**
	Starch 0.65 g/L					**Starch 1.30 g/L**				
Direct Red 28	40	49	53	65	69	39	67	79	94.8	96.5
Direct Red 7	15	28	64	64	67	30	45	66	86.4	88.4
Acid Blue 113	5	15	19	36	40	37	47	70	80.5	82.7
Direct Blue 53	20	49	62	65	66	33	50	64	83.8	85
Reactive Orange 107	3.3	4.9	5.6	5.8	5.8	3.3	4.8	7.2	10.4	11.3
Reactive Red 120	3.1	3.4	4.2	4.9	5.2	4	5.7	8	11.8	12.2

Consortium SKB-II without NaCl showed low growth (protein=0.465 and 0.499 mg/ml) with two dyes viz: Reactive Red 120 and Reactive Orange 107 after 120 hrs. Same dyes showed slight increase of 36 and 6.6% growth with 2% NaCl which further increased marginally by 2.6 and 4.4% compared to no salt with 4% NaCl after 120 hrs.

Maximum decolorization of 96% with Direct Red 28, followed by 90 % of Direct Red 7, 82% each of Acid Blue 113 & Direct Blue 53 and 11 & 10% each of Reactive Orange 107 and Reactive Red 120 was observed with 4% NaCl (w/v) by the consortium SKB-II after 120 hrs while with 2% NaCl (w/v) decolorization of 80, 79, 78, 74, 4 and 3% respectively with Direct Red 28, Direct Blue 53, Direct Red 7, Acid Blue 113, Reactive Orange 107 and Reactive Red 120 was observed after 120 hrs. Minimum color removal of 17% was observed with Direct Red 28 followed by 15% of Direct Red 7, 17 % of Acid Blue 113 and 19% of Direct Blue 53 while only 3% decolorization by the consortium was observed with Reactive Red 120 and Reactive Orange 107 when grown without NaCl (w/v) after 120 hrs (Table.4)

In this study it was observed that the consortium showed good growth and decolorization with four dyes Direct Red 28, Direct Red 7, Acid Blue 113 & Direct Blue 53 in presence of 4% NaCl whereas with dyes Reactive Red 120 and Reactive Orange 107 low growth and decolorization was observed in the presence of 4% NaCl.

4.3. Screening and Identification of bacterial isolates

The consortium on plating showed 53 differential colonies which were selected for batch culture decolorization studies on four azo dyes: Direct Red 28, Direct Red 7, Direct Blue 53 & Acid Blue 113 at a concentration of 10 mg/L (Fig. 7). Among these 53 isolates five isolates showed maximum decolorization with Direct Red 28 after 120 hrs and so were selected for identification. Gram staining showed morphology of St 6 was short segmented rods**,** St 43 & 47 was cocci with St 43 being diplococcus and St 47 occurring in clusters, while St 33 and St 53 occurred as gram positive rods. Molecular identification of these isolates, by analysis of the sequence similarity based on 16S rDNA sequence alignment and database comparison, identified: ST 6 as *Bacillus pumilus,* St33 as *Bacillus megaterium,* St 43 as *Bacillus cereus,* St 47 as *Bacillus vallismortis* while St 53 was identified as *Bacillus subtilis* (Fig.8.) by Microbial Type Culture Collection, IMTECH, Chandigarh, India.

Table.4. Decolorization (%) of azo dyes at varying NaCl concentrations by consortium SKB-II

Consortium SKB-II was grown in Bushnell Haas broth (BHB) medium with of starch (1.3 mg/L) and azo dyes (10 mg/L) along with varying concentration of NaCl (0, 2 and 4%) at 37ºC and 120 rpm. Decolorization was estimated at regular time intervals in the supernatant (as described in section 3.11.1.)

Dyes/Hrs.	**24**	**48**	**72**	**96**	**120**	**24**	**48**	**72**	**96**	**120**	**24**	**48**	**72**	**96**	**120**
	0% NaCl					**2% NaCl**					**4% NaCl**				
Direct Red 28	2.6	3.4	8.1	15.8	17.3	22.4	41.5	67.8	78.4	80.4	35.1	53.2	70.8	81.8	96.5
Direct Red 7	1.3	3.6	7.5	12.2	15.2	18.6	25.7	41.8	59.5	78.2	32.4	42.6	57.1	64.5	88.4
Acid Blue 113	1.9	6.4	11.2	15	16.8	17.7	27.4	43.7	62.3	74.2	33.6	47.9	55.2	63.2	82.7
Direct Blue 53	1.4	9.5	14.4	17	19.4	21.6	33.2	45.5	58.3	79.6	28.4	43.1	58.9	69.2	85
Reactive Orange 107	1.5	2.5	2.8	3	3.2	1.8	2.4	2.8	3	3.4	2	4.7	6.3	7	11.3
Reactive Red 120	1.7	2	2. 5	2.9	3.4	2	2.7	3.3	3.8	4.4	1.7	3.1	6.37	8.8	12.2

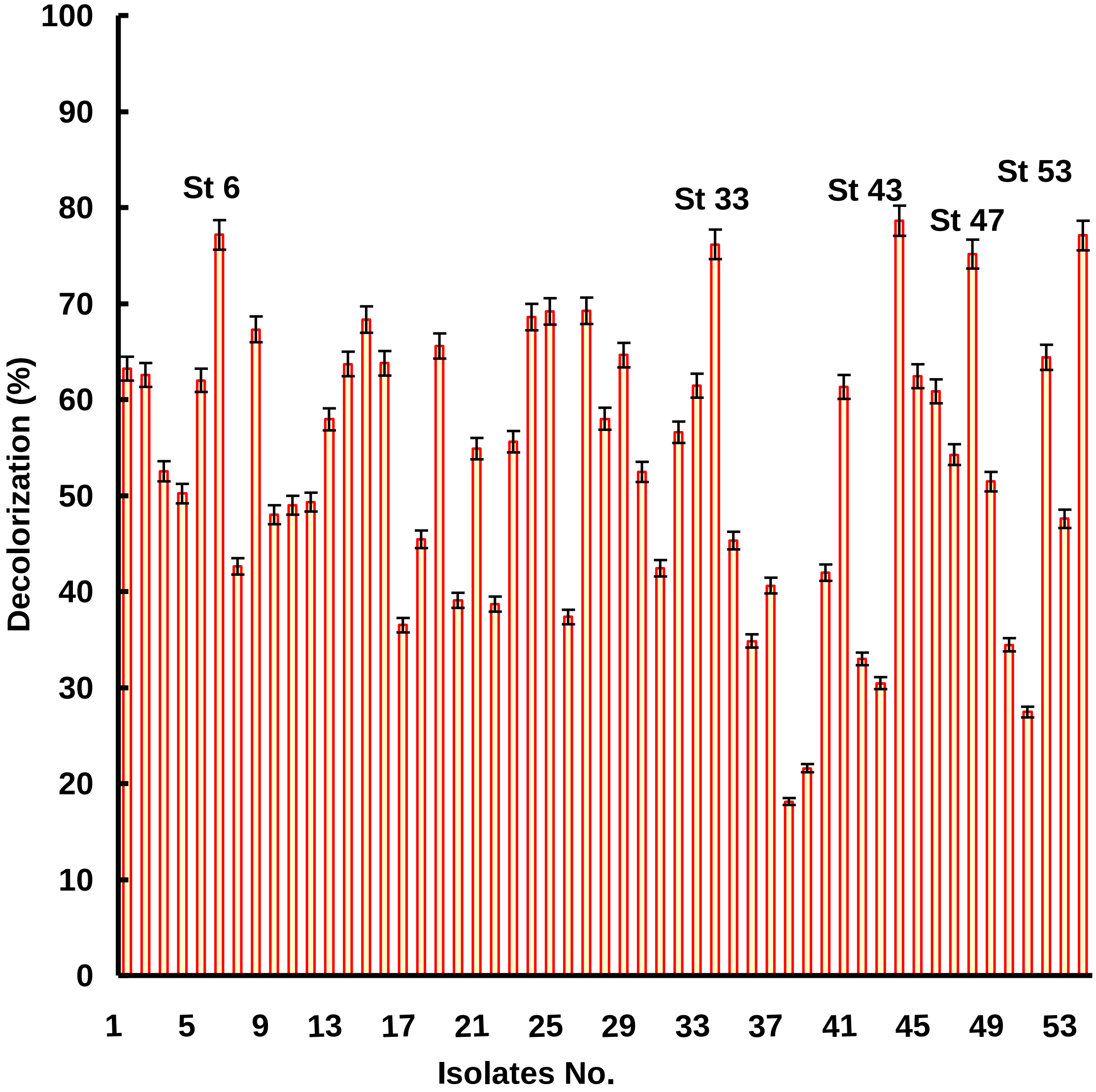


Fig.7. Decolorization of Direct Red 28 (10 mg/L) by fifthythree bacterial isolates

Consortium SKB-II was grown in Bushnell Haas agar (BHA) medium with of starch (1.3 mg/L), NaCl (4%) and azo dye (10 mg/L) at 37ºC and 120 rpm. Total of 53 differential colonies were selected for batch culture decolorization studies. Decolorization and growth (as protein) were estimated at 120 hrs. (as described in section 3.11.1.)

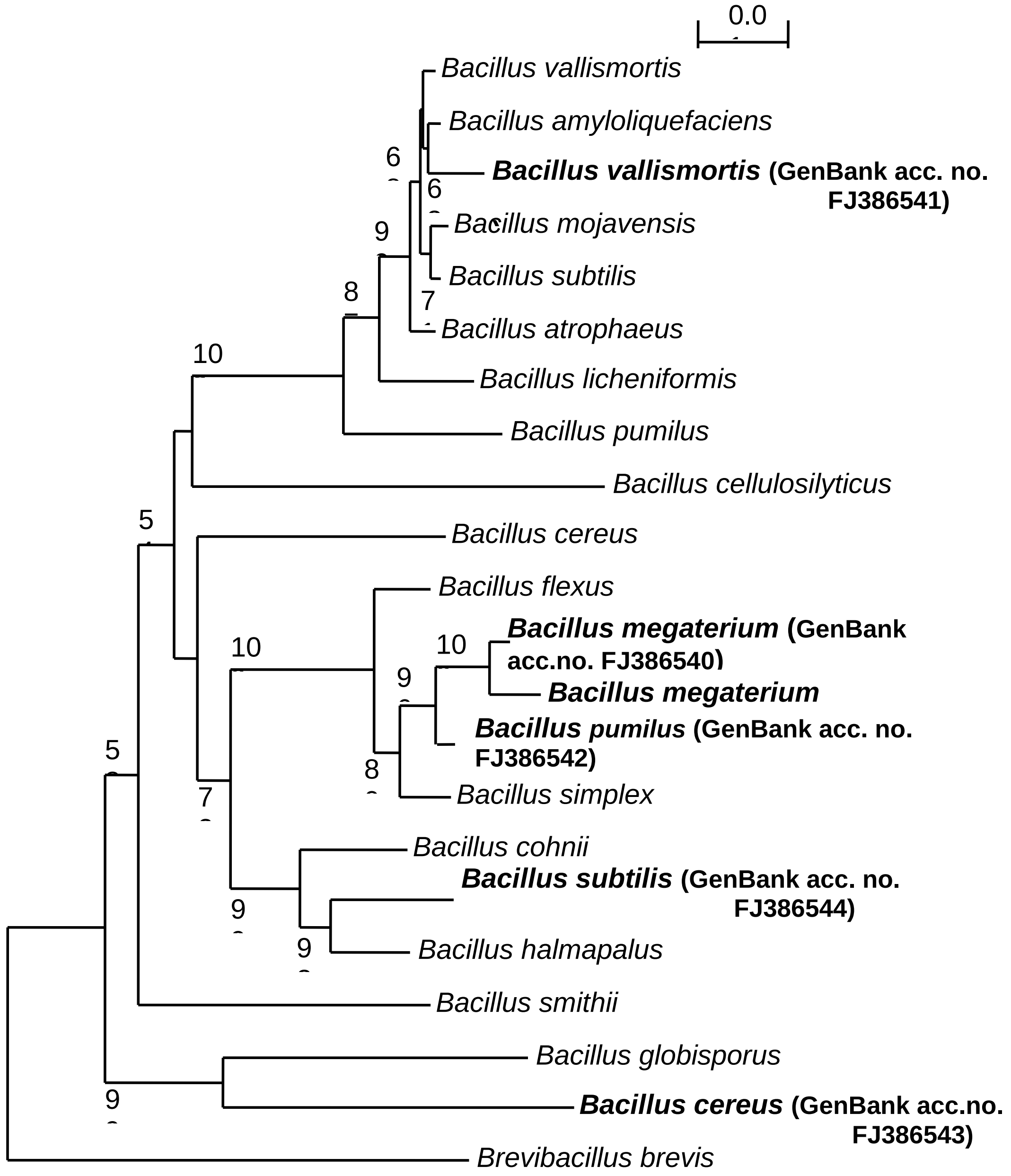


Fig.8. Phylogenetic tree indicating the position of different strains. Among other related species of the genus *Bacillus*. Bootstrap values (expressed as percentages of 1000 replications) greater than 50 % are shown at branch points. *Brevibacillus brevis* was used as an outgroup. Bar, 0.1 substitutions per site.

4.4 Decolorization potential of mixed culture (comprising of five bacterial isolates *viz*: ***Bacillus vallismortis, Bacillus megaterium, Bacillus cereus, Bacillus subtilis and Bacillus pumilus***) to decolorize individual dyes

The effect of various concentrations of the selected azo dyes ranging from 10-100 mg/L on decolorization efficiency of the mixed culture was studied in shake flasks at 37^{o}C. The mixed culture with Direct Red 28 dye after 120 hrs showed that as the concentration of the dye was increased from 10 to 100 mg/L, growth and decolorization decreased. At 10 mg/L maximum growth (as protein) of 1.38 mg/ml and maximum decolorization of 96% was observed whereas, growth (as protein) of 1.30mg/ml and decolorization of 86.8% was achieved at 20 mg/L. Further increase in dye concentration to 30 mg/L reduced the growth (as protein) to 1mg/ml) and decolorization to 65.6%. However as the dye concentration was increased to 50, 75 and 100 mg/L gradually decreased of growth (as protein) of 0.978, 0.89 and 0.831mg/ml and decolorization to 22, 9 and 7% respectively was observed. Hence it can be mentioned here that growth and decolorization were in correspondence with each other in between 20 to 30 mg/L wherein maximum inhibition of both growth and decolorization was observed (Fig.9).

The effect of increasing concentration of Direct Red 7 from 10 to100 mg/L on decolorization efficiency of the mixed culture was evaluated (Fig.10). At 10 mg/L growth (as protein) was 1.27mg/ml and decolorization was 90%. The growth (as protein) was reduced from 1.23, to 1.01 and then to 0.830 mg/ml when dye concentration was increased from 20 to 30 and then 50 mg/L, while decolorization reduced from 72 to 45% as the dye concentration was increased from 20 to 30 mg/L only. Not much decline in growth i.e. protein (0.83, 0.81 and 0.796 mg/ml) was noticed upon increasing the dye concentration from 50, to 75 and 100 mg/L. Decolorization however reduced gradually from 44, 34 and 29% from 50 to100 mg/L

Effect of varying concentration of Acid Blue 113 (10 to100 mg/L) on the mixed culture is shown in Fig.11. After 120 hrs., the mixed culture showed a growth as protein of 1.21 mg/ml and decolorization of 82% at 10 mg/L which decreased to 67 % at 20 mg/L. The growth (as protein) was 0.973 and 0.968 mg/ml and 63 and 61% for decolorization at 30 and 50 mg/L. Major reduction in decolorization (48 and 17%) was observed at 75 and 100 mg/L but not much difference in growth (protein=0.775 and 0.727 mg/ml) was observed. It was also observed that at 100 mg/L growth was very slow along with least decolorization of 13.3 to 16.4 % from 24 to 120 hours.

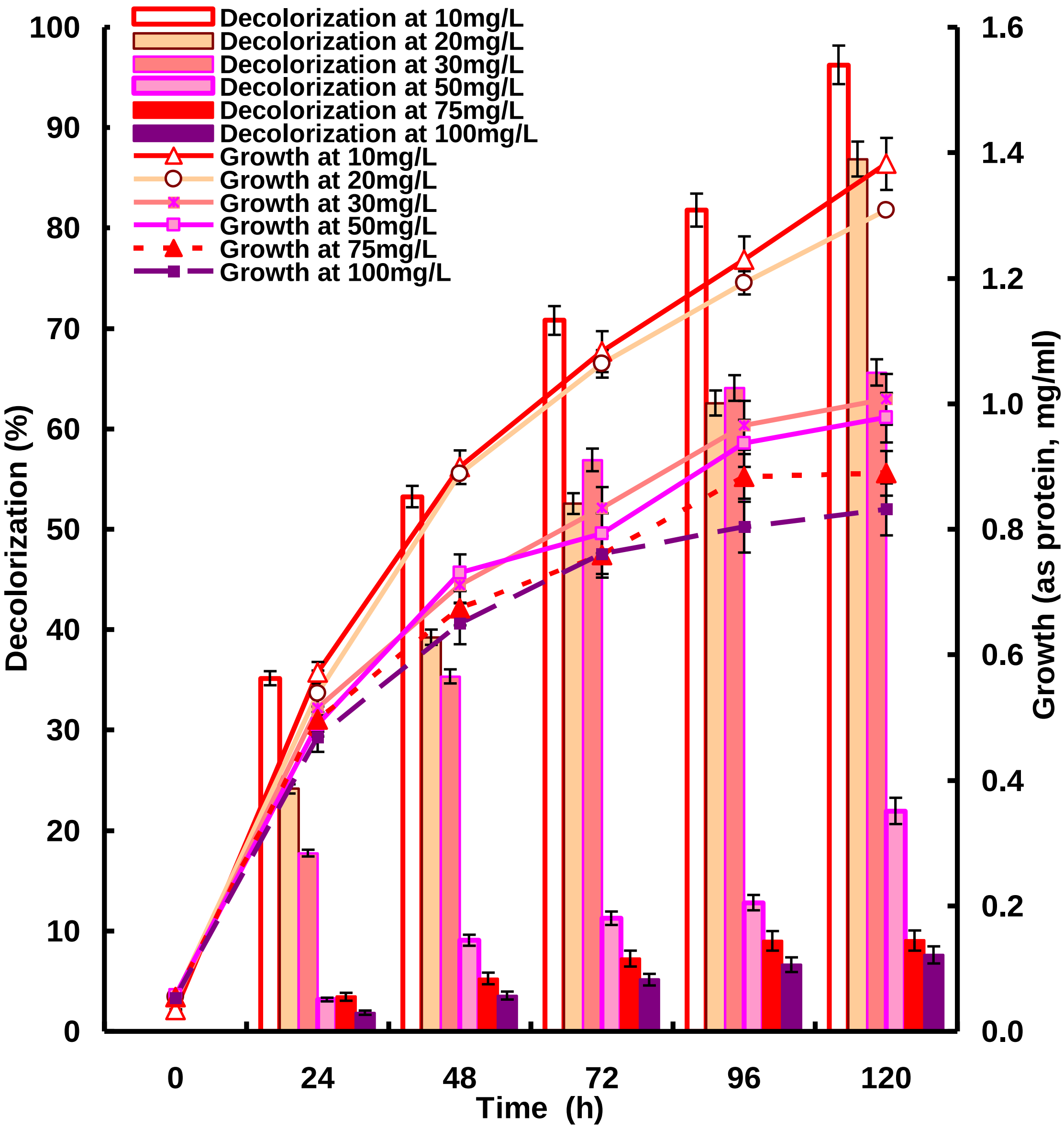


Fig.9. Decolorization (%) of azo dye Direct Red 28 (10-100 mg/L) by mixed culture comprising of five bacterial isolates

Mixed culture was grown at 37 °C on Bushnell Haas Medium (BHB) with starch (1.3 g/L), NaCl (4%) and varying dye concentration (10 to 100 mg/L) at pH 7. Decolorization and growth (as protein) were estimated at regular time intervals (as described in section 3.11.1.)

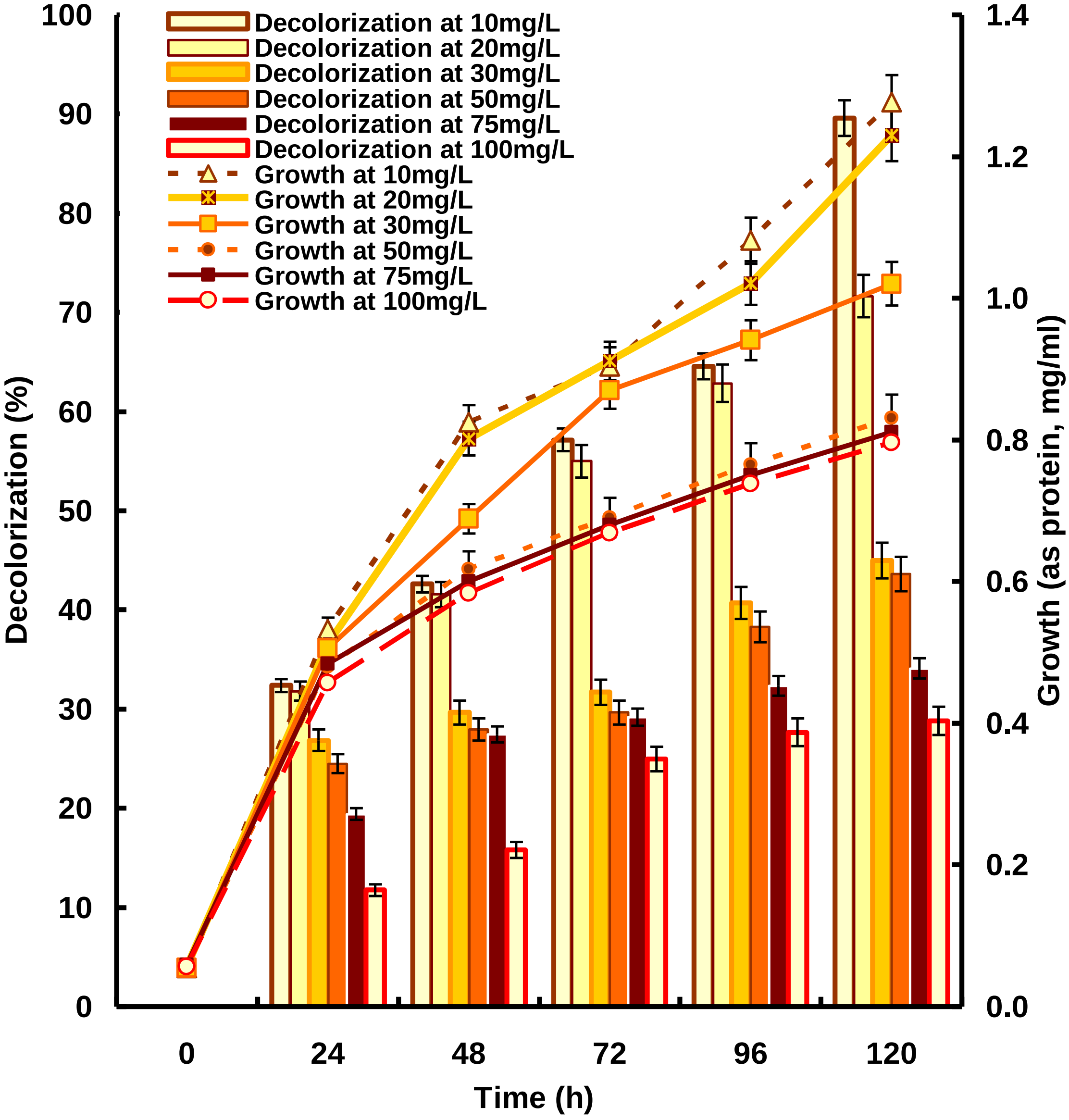


Fig.10. Decolorization (%) of azo dye Direct Red 7 (10-100 mg/L) by mixed culture comprising of five bacterial isolates

Mixed culture was grown at 37 °C on Bushnell Haas Medium (BHB) with starch (1.3 g/L), NaCl (4%) and varying dye concentration (10 to 100 mg/L) at pH 7. Decolorization and growth (as protein) were estimated at regular time intervals (as described in section 3.11.1.)

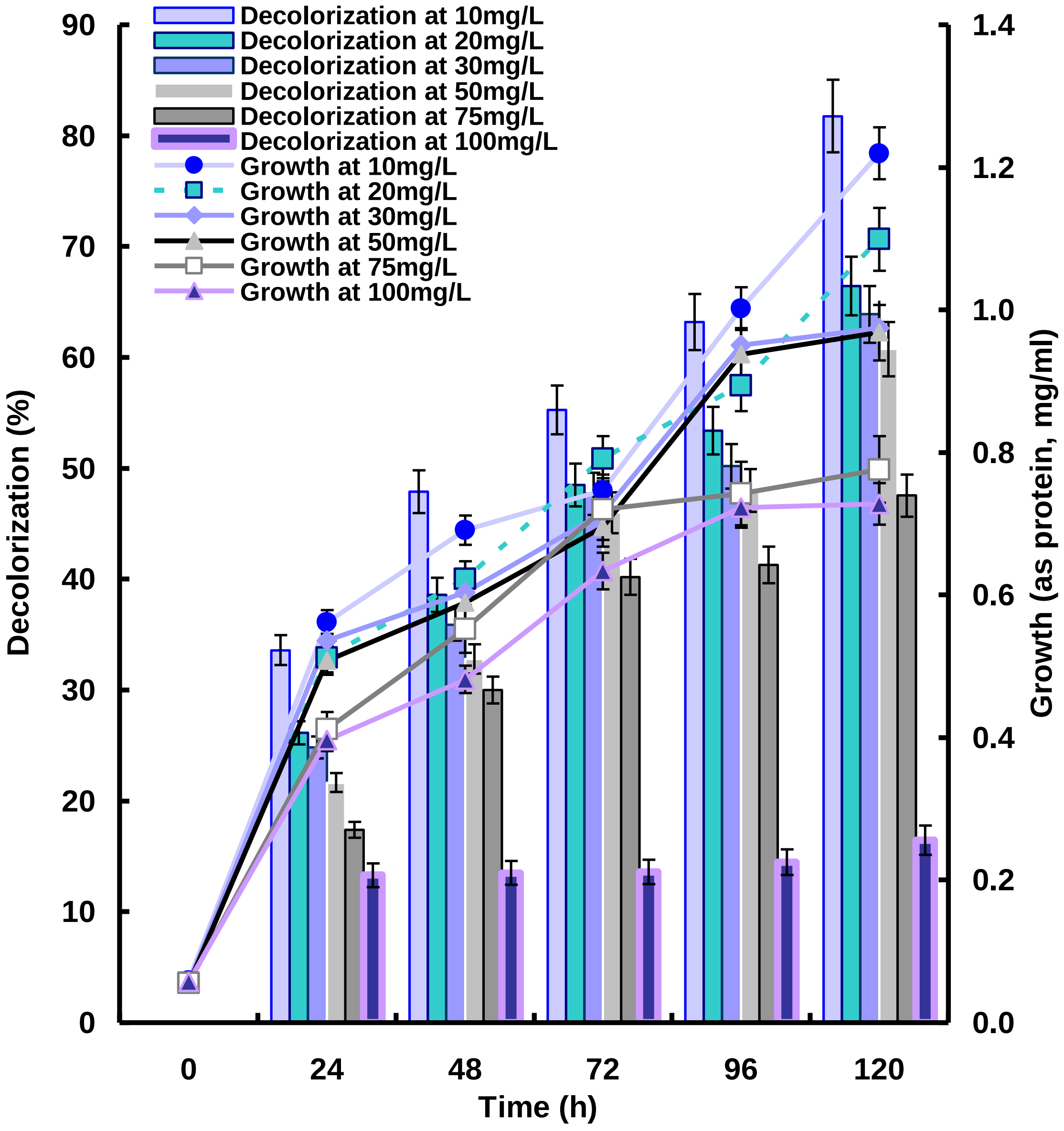


Fig.11. Decolorization (%) of azo dye Acid Blue 113 (10-100 mg/L) by mixed culture comprising of five bacterial isolates

Mixed culture was grown at 37 °C on Bushnell Haas Medium (BHB) with starch (1.3 g/L), NaCl (4%) varying dye concentration (10 to 100 mg/L) at pH 7. Decolorization and growth (as protein) were estimated at regular time intervals (as described in section 3.11.1.)

Varying concentration of Direct Blue 53 affected the decolorization efficiency and growth of the mixed culture as decolorization and growth decreased with increase in dye concentration (Fig.12). Highest decolorization of 82 % was achieved at 10 mg/L followed by gradual fall of decolorization to 68, 61, 53, 41 and 33% as the concentration was increased from 20 to100 mgL^{-1}. Growth of the mixed culture (as protein) at 10, 20 and 30 mg/L was 1.28, 1.27 and 1.20 mg/ml respectively after which inhibition of 1.04 mg/ml was observed at 50 mg/L and then decline of growth from 0.987 to 0.916 mg/ml protein with higher dye concentration of 75 &100 mg/L was observed.

4.5. Decolorization studies with mixture of dyes

Industrial effluent consists of a mixture of various dyes. Ability of our efficient mixed culture comprising of five bacterial isolates *viz*: *Bacillus vallismortis, Bacillus megaterium, Bacillus cereus, Bacillus subtilis and Bacillus pumilus* to decolorize different dyes was studied with mixture of two and four dyes at varying concentration of 10-100 mg/L. The decolorization of each dye was noted at their respective wavelength.

The capacity of the mixed culture to decolorize a mixture of two dyes (equal concentration) at 10 mg/L final concentration was studied at 37 °C and 120 rpm over 6 days. It was observed that among the two dyes , Direct Red 28 showed decolorization of 23% after 24hrs which increased to 53% at 72 hrs and then to 73% after 120 hrs while decolorization of Direct Red 7 was 18% at 24hrs and reached a maximum of 63 % after 120 hrs. Growth (as protein) of mixed culture in presence of two dyes at 24-120 hrs was 0.605, 0.853, 1.02, 1.12 and 1.19 mg/ml respectively (Fig.13). The effect of increasing concentration of the two dyes on the decolorization by mixed culture showed that as the concentration of the dyes was increased from 10 to 75 mg/L decolorization decreased gradually from 73 to 58% of Direct Red 28 and 63 to 39% of Direct Red 7 after 120 hrs (Fig.14). However, not much difference in the decolorization (as least as 22 and 21%) of the two dyes was noticed at 100 mg/L. As was observed in case of decolorization, growth of the mixed culture also declined from 1.19 mg/ml protein at 10 mg/L to 1.13, 0.962, 0.899 & 0.764 mg/ml protein from 20 to75 mg/L and at 100 mg/L only 50% of the growth i.e. 0.591 mg/ml protein as compared to growth at 10 mg/L was achieved.

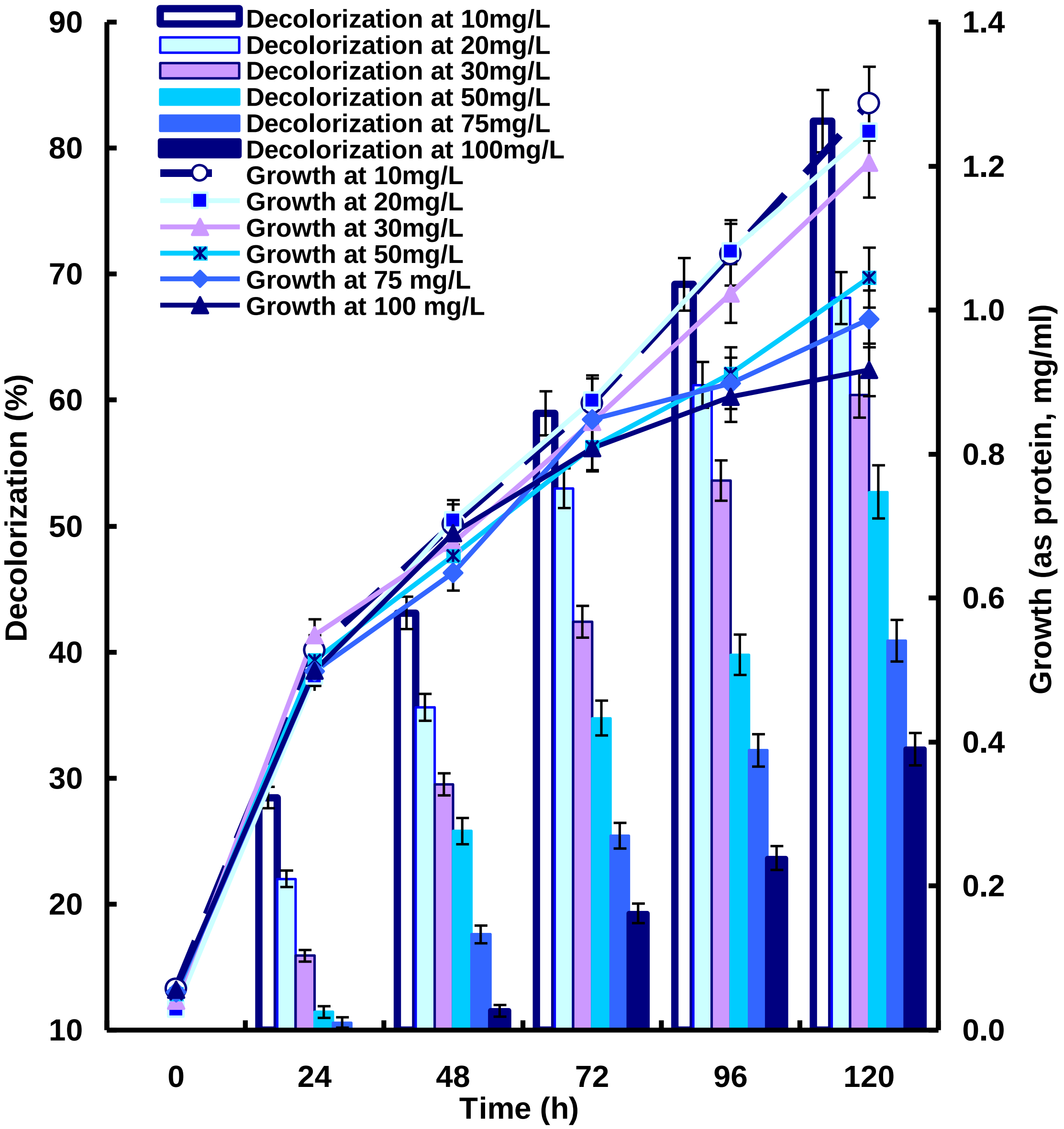


Fig.12. Decolorization (%) of azo dye Direct Blue 53 (10-100 mg/L) by mixed culture comprising of five bacterial isolates

Mixed culture was grown at 37°C on Bushnell Haas Broth (BHB) medium with starch (1.3 g/L), NaCl (4%) and varying dye concentration (10 to 100 mg/L) at pH 7. Decolorization and growth (as protein) were estimated at regular time intervals (as described in section 3.11.1.)

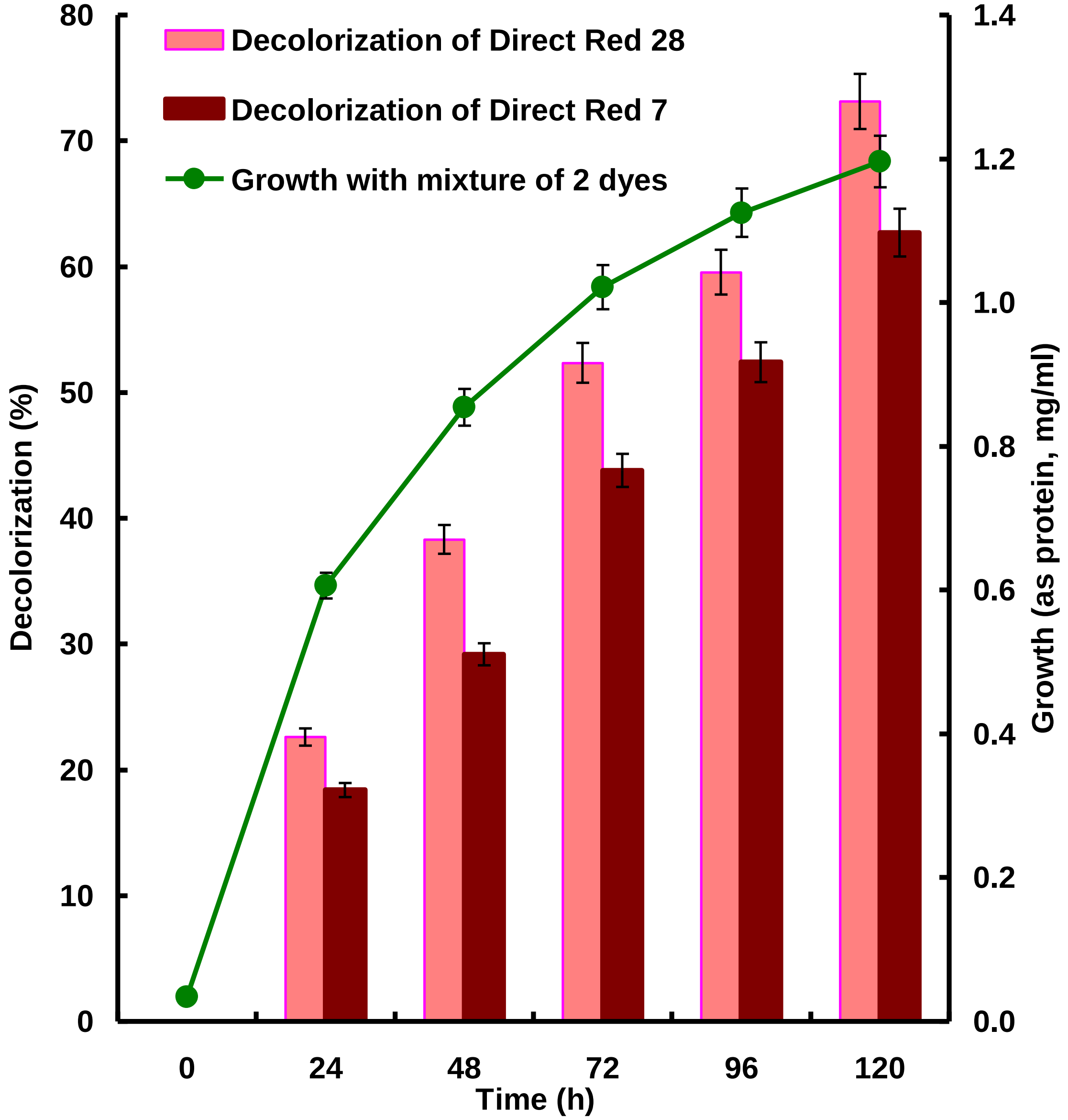


Fig.13. Decolorization (%) of mixture of Direct Red 28 and Direct Red 7 at 10 mg/L by mixed culture comprising of five bacterial isolates

Mixed culture was grown at 37°C on Bushnell Haas Broth (BHB) medium with starch (1.3 g/L), NaCl (4%) and mixture of two azo dyes in equal proportion at pH 7. Decolorization and growth (as protein) were estimated at regular time intervals (as described in section 3.11.1.)

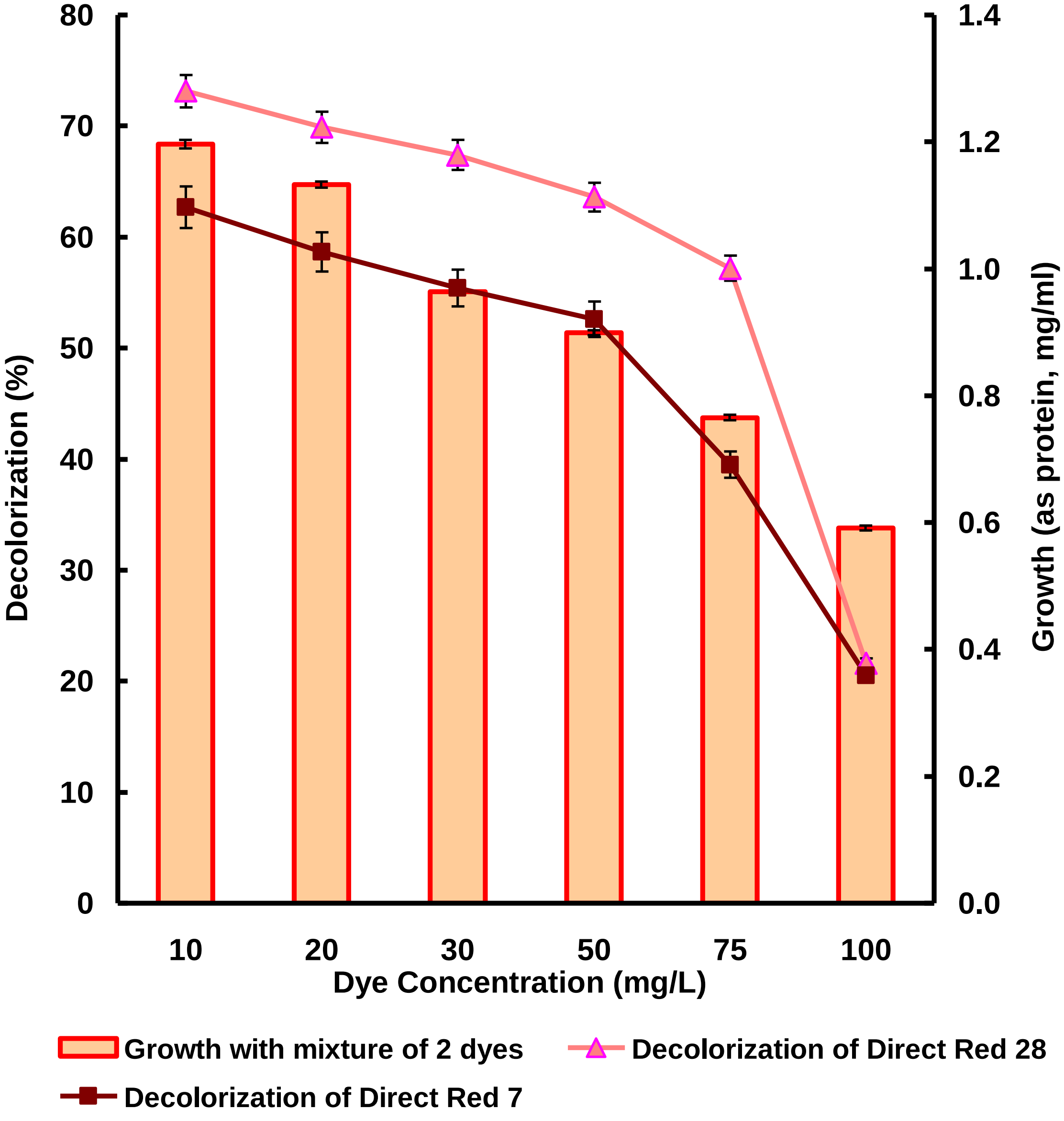


Fig.14. Decolorization (%) of mixture of Direct Red 28 and Direct Red 7 at 120 hrs by mixed culture comprising of five bacterial isolates

Mixed culture was grown at 37°C on Bushnell Haas Broth (BHB) medium with starch (1.3 g/L), NaCl (4%) and mixture of two azo dyes in equal proportion ranging from (10-100 mg/L) at pH 7. Decolorization and growth (as protein) were estimated at 120 hrs. (as described in section 3.11.1.)

Decolorization potential of the mixed culture in presence of 10 mg/L of mixture of two dyes Acid Blue 113 and Direct Blue 53 (equal concentration) was studied in a time course experiment at 37 °C and 120 rpm. Gradual increase in decolorization was observed with progression of time from 24 to 120 hrs. The decolorization of both the dyes was similar, increasing from 25% at 24 hrs to 53% after 120 hrs. Good growth (as protein) of 1.07 mg/ml was observed after 120 hrs (Fig.15). As concentration of the two dyes Acid Blue 113 and Direct Blue 53 (equal concentration) was increased from 10-100 mg/L decolorization decreased from 53 to 23 %. There was no difference in the decolorization between the two dyes. As was in case of decolorization, growth (as protein) of the mixed culture also gradually declined upto 50% (1.07 to 0.557 mg/ml) from 10-100 mg/L (Fig. 16.)

With a mixture of four dyes in equal proportion at 20 mg/L maximum decolorization was observed with Direct Red 28 and Direct Red 7 (6% of each) but only 2% with Acid Blue 113 and Direct Blue 53 after 24 hrs. However after 120 hrs Direct Red 28 was decolorized the maximum (45%) while not much difference in decolorization of rest three dyes (38, 36 and 32%) was observed. The mixture of four dyes did not have detrimental effect on the growth (as protein) of the mixed culture which was observed to be increasing from 0.486(24 hrs), to 0.637(48 hrs), to 0.876(72 hrs), to1.04 (96 hrs) and to 1.09 mg/ml at 120 hrs (Fig. 17).

As the concentration of the mixture of dyes was increased from 20 to 30 mg/L growth decreased by 25% after 120 hrs. Among the four dyes decolorization of Direct Red 28 by the mixed culture was affected the most; only 16 % decolorization at 30 mg/L instead of 45% at 20 mg/L was observed. Rest of the dyes, from 24-120 hrs showed gradual increase of 14% with Direct Red 7 and maximum 12 and 10% with Direct Blue 53 and Acid Blue 113 (Fig.18). At 100 mg/L the mixed culture showed least growth with 60% reduction as compared to growth observed at 30 mg/L after 120 hrs. Similarly, poor decolorization of 1.8-1.5% was observed with all four dyes. Overall, in this study least decolorization and growth were observed with 100 mg/L of mixture of dyes (Fig. 19.)

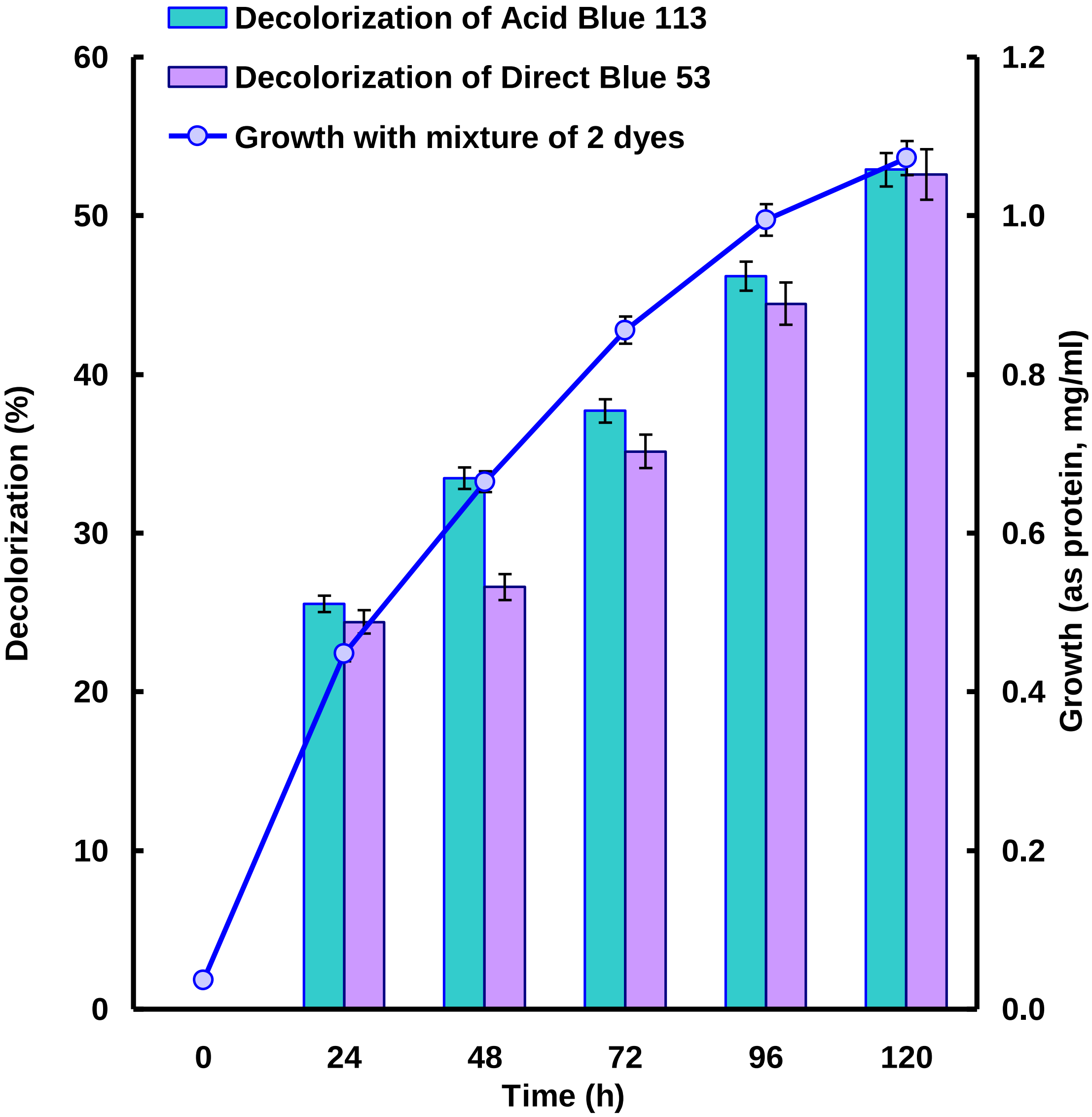


Fig.15. Decolorization (%) of mixture of Acid Blue 113 and Direct Blue 53 at 10 mg/L by mixed culture comprising of five bacterial isolates.

Mixed culture was grown at 37°C on Bushnell Haas Broth (BHB) medium with starch (1.3 g/L^{1}), NaCl (4%) and mixture of two azo dyes in equal proportion at pH 7. Decolorization and growth (as protein) were estimated at regular time intervals (as described in section 3.11.1.)

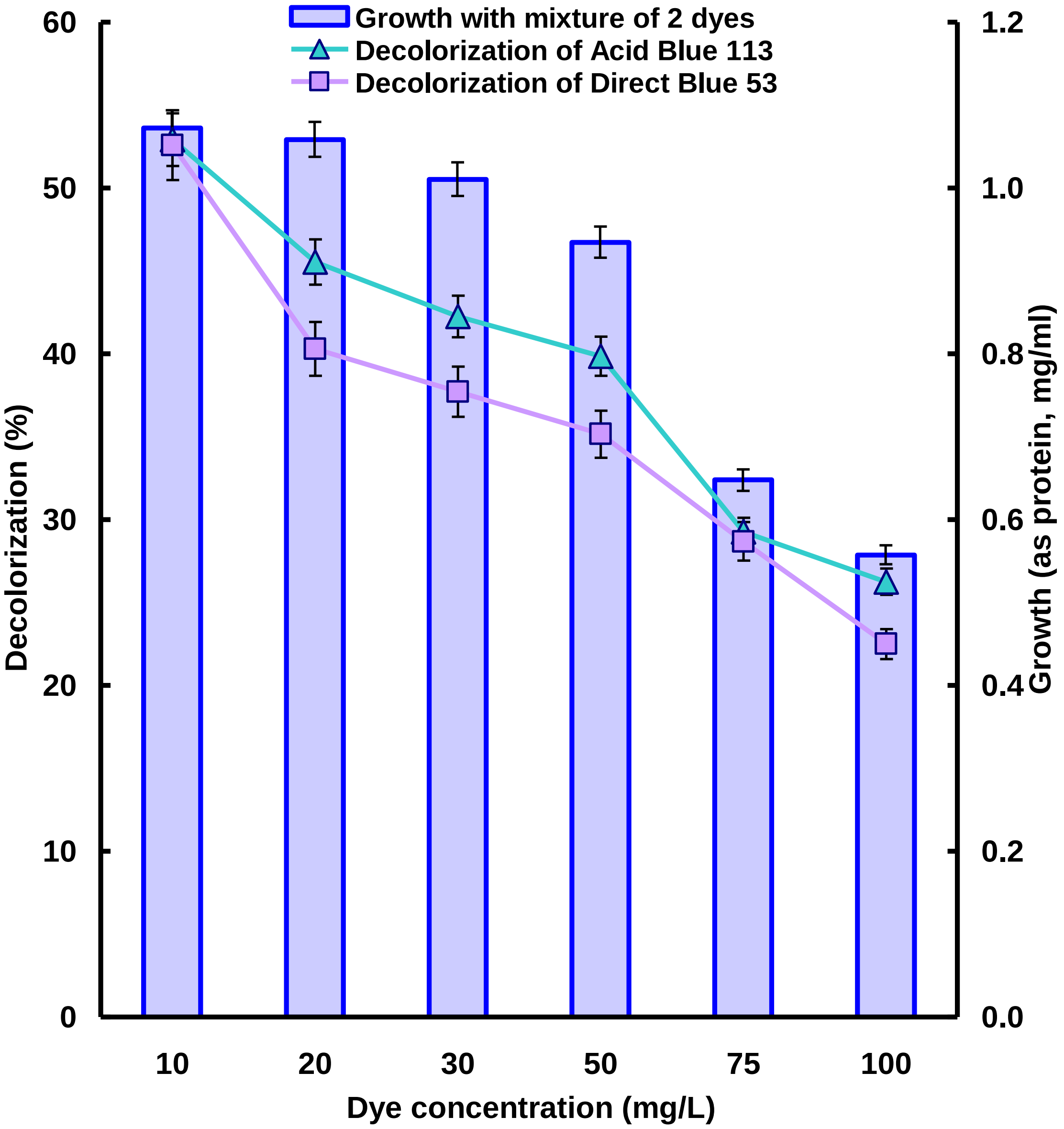


Fig.16. Decolorization (%) of mixture of Acid Blue 113 and Direct Blue 53 at 120 hrs. by mixed culture comprising of five bacterial isolates.

Mixed culture was grown at 37°C on Bushnell Haas Broth (BHB) medium with starch (1.3 g/L), NaCl (4%) and mixture of two dyes in equal proportion ranging from (10-100 mg/L) at pH 7. Decolorization and growth (as protein) were estimated at 120 hrs. (as described in section 3.11.1.)

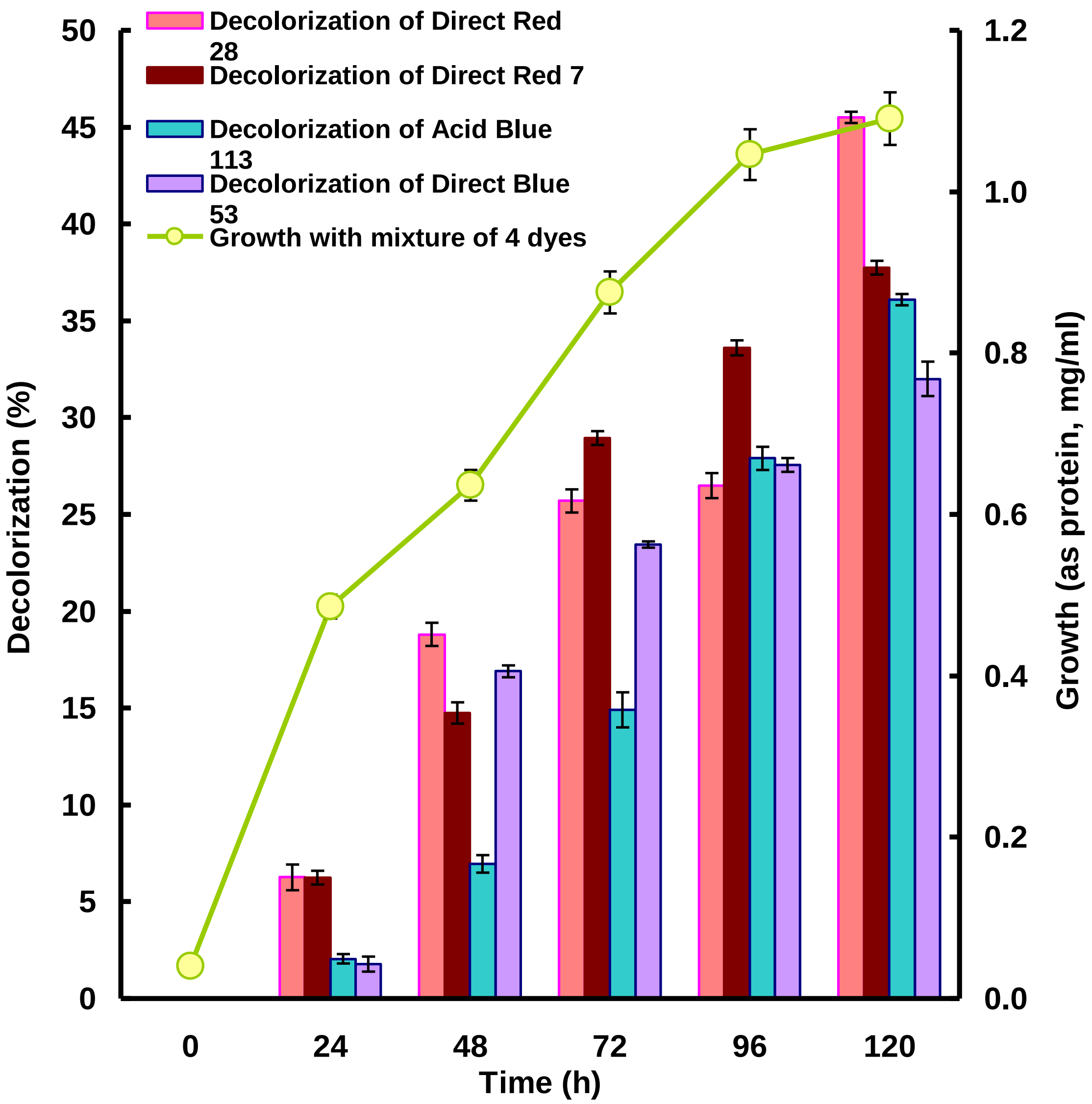


Fig.17. Decolorization (%) of mixture of four dyes: Direct Red 28, Direct Red 7, Acid Blue 113 & Direct Blue 53 (in equal proportion) at a final concentration of 20 mg/L by mixed culture comprising of five bacterial isolates.

Mixed culture was grown at 37°C on Bushnell Haas Broth (BHB) medium with starch (1.3 g/L), NaCl (4%) and mixture of four dyes at pH 7. Decolorization and growth (as protein) were estimated at regular time intervals (as described in section 3.11.1.)

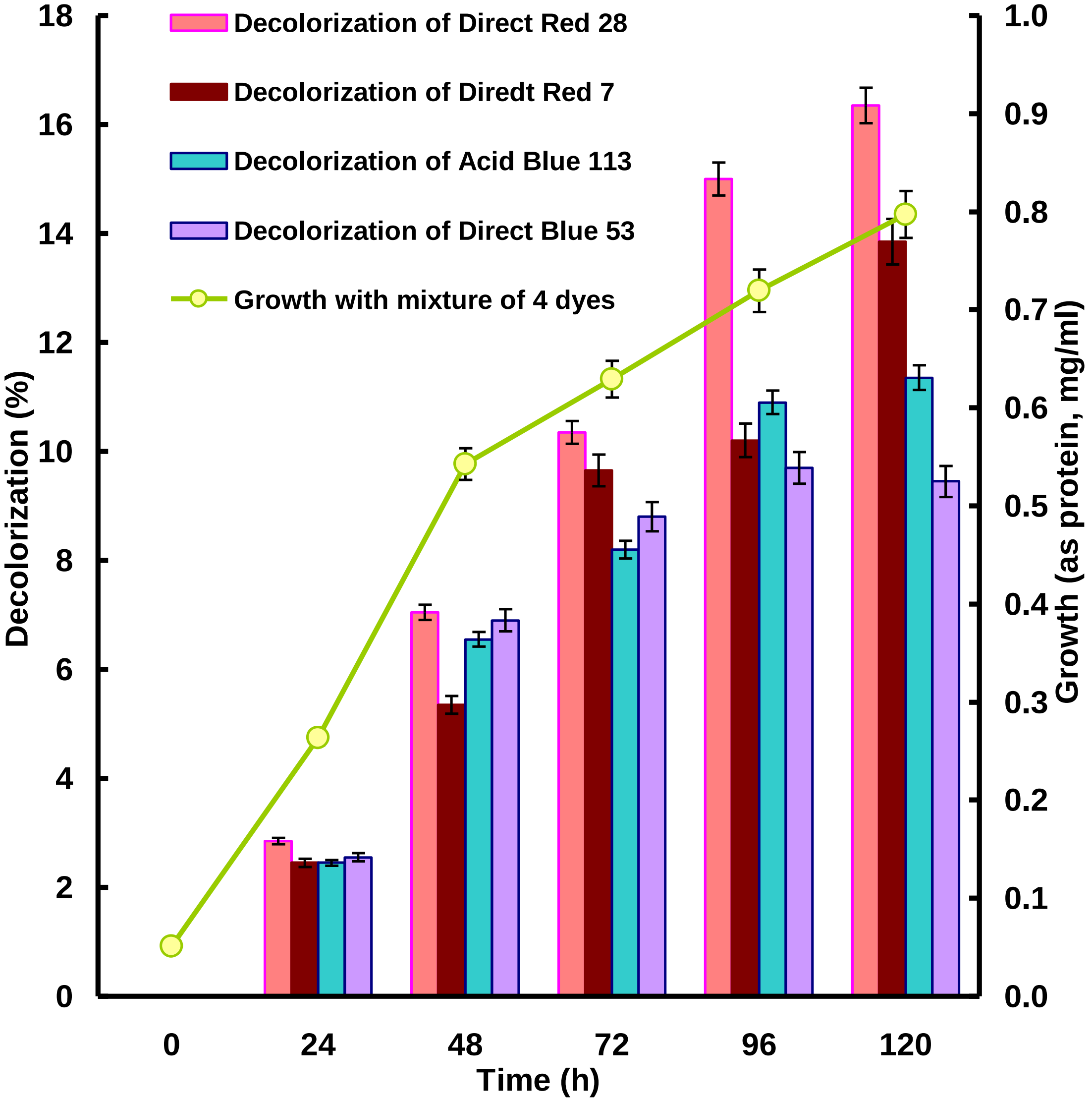


Fig.18. Decolorization (%) of mixture of four dyes: Direct Red 28, Direct Red 7, Acid Blue 113 & Direct Blue 53 (in equal proportion) at a final concentration of 30 mg/L by mixed culture comprising of five bacterial isolates.

Mixed culture was grown at 37°C on Bushnell Haas Broth (BHB) medium with starch (1.3 g/L), NaCl (4%) and mixture of four dyes at pH 7. Decolorization and growth (as protein) were estimated at regular time intervals (as described in section 3.11.1.)

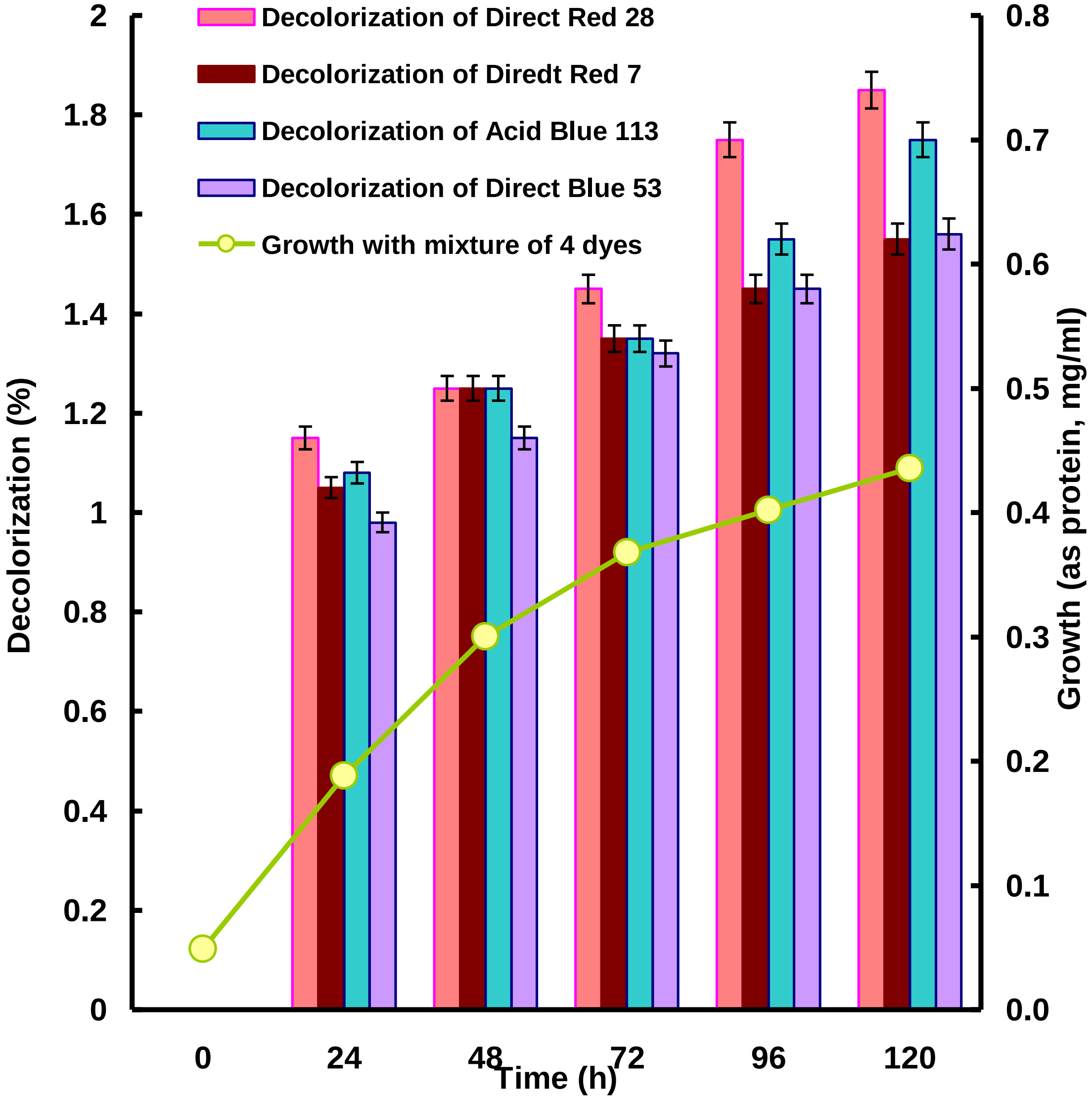


Fig.19. Decolorization (%) of mixture of four dyes: Direct Red 28, Direct Red 7, Acid Blue 113 & Direct Blue 53 in equal proportion at a final concentration of 100 mg/L by mixed culture comprising of five bacterial isolates

Mixed culture was grown at 37°C on Bushnell Haas Broth (BHB) medium with starch (1.3 g/L), NaCl (4%) and mixture of four dyes at pH 7. Decolorization and growth were estimated at regular time intervals (as described in section 3.11.1.)

Mixture of all four dyes at concentration ranging from 10 to 100 mg/L resulted in maximum decolorization of Direct Red 28, and Acid Blue113 followed by Direct Red 7 and Direct Blue 53. At 24 hrs (with 10 mg/L of four dyes each) 57 & 52% Direct Red 28 & Direct Red 7 and 48 & 46% of Direct Blue 53 and Acid Blue113 respectively were decolorized by the mixed culture after 120 hrs. At 100 mg/L decolorization was drastically reduced to a maximum of 2% only with all four dyes. From 10 to 20 mg/L gradual decrease in growth and decolorization was observed however increase in dye concentration from 20 to 30 mg/L resulted in sudden 60% decline of growth as protein from 1.09 to 0.798 mg/ml and decolorization of 16% Direct Red 28, 14% Direct Red 7, 11% Acid Blue113 and 9% Direct Blue 53. Thereafter from 30-100 mg/L there was gradual reduction of more than 50% in growth and decolorization (Fig. 20.)

4.6. Decolorization studies with raw untreated effluent procured from local dyeing houses and textile dyeing industries

The UV/visible spectrum of the raw untreated effluent (blue in color) showed maximum absorption peaks at 551, 665 and 480 nm. Shake flask experiments conducted at 37 °C and 120 rpm showed that as the concentration of the raw untreated effluent was increased from 1, 5 and 10%, growth of the mixed culture was 1.18 & 0.957 mg/ml protein at 1 & 5% raw untreated effluent respectively after 120 hrs. At 10% raw untreated effluent an inhibition of 72.5% in growth as compared to growth obtained at 1% was observed (Fig.21). With 1% raw untreated effluent, decolorization of the dyes (showing maxima at 551 nm) increased gradually from 26-44.4% from 24 to120 hrs. At a concentration of 5% raw untreated effluent, sudden increase in decolorization (4 to 25%) after 24 to 48 hrs was observed which further increased marginally to 31% at 120 hrs. Drastically lower decolorization (0.1 to 3.2%) from 24 to120 hrs was achieved with 10% concentration of raw untreated effluent.

The mixed culture with 1% raw untreated effluent (showing absorption maxima of 665 nm) showed gradual increase in decolorization from 33 to 56% from 24 hrs to 120 hrs. Similar decolorization trends were observed at 665 nm as was observed with 551 nm, wherein at 5% raw untreated effluent concentration sudden increase in decolorization from 6% to 26% was observed from 24 to 48 hrs and thereafter marginal increase to 47% at 120 hrs was achieved. Decolorization of 1.4 to 7% was obtained with 10% raw untreated effluent concentration as the time was increased from 24 to 120 hrs (Fig.21).

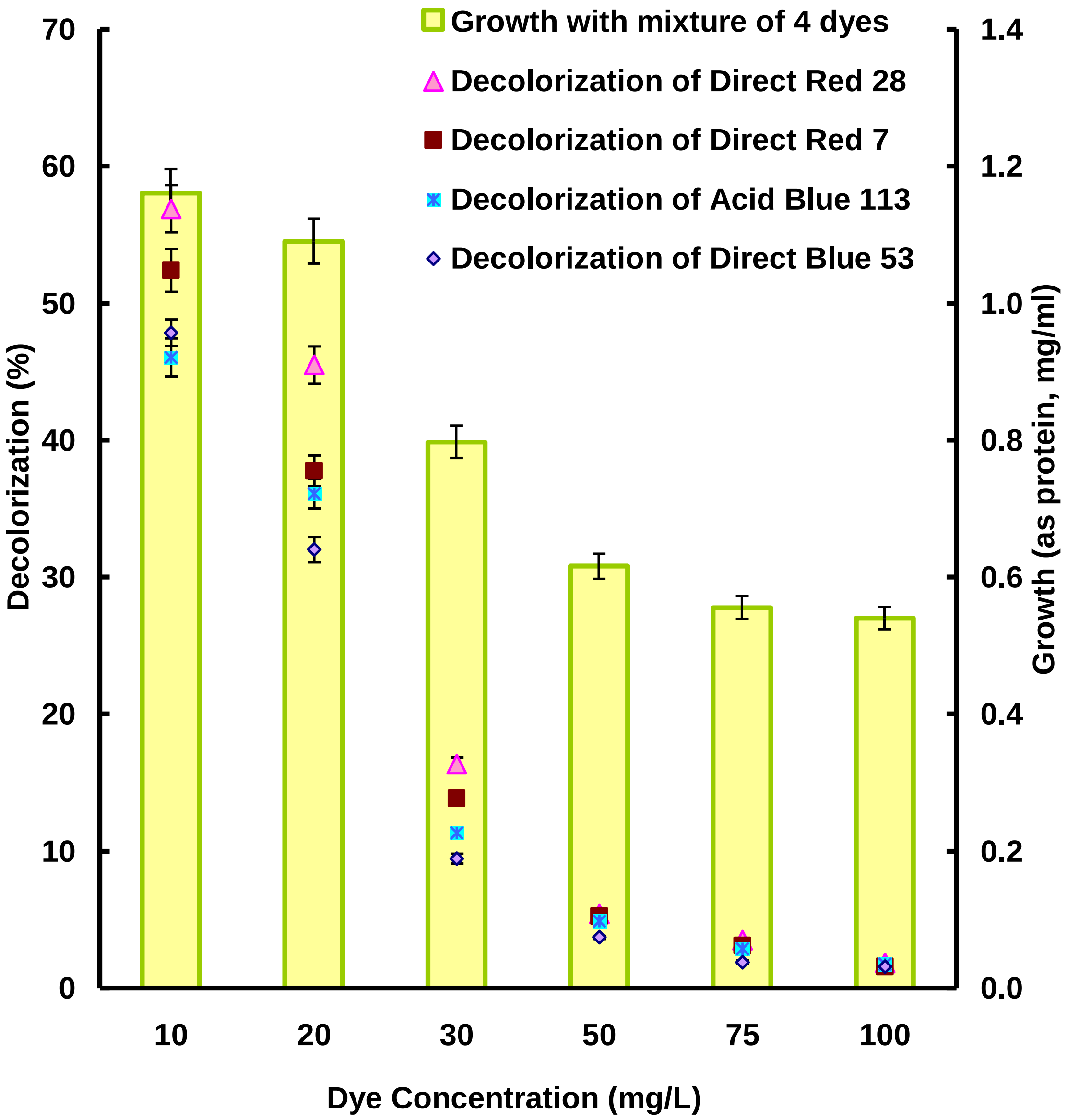


Fig.20. Decolorization (%) of mixture of four dyes: Direct Red 28, Direct Red 7, Acid Blue 113 & Direct Blue 53 (ranging from concentration 10-100 mg/L) by mixed culture comprising of five bacterial isolates.

Mixed culture was grown at 37°C on Bushnell Haas Broth (BHB) medium with starch (1.3 g/L), NaCl (4%) and mixture of four dyes at pH 7. Decolorization and growth (as protein) were estimated at 120 hrs. (as described in section 3.11.1.)

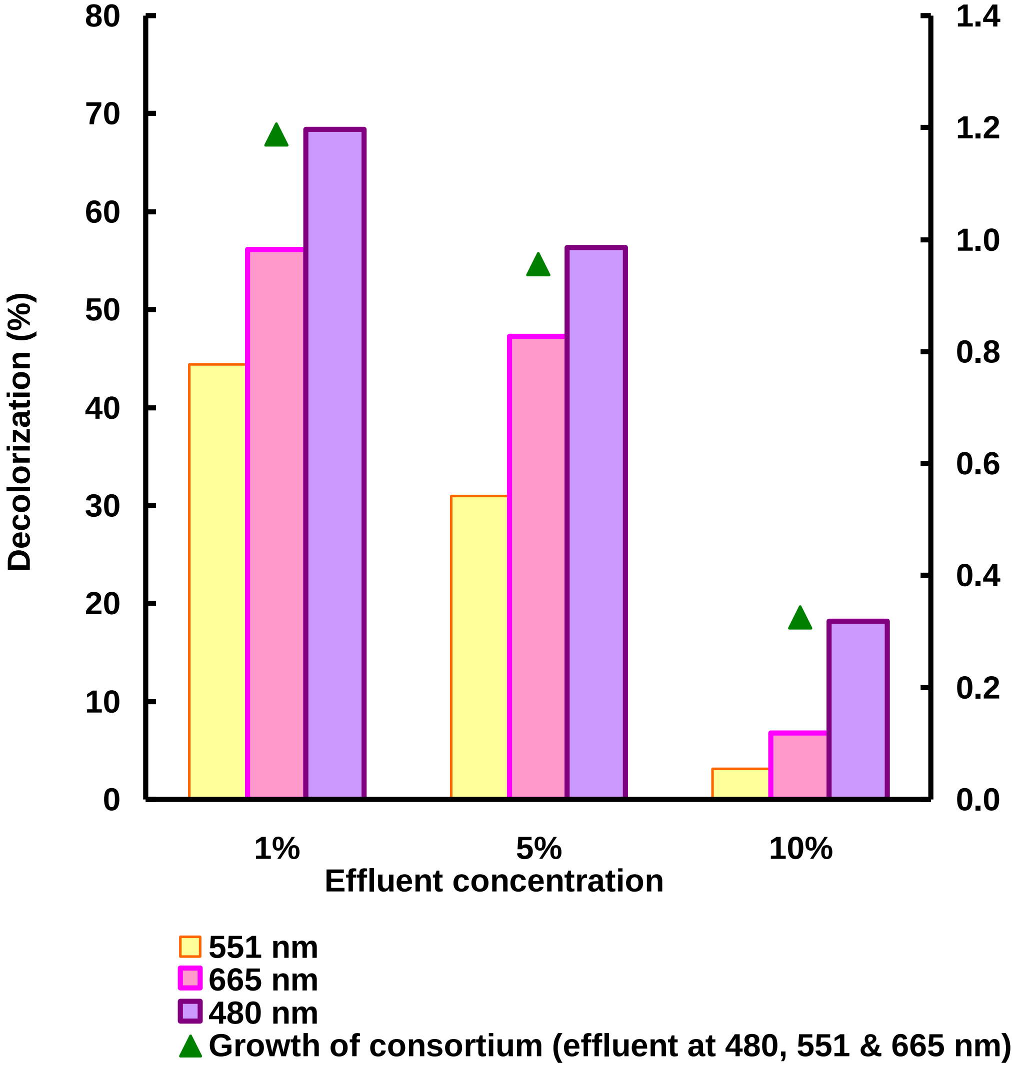


Fig.21. Decolorization (%) of raw untreated effluent at three wavelengths by mixed culture comprising of five bacterial isolates.

Mixed culture was grown at 37ºC in sterile Bushnell Haas Broth (BHB) medium with starch (1.3 g/L), NaCl (4%) and varying concentration of effluent sample (1to10%). Decolorization and growth (as protein) were estimated at 120 hrs. (as described in section 3.11.1.)

With 1% raw untreated effluent (absorption maxima of 480 nm) the mixed culture was able to decolorize 34 to 68.4% effluent from 24 to 120 hrs. With 5% concentration as mentioned in above two cases (5% raw untreated effluent with absorption maxima of 551 & 665 nm) sudden increase in decolorization from 20 to 47% after 24 to 48 hrs followed by 56% after 120 hrs was observed. At higher concentration (10%) of raw untreated effluent maximum decolorization of 9-18% was observed from 24 to 120 hrs (Fig.21).

Comparative study of three concentrations of raw untreated effluent (at absorbance maxima: 551, 665 and 480 nm) showed growth of the mixed culture (protein=1.18 & 0.955 mg/ml) at 1& 5% raw untreated effluent. Maximum inhibition of growth of mixed culture (protein=0.323 mg/ml) was observed with 10% raw untreated effluent. Maximum decolorization of 68.4% (at 480 nm) followed by 56 and 44% (at 665 & 551 nm) at 1% raw effluent concentration by the mixed culture was observed after 120 hrs. Increasing the concentration to 5% raw untreated effluent resulted in lower decolorization of 56, 47 & 31% respectively followed by even lower decolorization of 18, 7 & 3% respectively at 480, 665 & 551 nm respectively at 10% raw untreated effluent (Fig.21). It is noteworthy to mention here that inspite of the number of trials conducted, maximum decolorization of 11 to 27% was observed with real raw untreated effluents procured from textile industries. Characterization of the effluents showing maximum decolorization procured from local dyeing house and textile industries located in varies localities/areas is mentioned in Table 5.

Table.5. Wastewater characterization of real raw untreated effluent procured from local dyeing house and textile industries located in various localities/areas (as described in section 3.11.1.)

	pH	TSS	COD	BOD
Effluent from local dyeing house	9.56	2293	3600	1860
Effluent from textile industry	13	545	4937	1128

4.7 BIOREACTOR STUDIES

4.7.1 Biofilm development on different immobilization supports

The experimental set up for the bioreactor studies is shown in Fig. 22. Development of biofilm by mixed culture comprising of five bacterial isolates *viz: Bacillus pumilus, Bacillus cereus, Bacillus subtilis, Bacillus vallismortis* and *Bacillus megaterium* on different support materials is evident from SEM studies (Fig. 23-24). The brief description of the SEM studies carried out on different supports is given below:

The SEM of the control plastic shows smooth surface area (Fig. 23) while SEM of the support particle after biofilm formation in the bioreactor shows mainly rod shaped bacterial cells attached on the surface of the support material. However some un-colonized surface area on the support particle is also visible. As compared to plastic clips the SEM of the control marble chips shows highly rough surface with cavities and crevices (Fig.24) and SEM of the support particle after biofilm formation in the bioreactor show clusters of bacterial cells embedded in cavities and crevices of the marble chips however some un-colonized patches on the support particle were also visible.

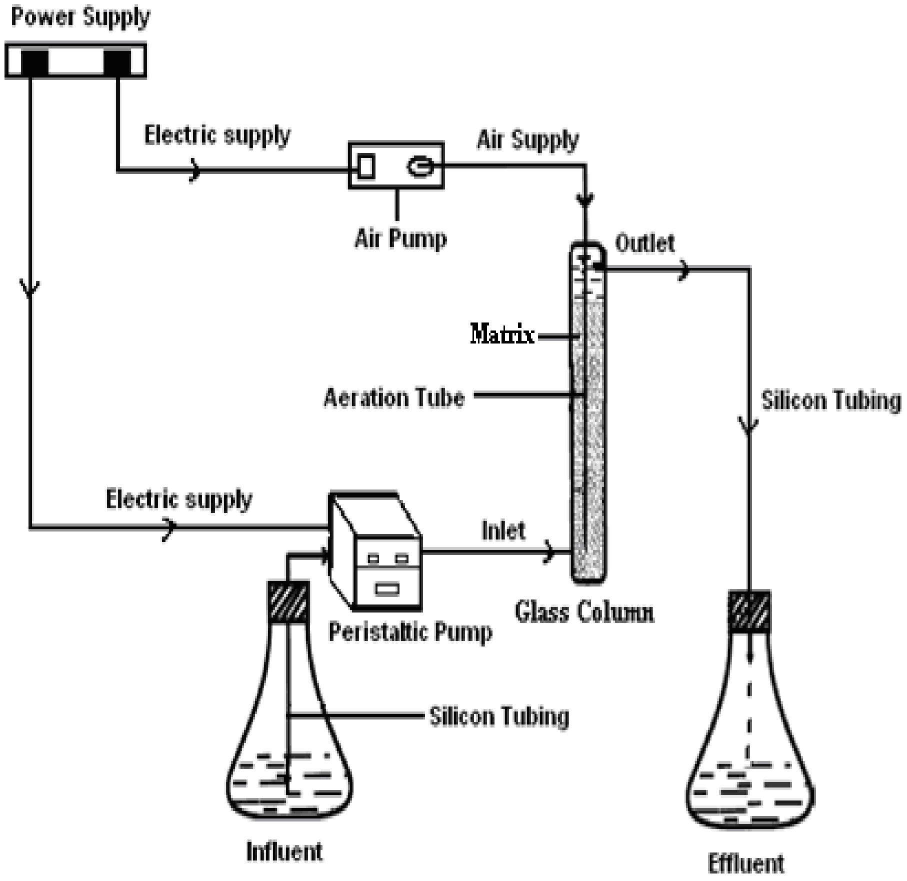


Fig.22(a).Graphical Representation of aerobic immobilized up-flow packed bed bioreactor

Mixed culture comprising of five bacterial isolates was used for biofilm formation to decolorize Direct Red 28 dye (10 mg/L) in BHB medium at 37°C with starch (1.3 g/L) and NaCl (4%) at pH 7 in packed bed reactor. Decolorization and growth (as protein) were estimated at regular time intervals (as described in section 3.11.1.)

Fig. 22(b). Experimental set up for the bioreactor studies with plastic clips as matrix

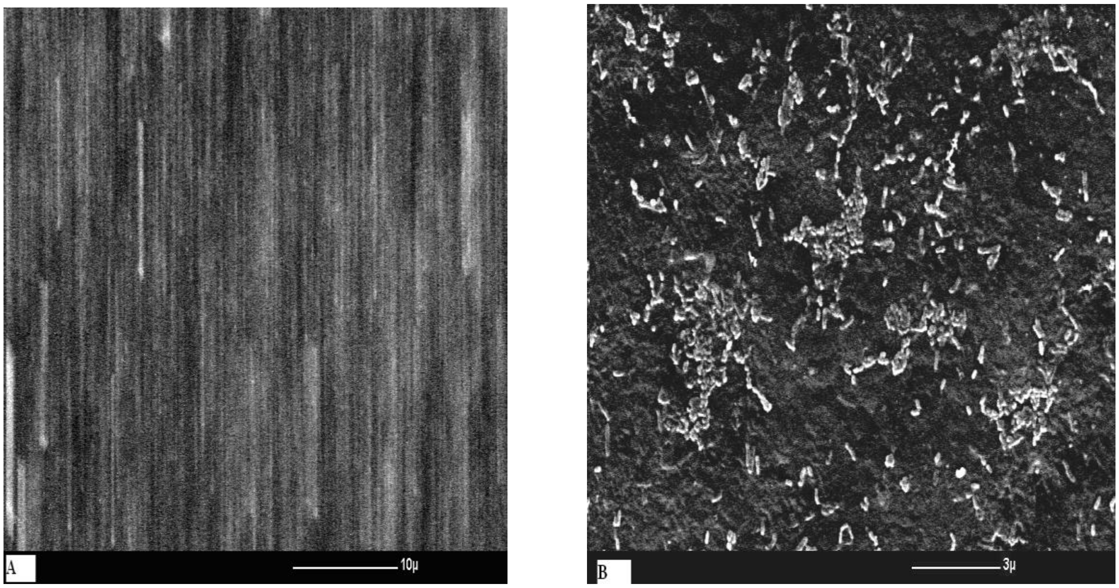


Fig.23. Scanning Electron Microscopic studies for biofilm formation on plastic-clips

Plastic clips were collected from the bioreactor after biofilm formation (A: Sterile un-inoculated support particle showing different surface zones (x1,500); B: Support particle completely covered with a layer of rod-shaped bacterial cells (x 4000) formed by mixed culture comprising of comprising of five bacterial isolates (*viz: Bacillus vallismortis, Bacillus megaterium, Bacillus cereus, Bacillus subtilis* and *Bacillus pumilus.)* and were fixed with 4% (v/v) glutaraldehyde in 0.1 M phosphate buffer (as described in section 3.13.)

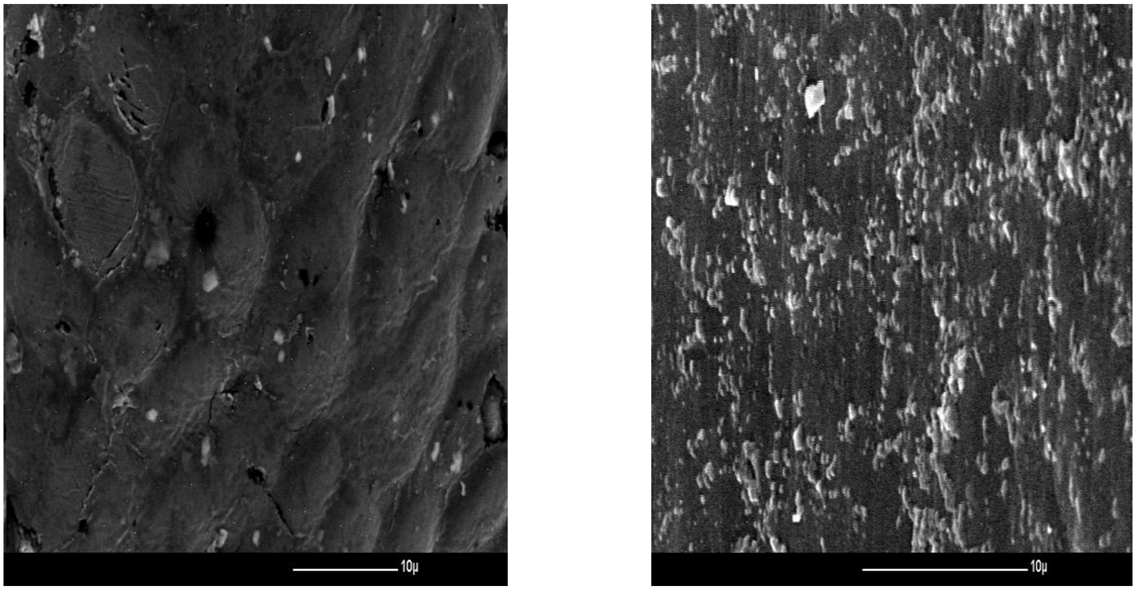


Fig.24. Scanning Electron Microscopic studies for biofilm formation on marble chips

Marble chips were collected from the bioreactor after biofilm formation (A: Sterile un-inoculated support particle showing different surface zones (x1,500); B: Support particle completely covered with a layer of rod-shaped bacterial cells (x 4000) formed by mixed culture comprising of comprising of five bacterial isolates (*viz: Bacillus vallismortis, Bacillus megaterium, Bacillus cereus, Bacillus subtilis* and *Bacillus pumilus.)* and were fixed with 4% (v/v) glutaraldehyde in 0.1 M phosphate buffer (as described in section 3.13.)

4.7.2. Optimization of parameters of the up-flow immobilized packed bed bioreactor

The effect different hydraulic retention time (11, 7.33 and 5.5 hrs for plastic clips and 8.16, 5.4, 4.08 hrs for marble chips) on the input stream (containing Direct Red 28 dye; 10 mg/L in BHB medium) with two aeration rates (0.4 & 0.6 mmoles/min.) using two different immobilization support on decolorization efficiency and biofilm stability of mixed culture in the bioreactor was studied. At different time intervals the sample from the outlet of the bioreactor were collected for growth of the mixed culture and decolorization of the dyes containing water.

With plastic clips as immobilization support, a stable biofilm formation by mixed culture comprising of five bacterial isolates (*Bacillus vallismortis, Bacillus megaterium, Bacillus cereus, Bacillus subtilis* and *Bacillus pumilus*) was observed after 57 hours at aeration rate of 0.4 mmoles/min. and hydraulic retention time of 11 hrs (Fig. 25a). Sudden decline in growth (as protein) of the mixed culture (0.980 to 0.279 mg/ml) was observed from 74-134 hrs when dye in BHB was introduced into the bioreactor after biofilm formation. The protein concentration increased to 0.414 mg/ml at 146 hrs and remained at a protein concentration of 0.546 to 0.732 mg/ml till the end of the experiment 291 hrs. After 74 hrs of the experiment i.e.17 hrs after the introduction of the dye containing BHB medium through the bioreactor, 68.4% decolorization was observed which remained constant till 134 hrs. Slight increase in decolorization to 74% was achieved between 134 and 146 hrs and after that decolorization varied from 66% to 84% from 160 to 291 hrs (Fig. 25b).

At hydraulic retention time of 7.3 hrs and aeration rate of 0.4 mmoles/min., with plastic clips as support particle, stable biofilm was formed after 65 hours by mixed culture comprising of five bacterial isolates (Fig.26a). Introduction of the Direct Red 28 dye in BHB medium into the bioreactor significantly reduced the growth (as protein) from 0.987 to 0.378 mg/ml (89 to 139 hrs) and thereafter, increase in growth (protein=0.531 to 0.761 mg/ml was noticed at 164 hrs which remained constant till the end of the experiment i.e. 282 hrs. At 89th hr i.e. after 24 hrs of treating the dye in the bioreactor 66% decolorization was obtained. Further with progression of time, random increase and decrease in percent decolorization was observed from 98 hrs to the end of the experiment (296 hrs) with a maximum decolorization of 82% (Fig.26b)

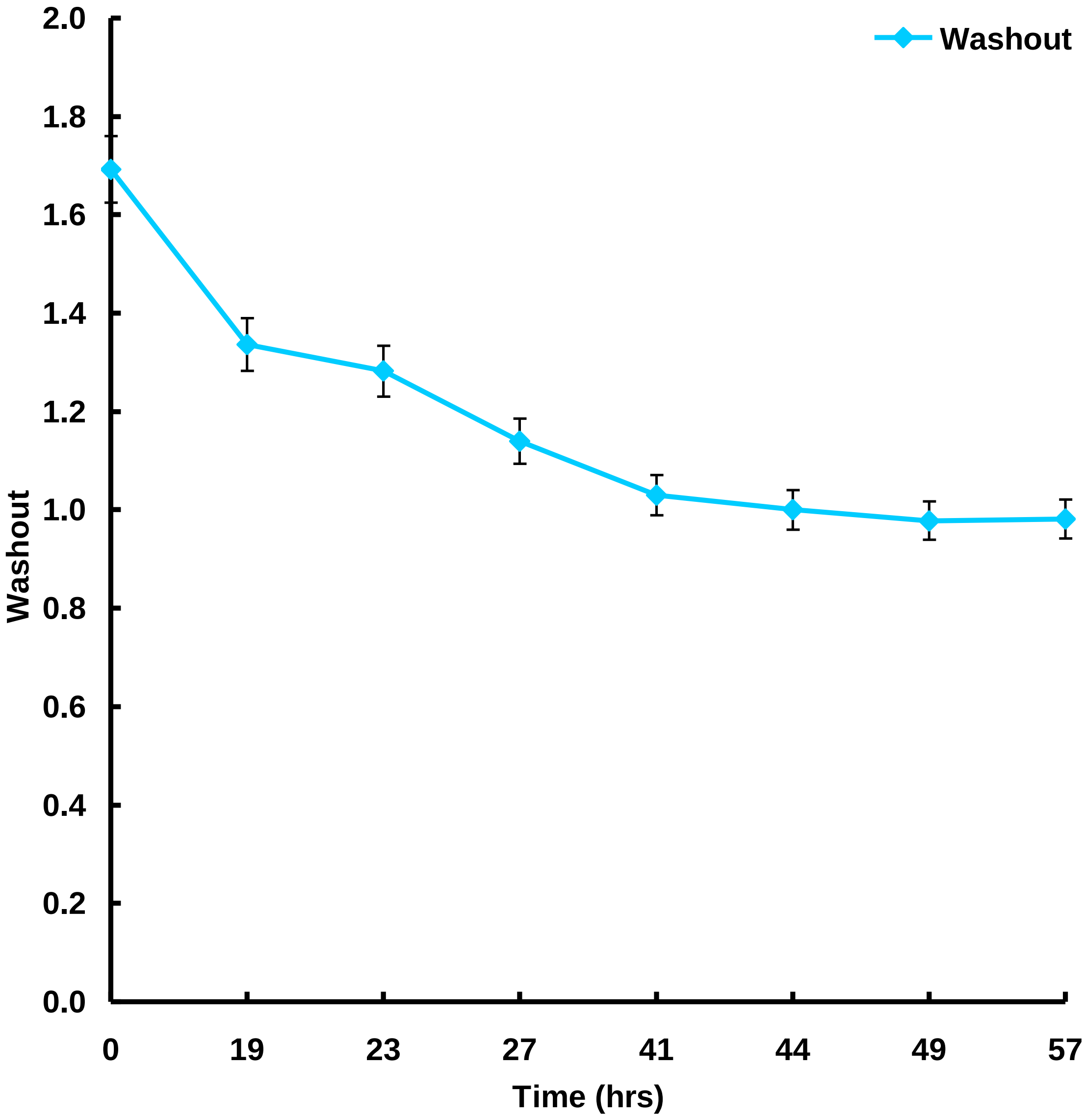


Fig.25a. Stability (washout at 550 nm) of the immobilized mixed culture on plastic clips in a packed bed bioreactor at an aeration rate of 0.4 mmoles/min. and hydraulic retention time (HRT) of 11hrs.

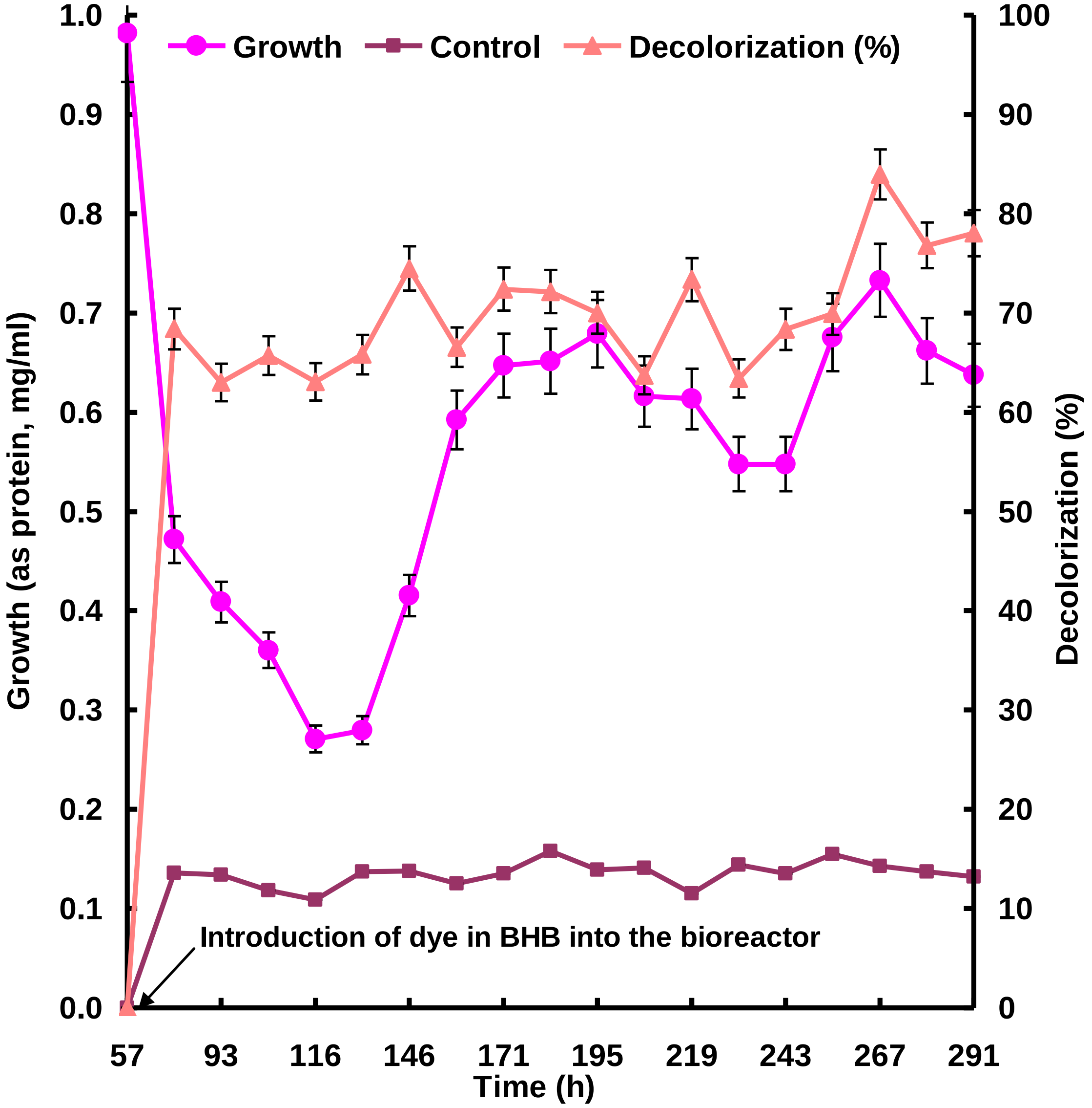


Fig.25b. Decolorization (%) of Direct Red 28 (10 mg/L) in a packed bed bioreactor with and without (control) mixed culture and growth (as protein) of mixed culture on plastic clips at aeration rate of 0.4 mmoles/min. and hydraulic retention time of 11 hrs.

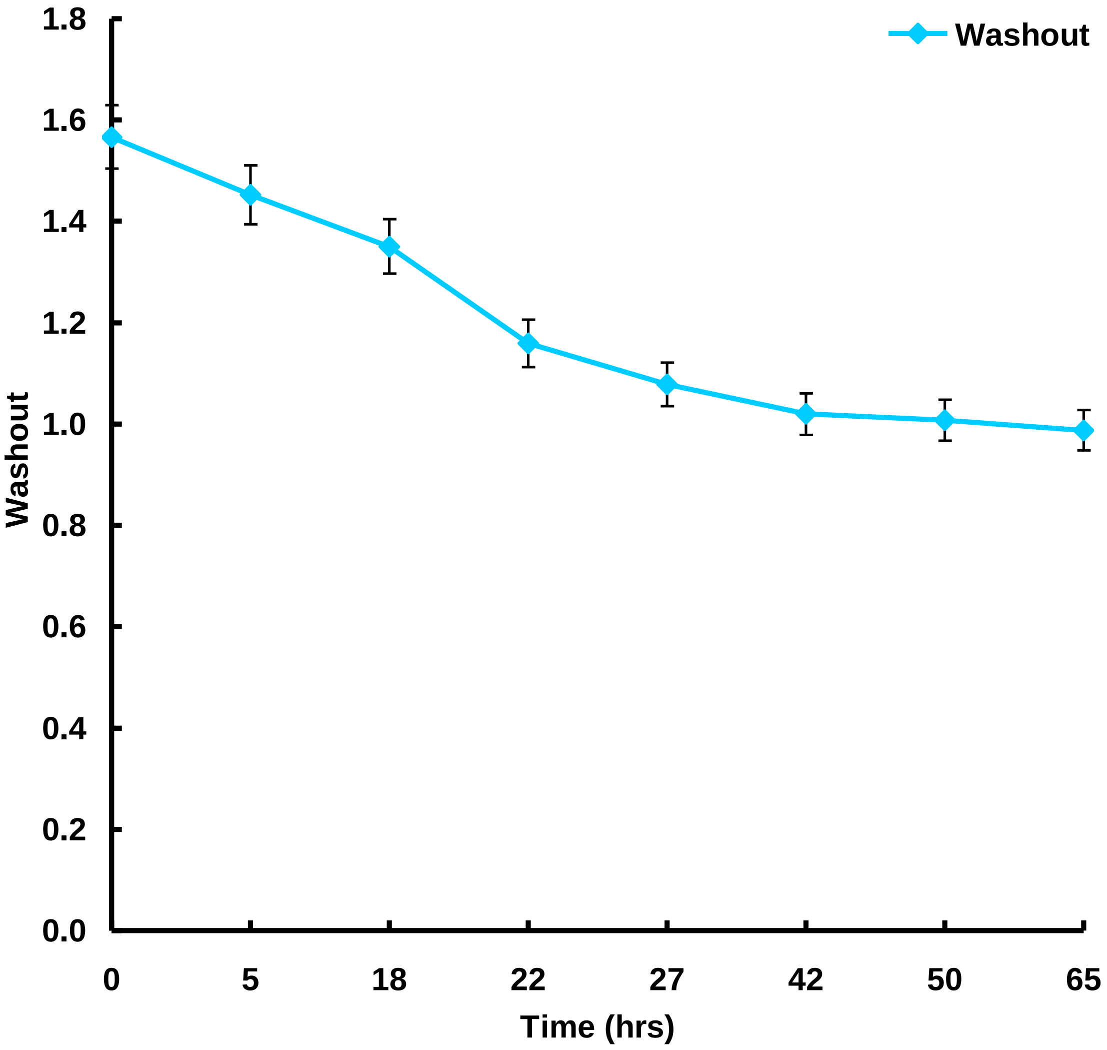


Fig.26a. **Stability (washout at 550 nm) of the immobilized mixed culture on plastic clips in a packed bed bioreactor at an aeration rate of 0.4 mmoles/min. and hydraulic retention time (HRT) of 7.3 hrs.**

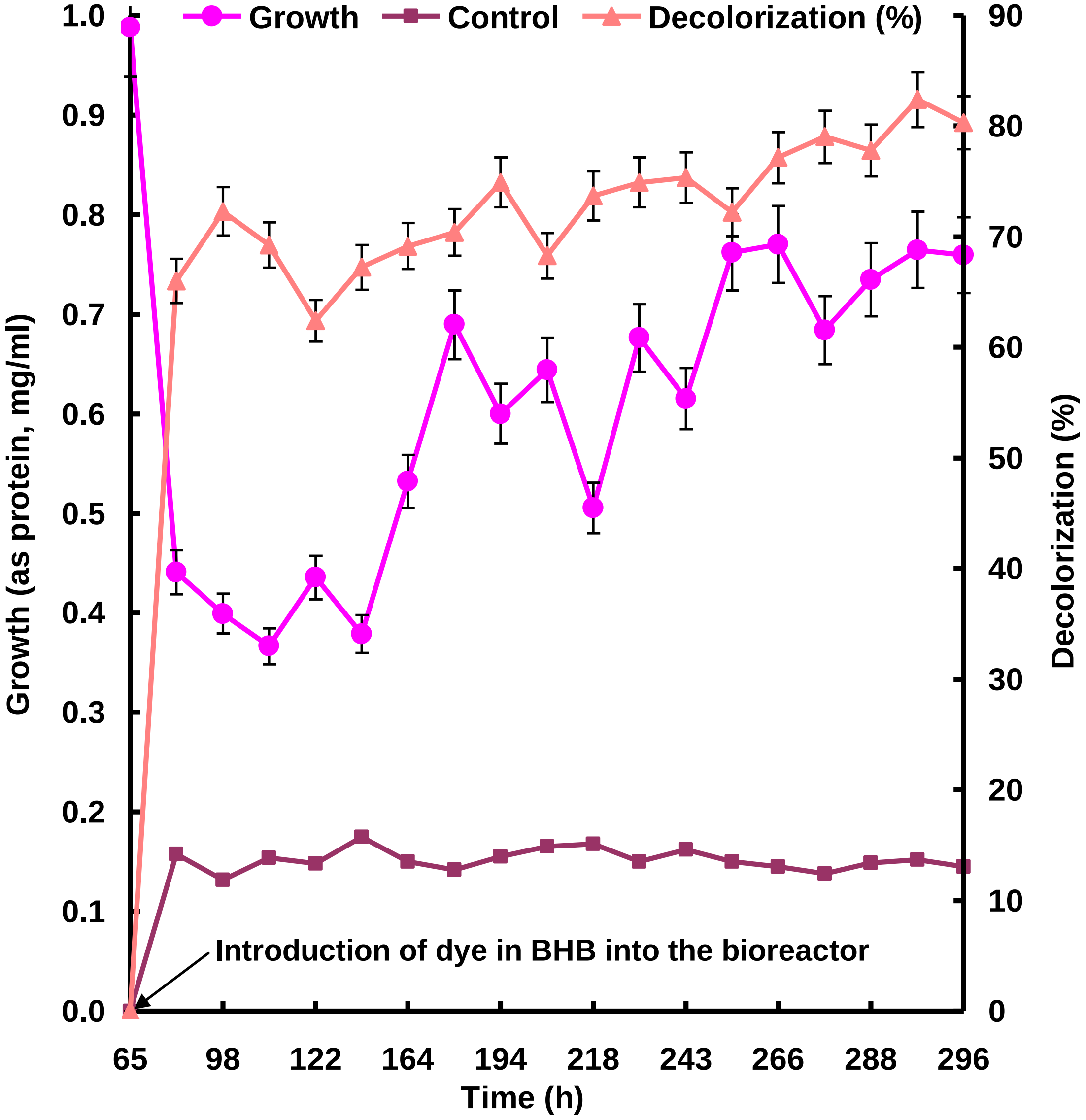


Fig.26b. Decolorization (%) of Direct Red 28 (10 mg/L) in a packed bed bioreactor with and without (control) mixed culture and growth (as protein) of mixed culture on plastic clips at aeration rate of 0.4 mmoles/min. and hydraulic retention time of 7.3 hrs.

The mixed culture comprising of five bacterial isolates, at hydraulic retention time of 5.5 hrs and aeration of 0.4 mmoles/min., required maximum and longest time of 73 hrs for stable biofilm formation as compared to the time taken at above mentioned two parameters (aeration 0.4 mmoles/min. and HRT of 11 and 7.3 hrs.) (Fig.27a). Upon introduction of the dye (in BHB) into the bioreactor, the growth (as protein) of the mixed culture dropped from 0.702 mg/ml at 73 hours to 0.366 mg/ml at 75 hrs and remained in the range of 0.690 to 0.424 mg/ml till 131 hrs. A slight increase in growth (as protein) 0.584 mg/ml was observed after 147 hrs which again dropped to 0.484 mg/ml at 171 hrs and then increased to a maximum of 0.631 mg/ml till the end of the study at 236 hrs. Minimum decolorization of 66% was observed at 75 hrs after which decolorization varied from 64% (at 82 hrs) to a maximum of 76% at 180 hrs and thereafter the decolorization trend remained the same, without much variation till 236 hrs (Fig.27b).

At hydraulic retention time of 11 hrs., and increasing the aeration rate to 0.6 mmoles/min from 0.4 mmoles/min using plastic clips as support particle, biofilm formation took place after 57 hours with protein concentration of 1.09mg/ml by the mixed culture comprising of five bacterial isolates (Fig.28a). Addition of the dye (in BHB) reduced the protein to 0.557mg/ml within 17 hrs of introducing the dye through the bioreactor after which a slight increase in growth (as protein) 0.617 mg/ml at 111 hrs was observed. Thereafter the growth (as protein) remained in the range of 0.531 to 0.708 mg/ml till the end of the experiment. Starting from 73.6% at 74 hrs, decolorization of Direct Red 28 varied within a range of minimum 62% to maximum of 92% till 291 hours (Fig.28b).

With hydraulic retention time of 7.3 hrs, and aeration rate of 0.6 mmoles/min., longer time period of 78 hrs. was required for stable biofilm formation on the plastic clips by the mixed culture comprising of five bacterial isolates after which dye (in BHB) was introduced into the bioreactor (Fig.29a). Growth (as protein) of the mixed culture drastically dropped by 65% i.e. from 1.32 at 78 hrs to 0.460mg/ml within 10 hrs of introducing the dye (in BHB) into the bioreactor which further got reduced to 0.346mg/ml at 98 hrs. Growth (protein=0.401 to 0.782 mg/ml) was observed to increase again between 113 to 282 hrs. Decolorization ranged between 63% at 88 hrs to 78% at 296 hrs (Fig.29b).

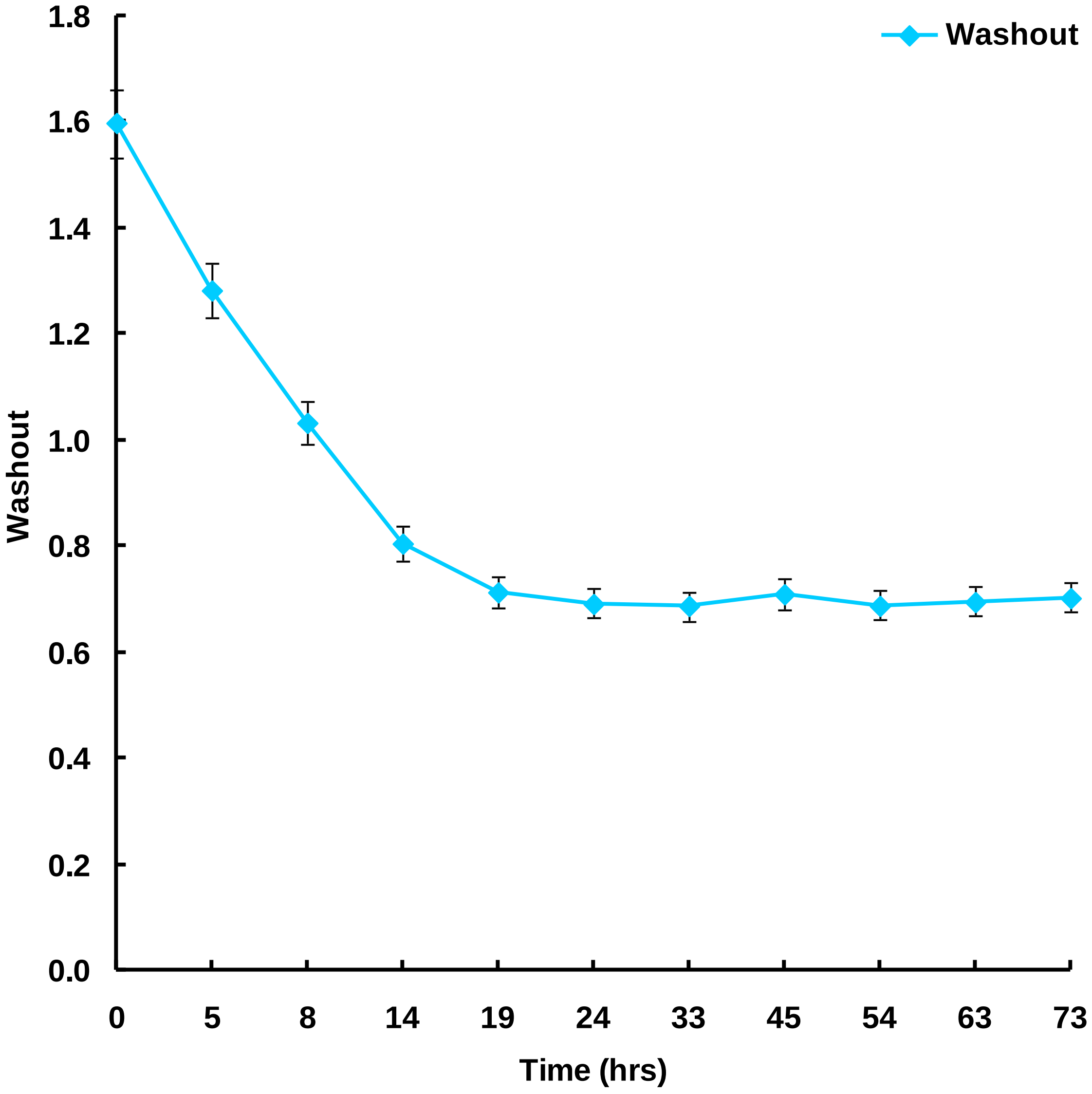


Fig.27a. Stability (washout at 550 nm) of the immobilized mixed culture on plastic clips in a packed bed bioreactor at an aeration rate of 0.4 mmoles/min. and hydraulic retention time (HRT) of 5.5 hrs.

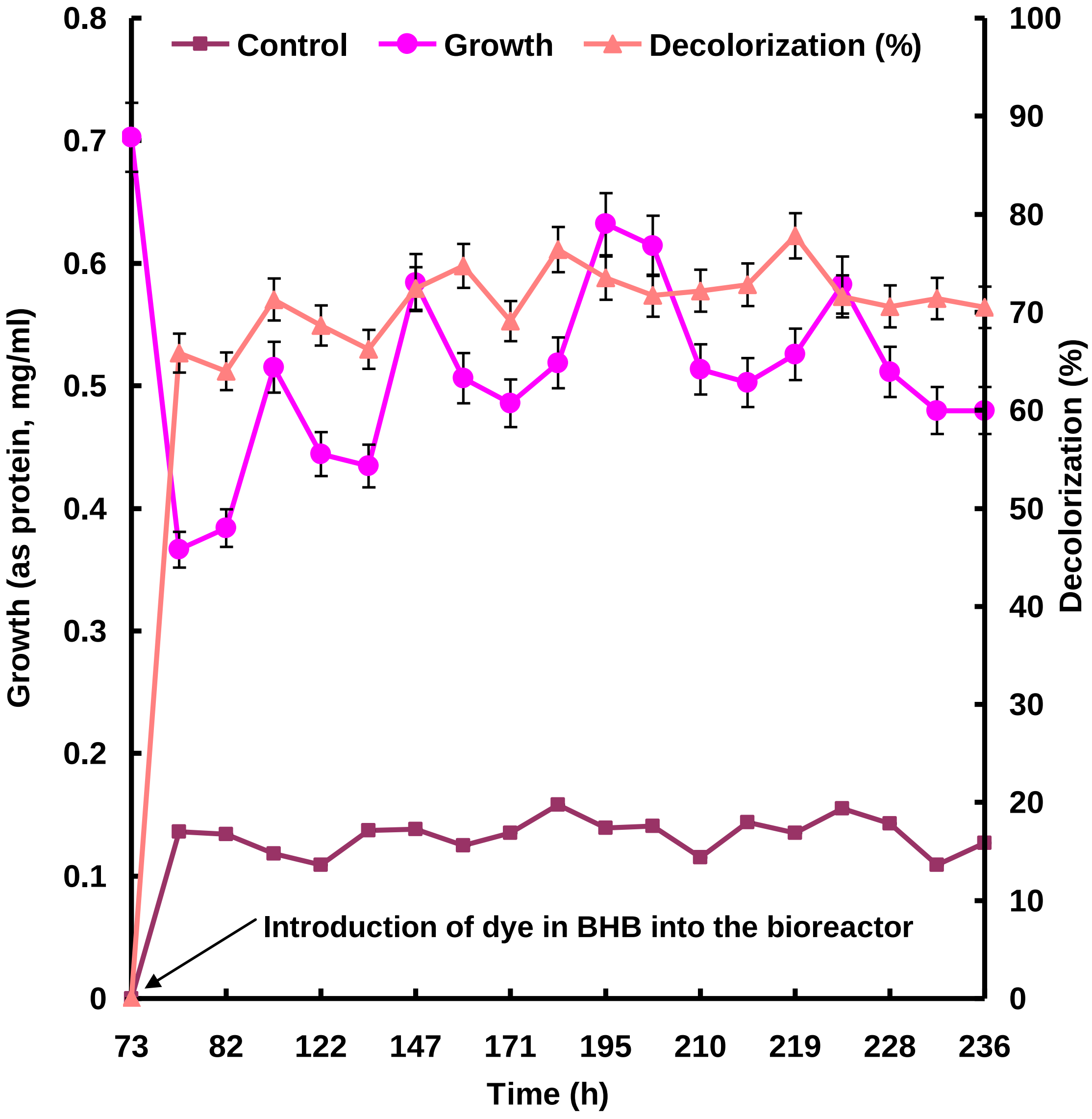


Fig.27b. Decolorization (%) of Direct Red 28 (10 mg/L) in a packed bed bioreactor with and without (control) mixed culture and growth (as protein) of mixed culture on plastic clips at aeration rate of 0.4 mmoles/min. and hydraulic retention time of 5.5 hrs.

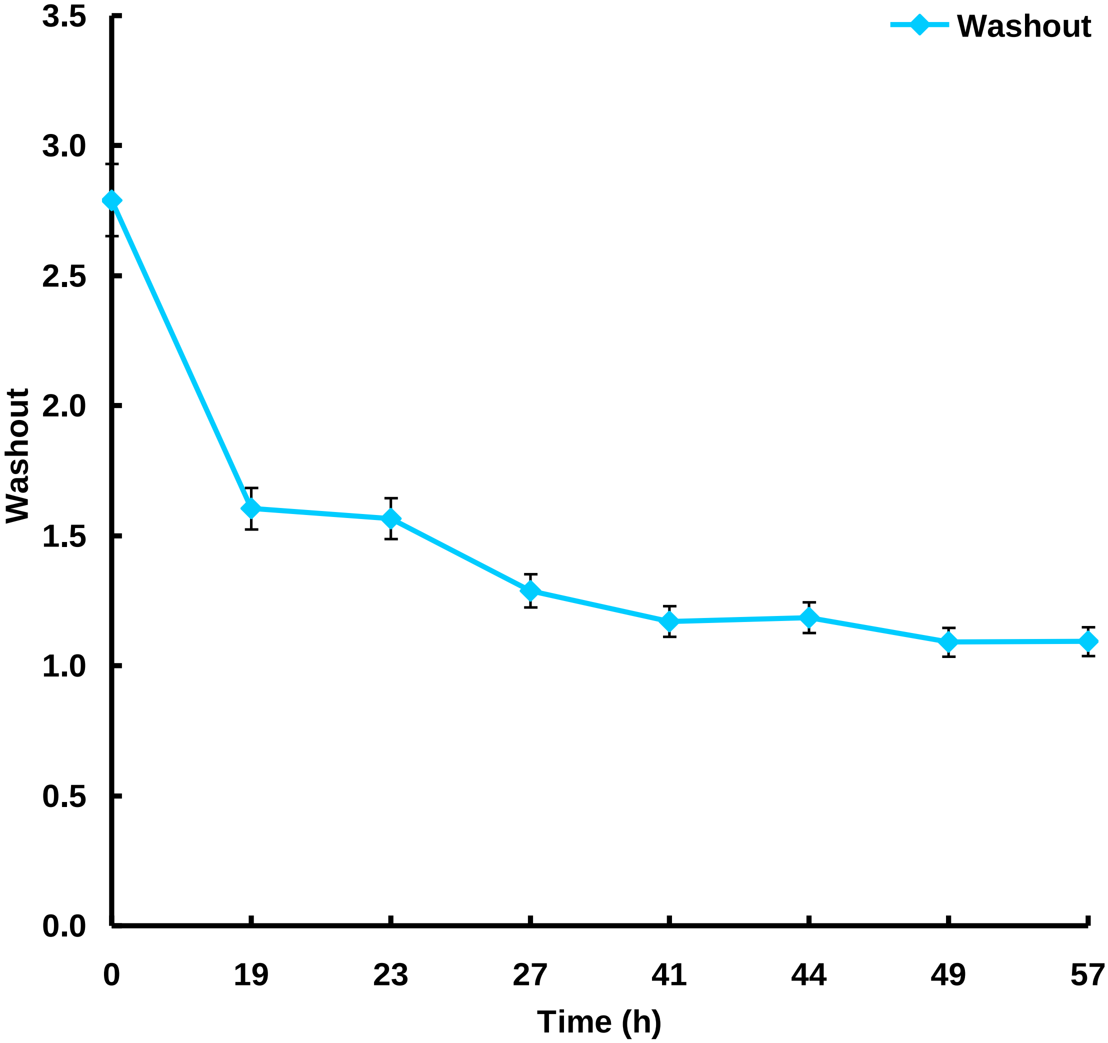


Fig.28a. Stability (washout at 550 nm) of the immobilized mixed culture on plastic clips in a packed bed bioreactor at an aeration rate of 0.6 mmoles/min. and hydraulic retention time (HRT) of 11 hrs.

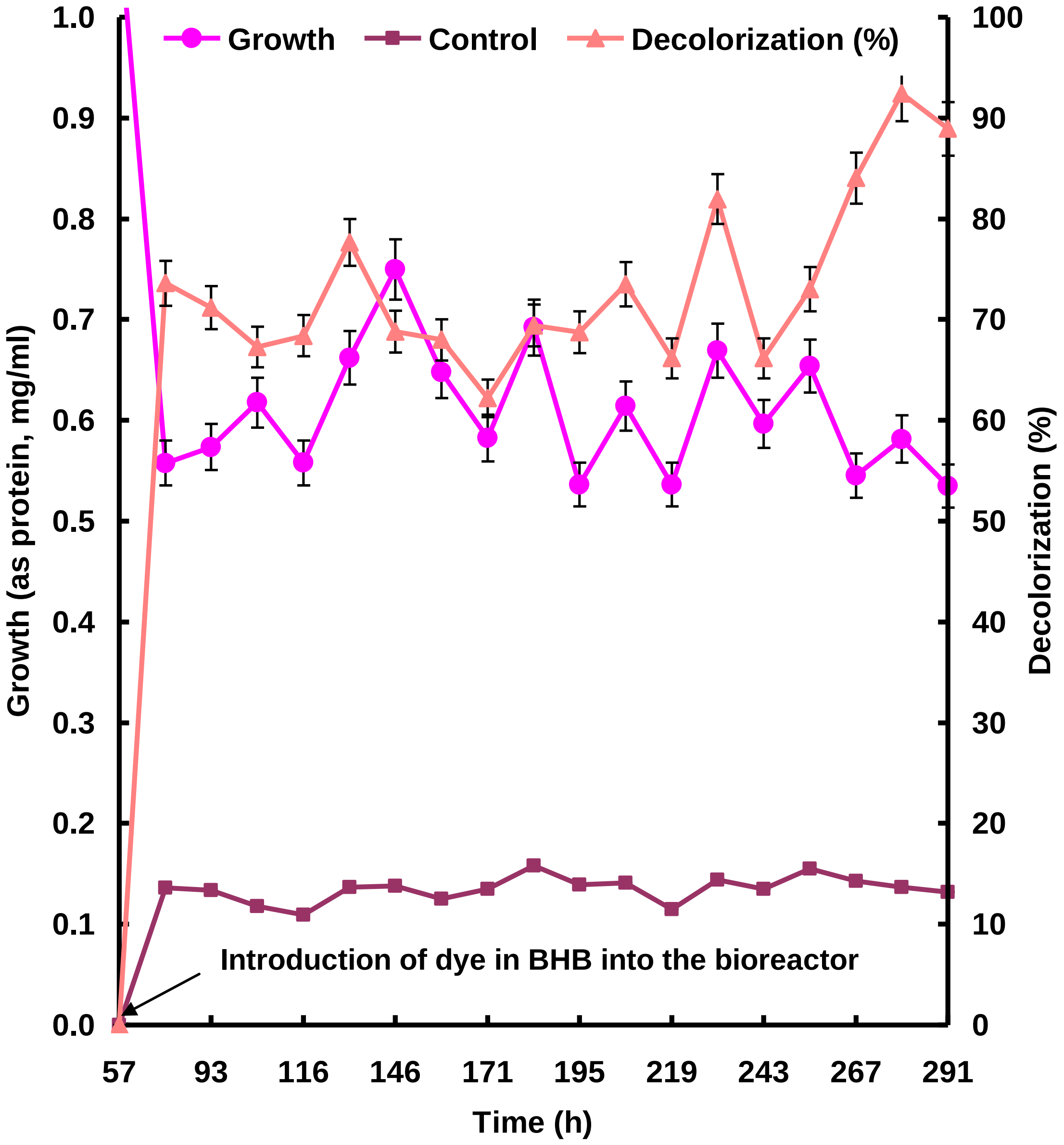


Fig.28b. Decolorization (%) of Direct Red 28 (10 mg/L) in a packed bed bioreactor with and without (control) mixed culture and growth (as protein) of mixed culture on plastic clips at aeration rate of 0.6 mmoles/min. and hydraulic retention time of 11 hrs.

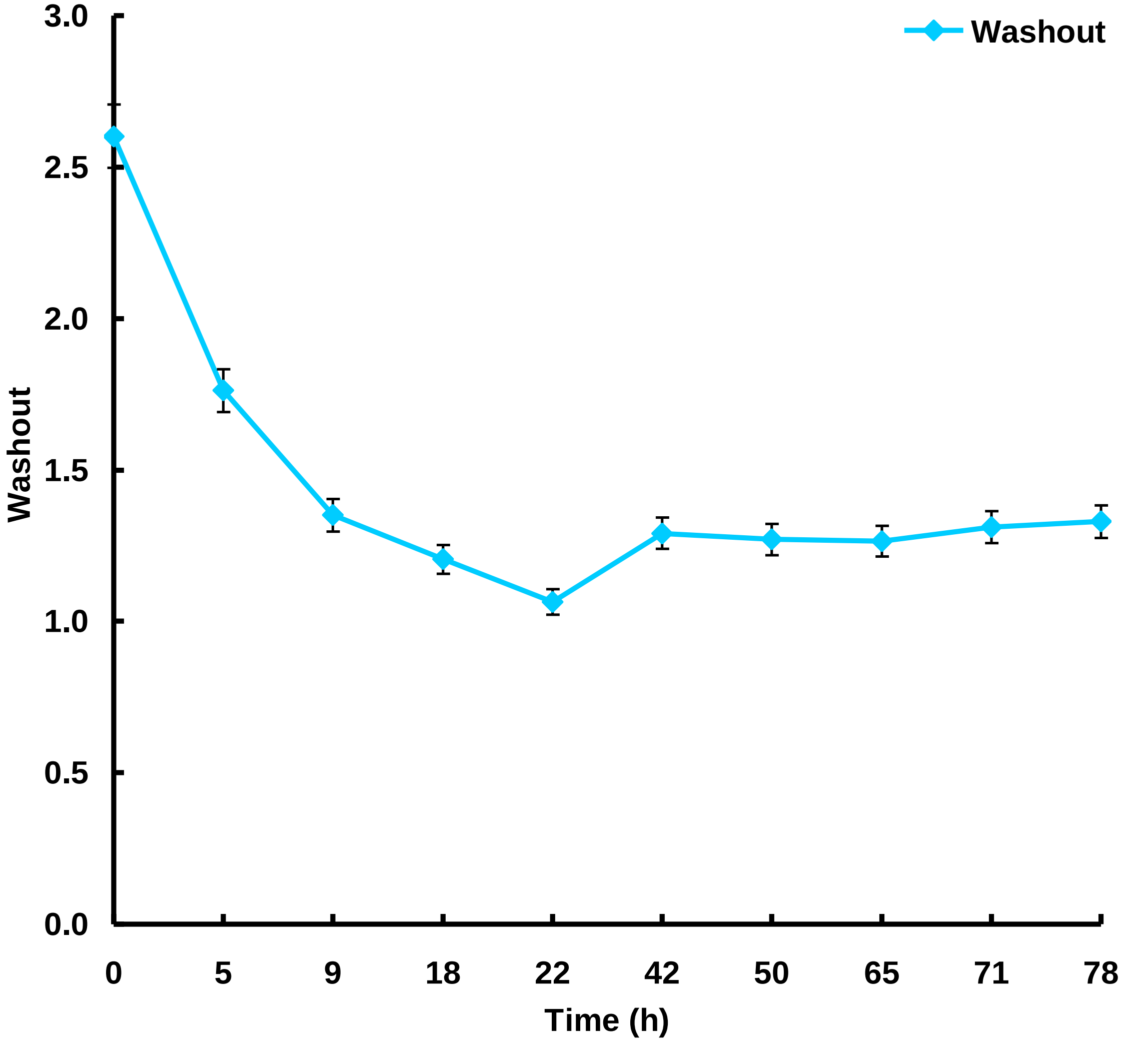


Fig.29a. Stability (washout at 550 nm) of the immobilized mixed culture on plastic clips in a packed bed bioreactor at an aeration rate of 0.6 mmoles/min. and hydraulic retention time (HRT) of 7.3 hrs.

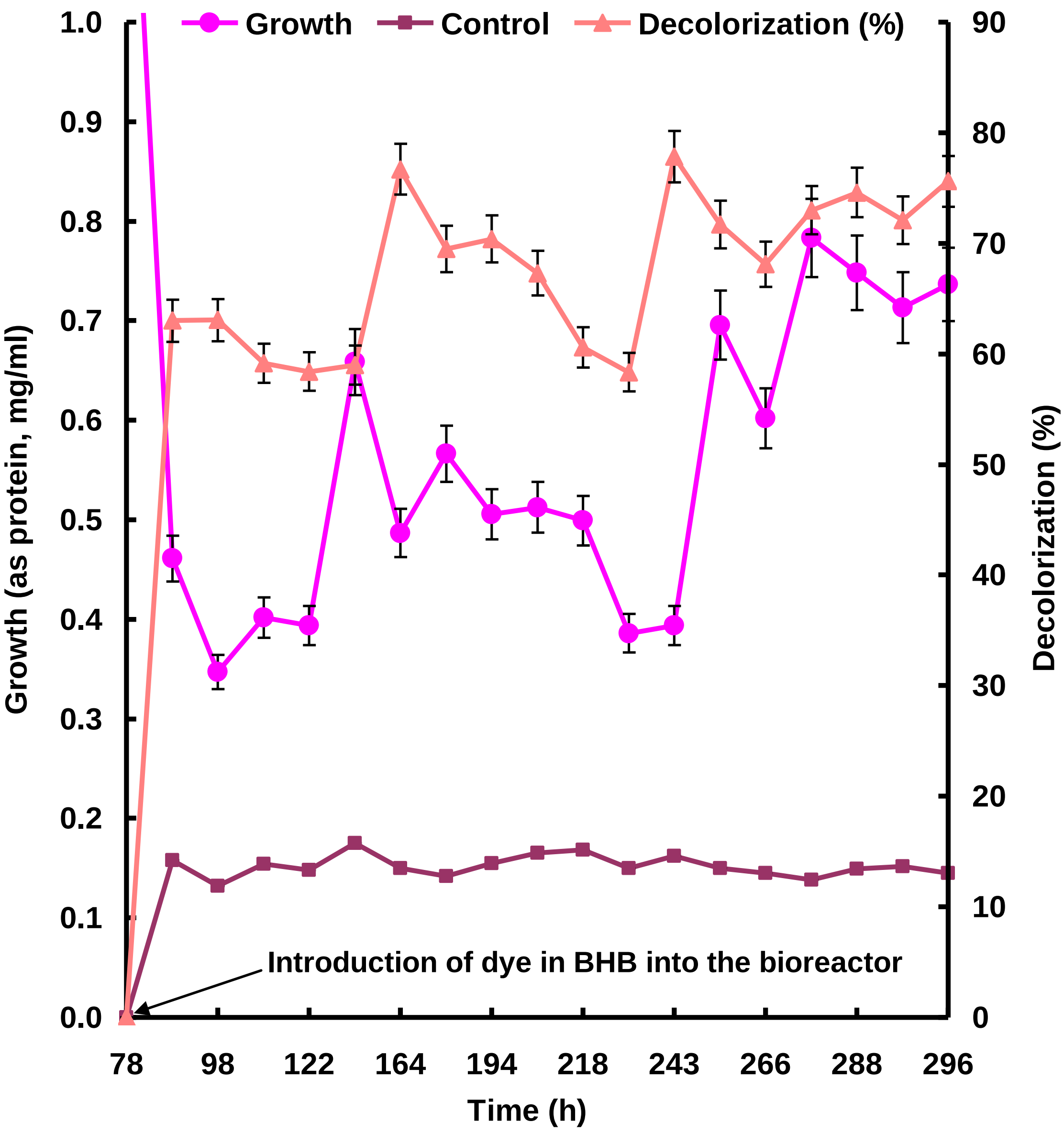


Fig.29b. Decolorization (%) of Direct Red 28 (10 mg/L) in a packed bed bioreactor with and without (control) mixed culture and growth (as protein) of mixed culture on plastic clips at aeration rate of 0.6 mmoles/min. and hydraulic retention time of 7.3 hrs.

A stable biofilm formation required 82 hrs by the mixed culture comprising of five bacterial isolates at hydraulic retention time of 5.5 hrs and an aeration rate of 0.6 mmoles/min on plastic clips as support particle (Fig.30a). Upon introduction of the dye in BHB medium, growth (as protein) of the mixed culture declined from 0.493 to 0.313 mg/ml from 82.3 to 122 hrs. Increase in growth (as protein) to 0.608 mg/ml was observed at 128 hrs however which decreased to 0.366 mg/ml at 131 hrs., and was observed to be in the range of 0.354 to 0.796 mg/ml from 147 to 210 hrs. Minimum decolorization of 45% was observed at 122 hrs which increased to a maximum of 77% at 147 hrs. and the experiment ended at 237 hrs (Fig.30b).

By comparing the λ max., of the initial and final values of the treated dye before and after the treatment, maximum decolorization of Direct Red 28 dye in BHB medium was observed at aeration rate of 0.6 mmoles/min and hydraulic retention time of 11 hrs. using plastic clips as matrix (Fig. 31).

4.7.3 Optimization of parameters of the up-flow immobilized packed bed bioreactor using marble chips as matrix

The effect different hydraulic retention time (8.16, 5.4, 4.08 hrs) along with two aeration rates (0.4 & 0.6 mmloes/min.) on the input stream (Direct Red 28 dye (10 mg/L) in BHB medium) by using marble chips as matrix in the bioreactor was studied. At different time intervals the sample from the outlet of the bioreactor were collected for growth of the defined mixed culture and decolorization of the dyes containing water.

At aeration rate of 0.4 mmoles/min., and hydraulic retention time of 8.16 hrs, mixed culture comprising of five bacterial isolates formed stable biofilm on marble chips (matrix) at 62 hours (Fig.32a).Upon introduction of the dye (in BHB) into the bioreactor, growth (as protein) of the mixed culture declined sharply (1.06 to 0.283 mg/ml) from 62 to110 hrs which after 200 hrs. remained in the range of 0.354 to 0.554 mg/ml. After 85 hrs of the experiment (i.e.38 hrs after the introduction of the dye) 95.6% decolorization was observed which decreased to 79% at 229 hrs. and thereafter 95-100% decolorization was observed till the end of 290 hrs. experiment (Fig.32b).

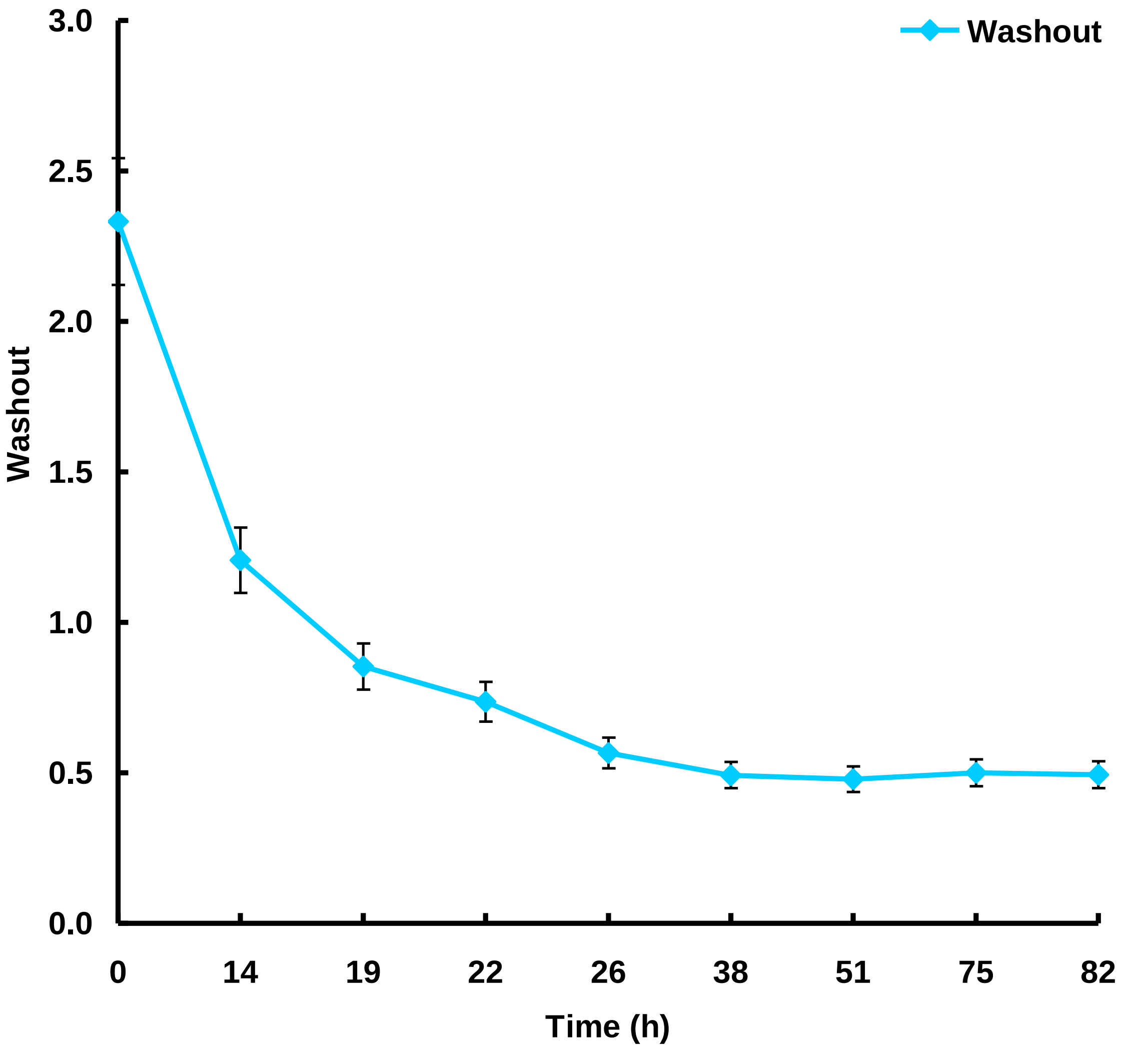


Fig.30a. Stability (washout at 550 nm) of the immobilized mixed culture on plastic clips in a packed bed bioreactor at an aeration rate of 0.6 mmoles/min. and hydraulic retention time (HRT) of 5.5 hrs.

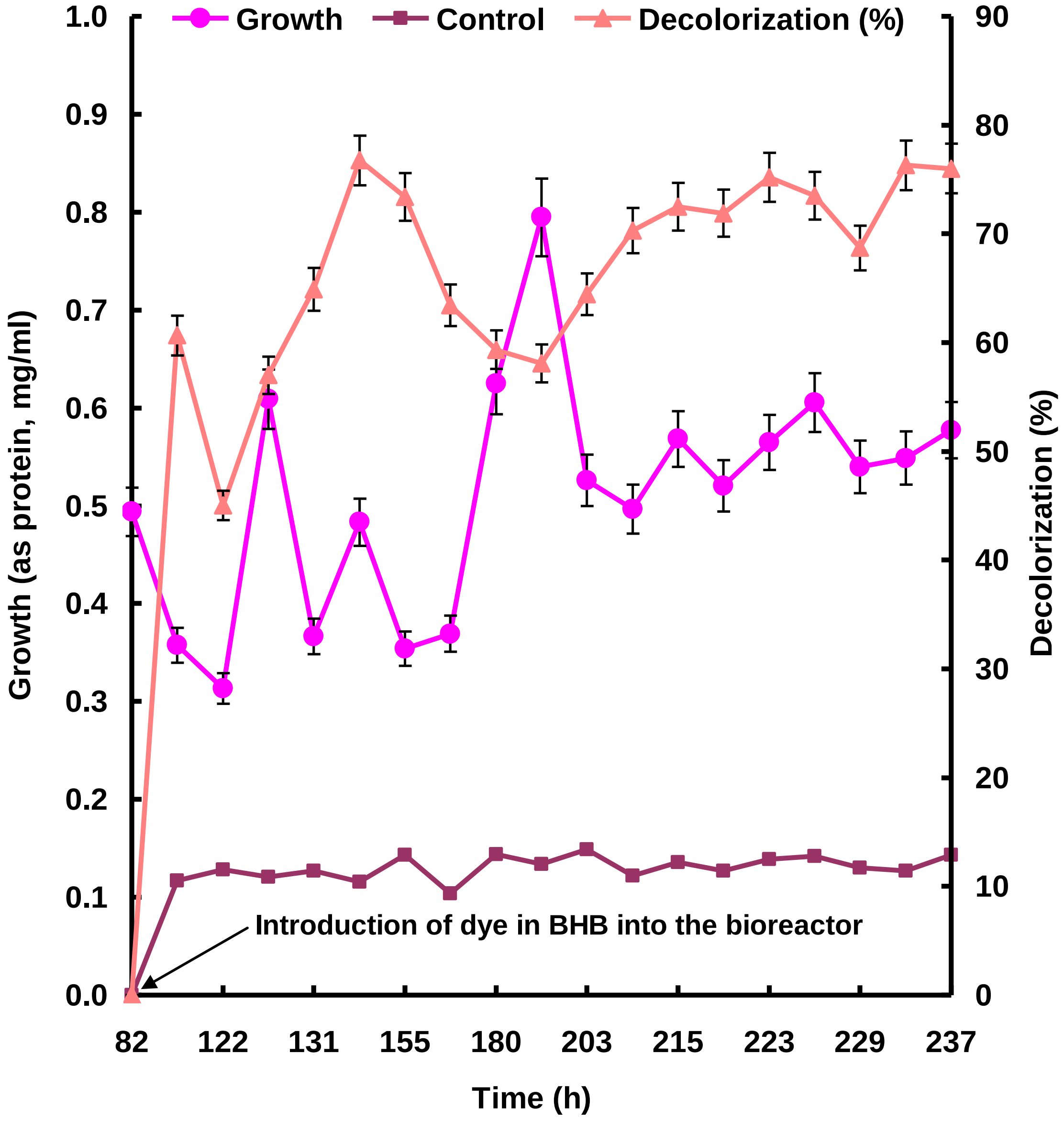


Fig.30 b. Decolorization (%) of Direct Red 28 (10 mg/L) in a packed bed bioreactor with and without (control) mixed culture and growth (as protein) of mixed culture on plastic clips at aeration rate of 0.6 mmoles/min. and hydraulic retention time of 5.5 hrs.

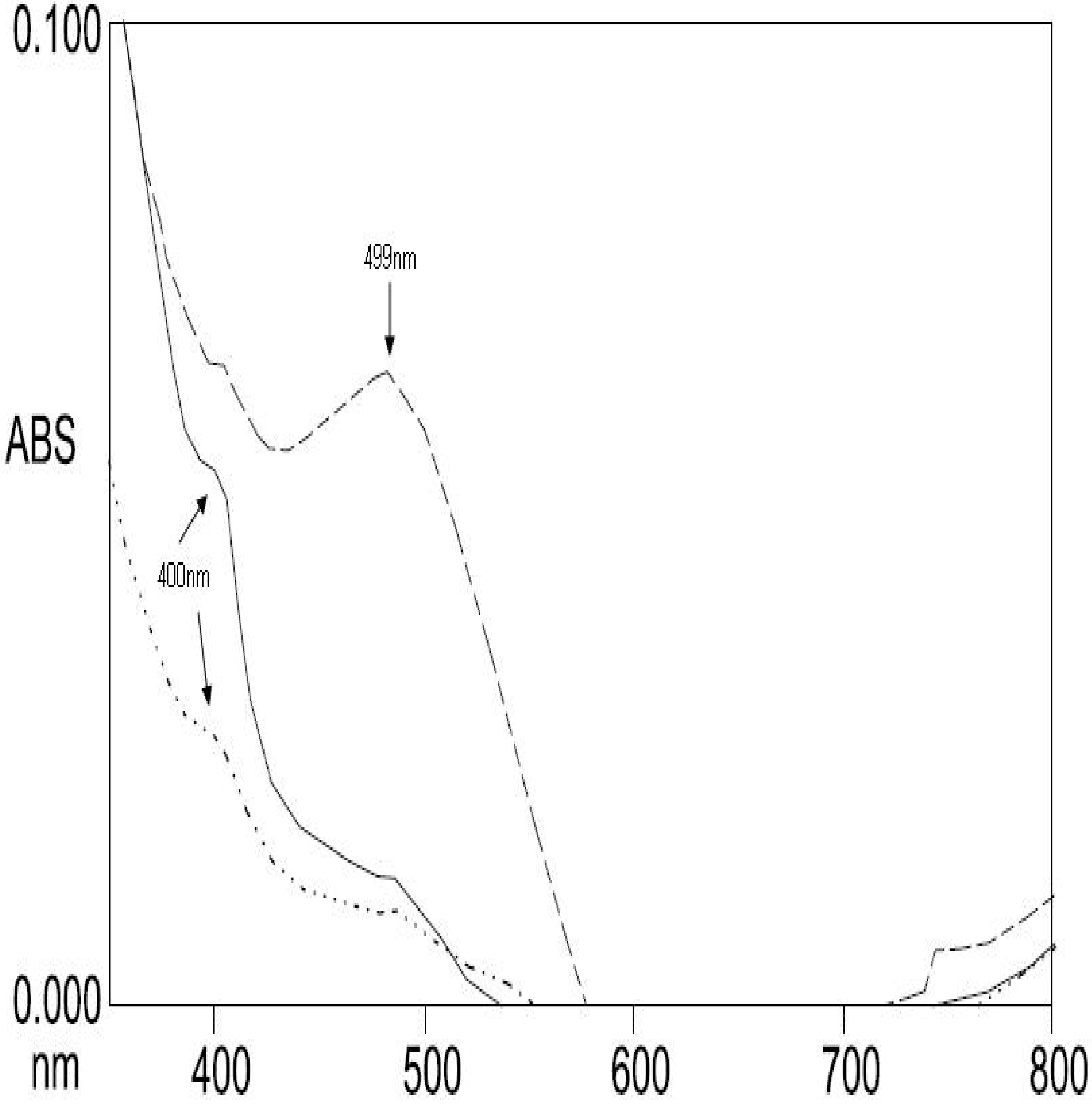


Fig.31. Absorption spectra of Direct Red 28 dye treated in the bioreactor using plastic clips as matrix at an aeration rate of 0.6 mmoles/min. and hydraulic retention time of 11 hrs. Input feed of the bioreactor 499 nm kmax of the dye (---), output feed of the bioreactor(___),output feed of the duplicate bioreactor(.....).

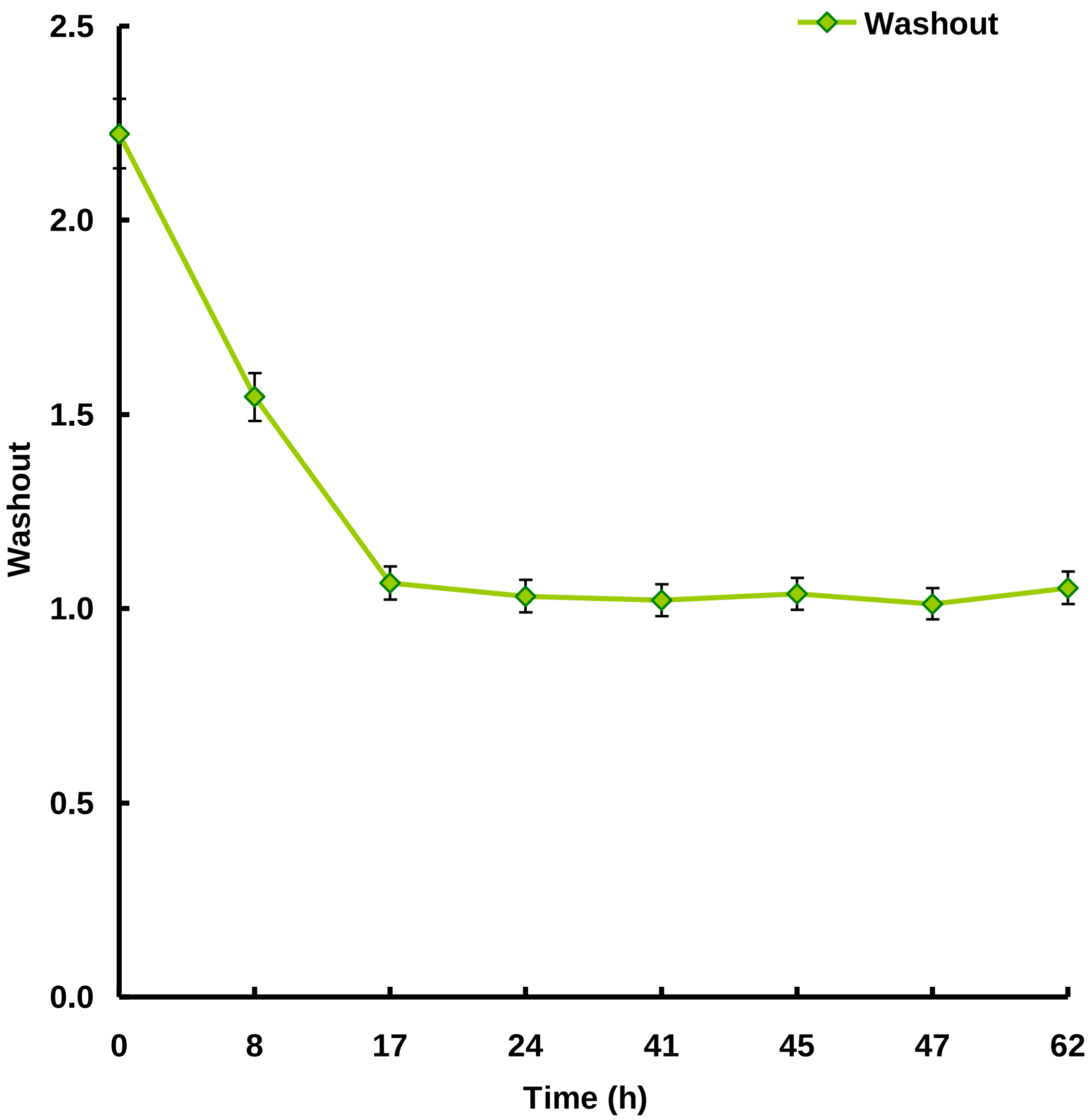


Fig.32a. Stability (washout at 550 nm) of the immobilized mixed culture on marble chips in a packed bed bioreactor at an aeration rate of 0.4 mmoles/min. and hydraulic retention time (HRT) of 8.16 hrs.

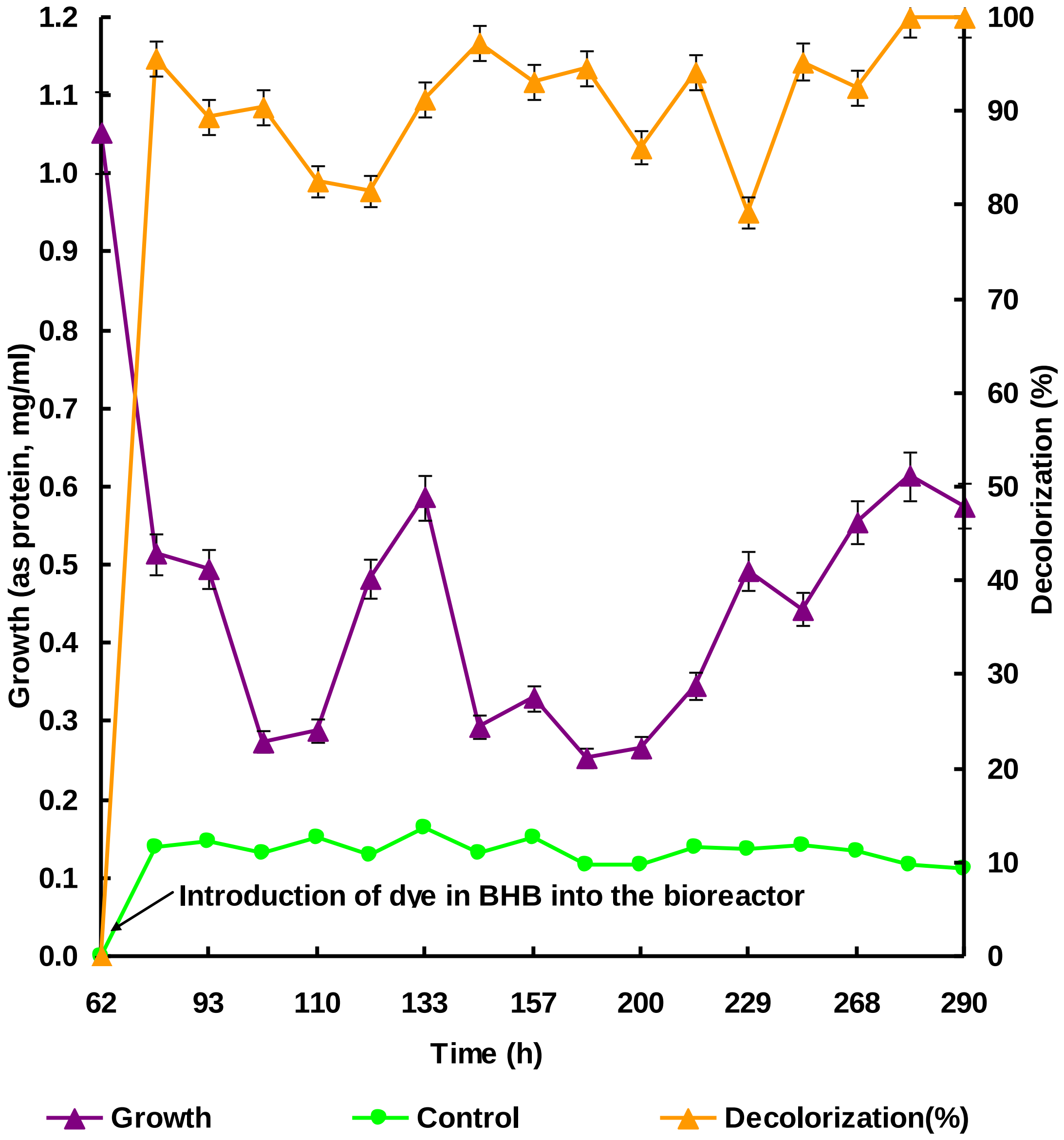


Fig.32b. Decolorization (%) of Direct Red 28 (10 mg/L) in a packed bed bioreactor with and without (control) mixed culture and growth (as protein) of mixed culture on marble chips at aeration rate of 0.4 mmoles/min. and hydraulic retention time of 8.16 hrs.

At HRT of 5.4 hrs. and aeration rate of 0.4 mmoles/min. using marble chips as support particle, the mixed culture comprising of five bacterial isolates formed stable biofilm after 85 hours (Fig.33a). After introduction of the dye Direct Red 28(in BHB) into the bioreactor, the growth (as protein) declined sharply from 0.895 to 0.442 mg/ml (85 to 94 hrs) and remained so till 158 hrs and thereafter, increase in growth (0.477 to 0.548 mg/ml protein) was observed from 162 to 266 hrs. At 94th hr of the experiment (i.e. after 9 hrs introduction of the dye in the bioreactor) 80% decolorization was observed. Random increase and decrease of 76 to 87% was noticed between 94 to 266 hrs.(Fig.33b).

For stable biofilm formation at HRT of 4 hrs. and aeration of 0.4 mmoles/min. 96 hrs. were required (Fig.34a). After 9.5 hrs of introducing the dye (in BHB) into the bioreactor, growth (as protein) of the mixed culture dropped from 0.486 to 0.325 mg/ml from 105 to 146 hrs. Increase in growth (0.433 mg/ml as protein) was observed after 154 hrs. Variation in decolorization from 82 to 50% (105 to 204 hrs.) was observed (Fig.34b).

Increase in aeration from 0.4 to 0.6 mmoles/min. and with HRT of 8.16 hrs. stable biofilm was formed after 62 hrs (Fig.35a). At 85th hr of the experiment (i.e. after 23 hrs of introduction of dye (in BHB) into the bioreactor) sharp reduction in growth (as protein) from 1.11 to 0.566 mg/ml was observed which decreased to minimum of 0.247 mg/ml at 222 hrs. After 290 hrs, growth (as protein) gradually increased to maximum of 0.938 mg/ml and maximum decolorization of 100% was observed at this parameter (Fig.35b).

At HRT of 5.4 hrs., and constant aeration rate of 0.6 mmoles/min. 89hrs was required for stable biofilm formation on the marble chips by the mixed culture (Fig.36a). After dye (in BHB) was introduced into the bioreactor (i.e.16 hrs. of passing the dye through the bioreactor) growth (as protein) of the mixed culture was reduced from 0.667 to 0.442 mg/ml. Variation in decolorization (72 to 87%) was observed at this parameter with average decolorization of 78% (Fig.36b).

For stable biofilm formation by the mixed culture 90 hrs. were required at HRT of 4 hrs. and aeration rate of 0.6 mmoles/min (Fig.37a).Upon introducing the dye (in BHB), growth (as protein) of the mixed culture declined from 0.796 to 0.318 mg/ml (from 90 to 105 hrs.). Increase in growth from 0.389 to 0.442 mg/ml protein was observed after 114 hrs to 129 hrs. The protein ranged between 0.268 to 0.531 mg/ml with maximum decolorization of 84% (Fig.37b).

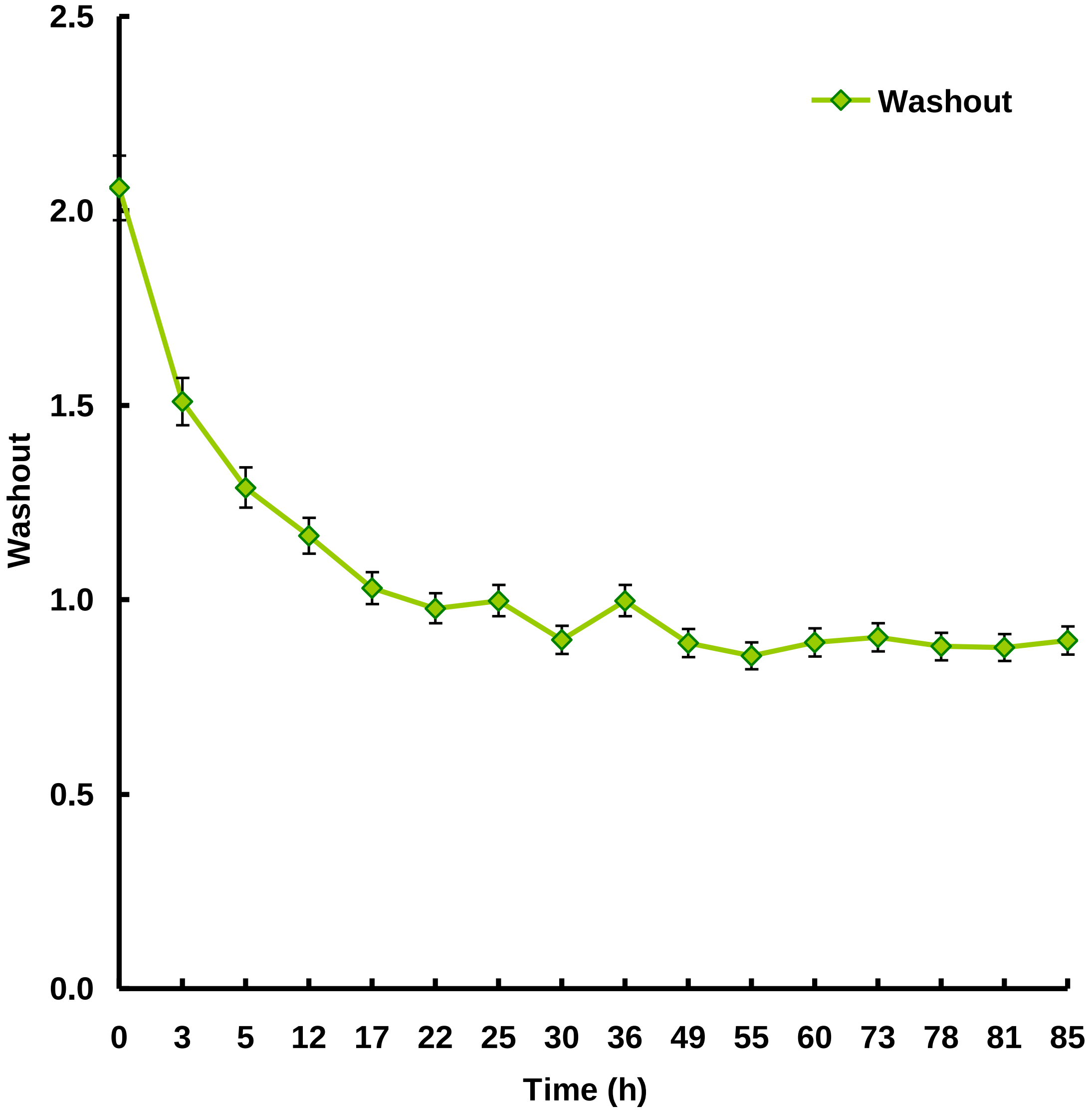


Fig.33a . Stability (washout at 550 nm) of the immobilized mixed culture on marble chips in a packed bed bioreactor at an aeration rate of 0.4 mmoles/min. and hydraulic retention time (HRT) of 5.4 hrs.

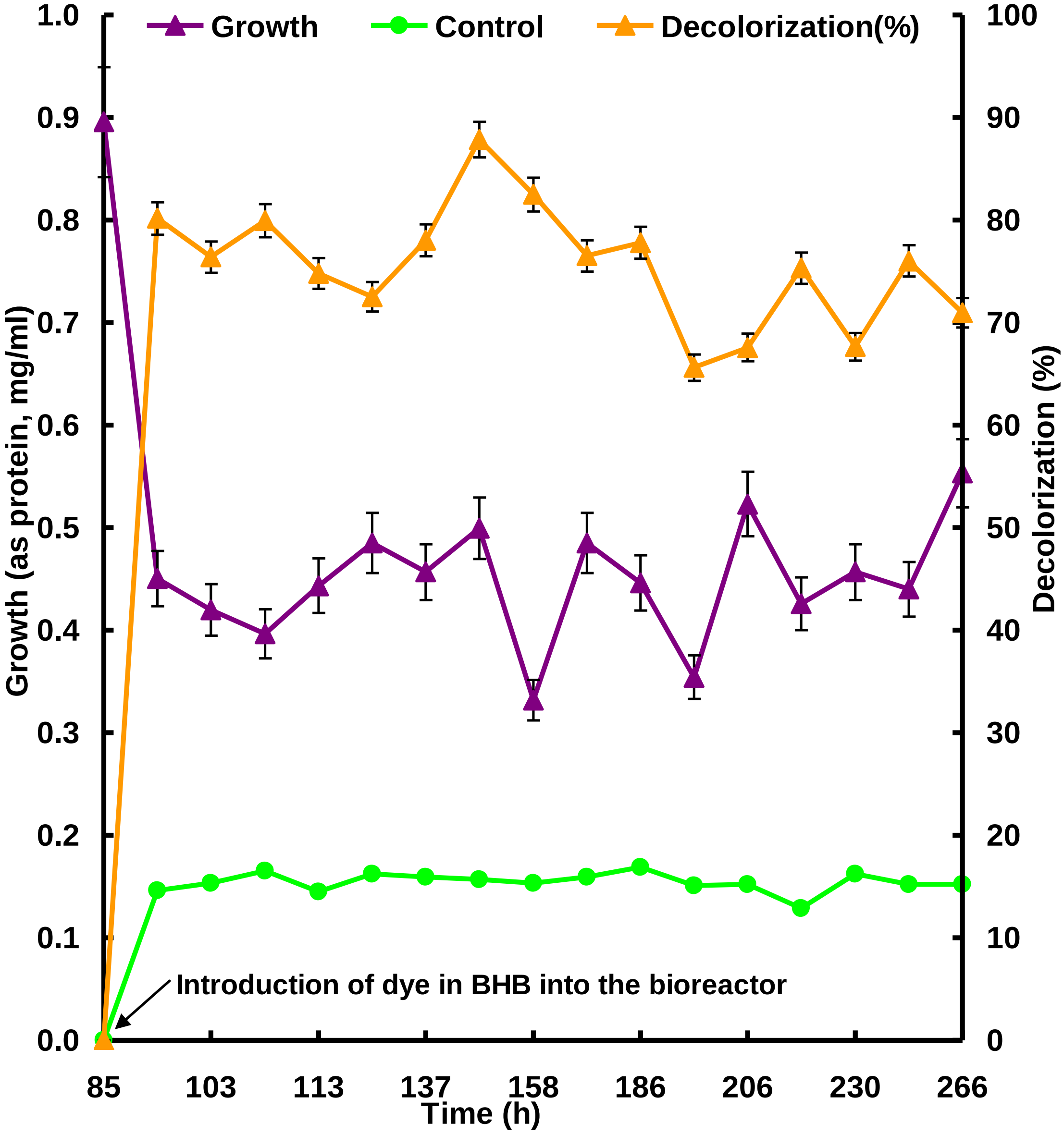


Fig.33b. Decolorization (%) of Direct Red 28 (10 mg/L) in a packed bed bioreactor with and without (control) mixed culture and growth (as protein) of mixed culture on marble chips at aeration rate of 0.4 mmoles/min. and hydraulic retention time of 5.4 hrs.

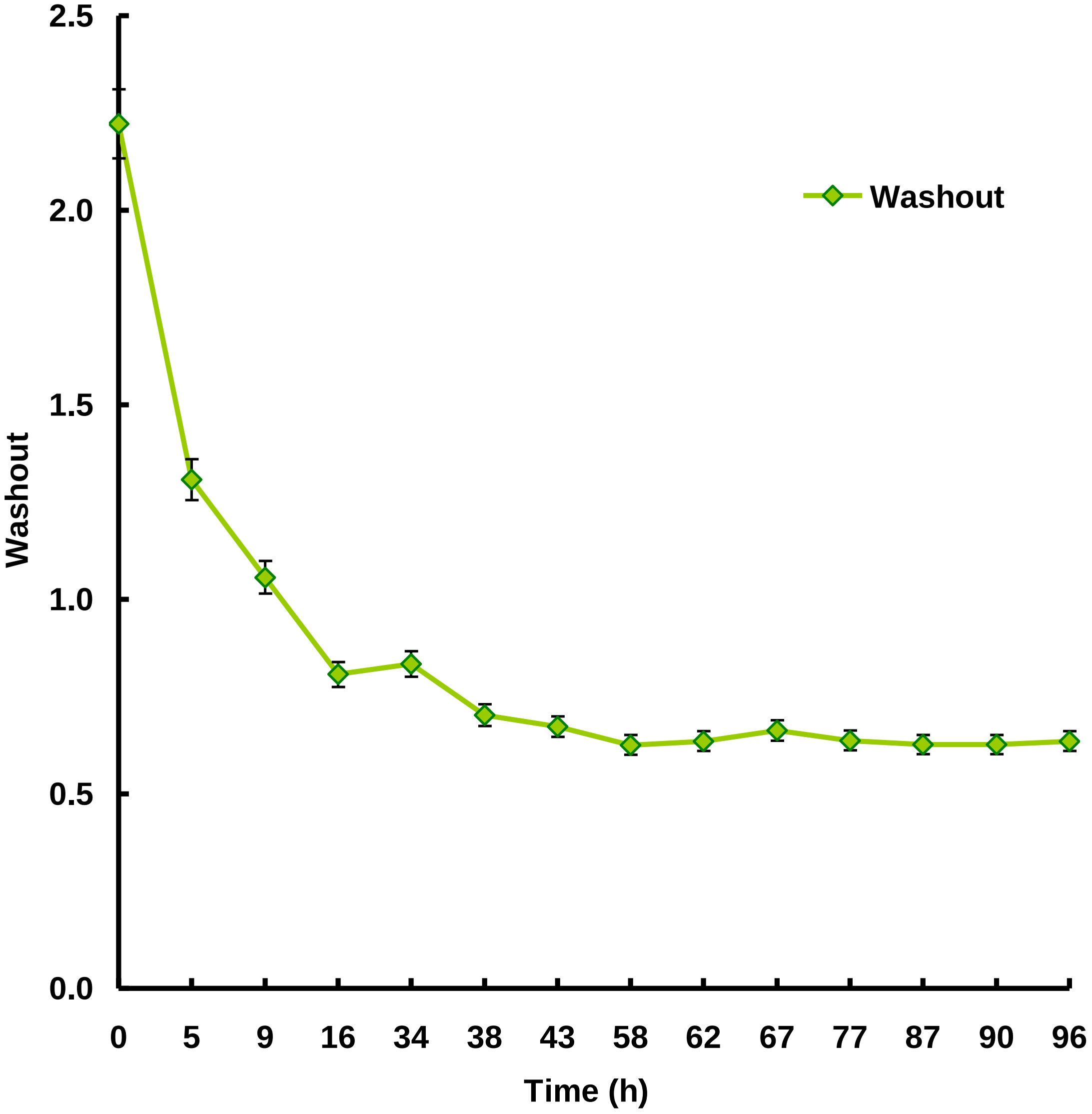


Fig.34a . Stability (washout at 550 nm) of the immobilized mixed culture on marble chips in a packed bed bioreactor at an aeration rate of 0.4 mmoles/min. and hydraulic retention time (HRT) of 4 hrs.

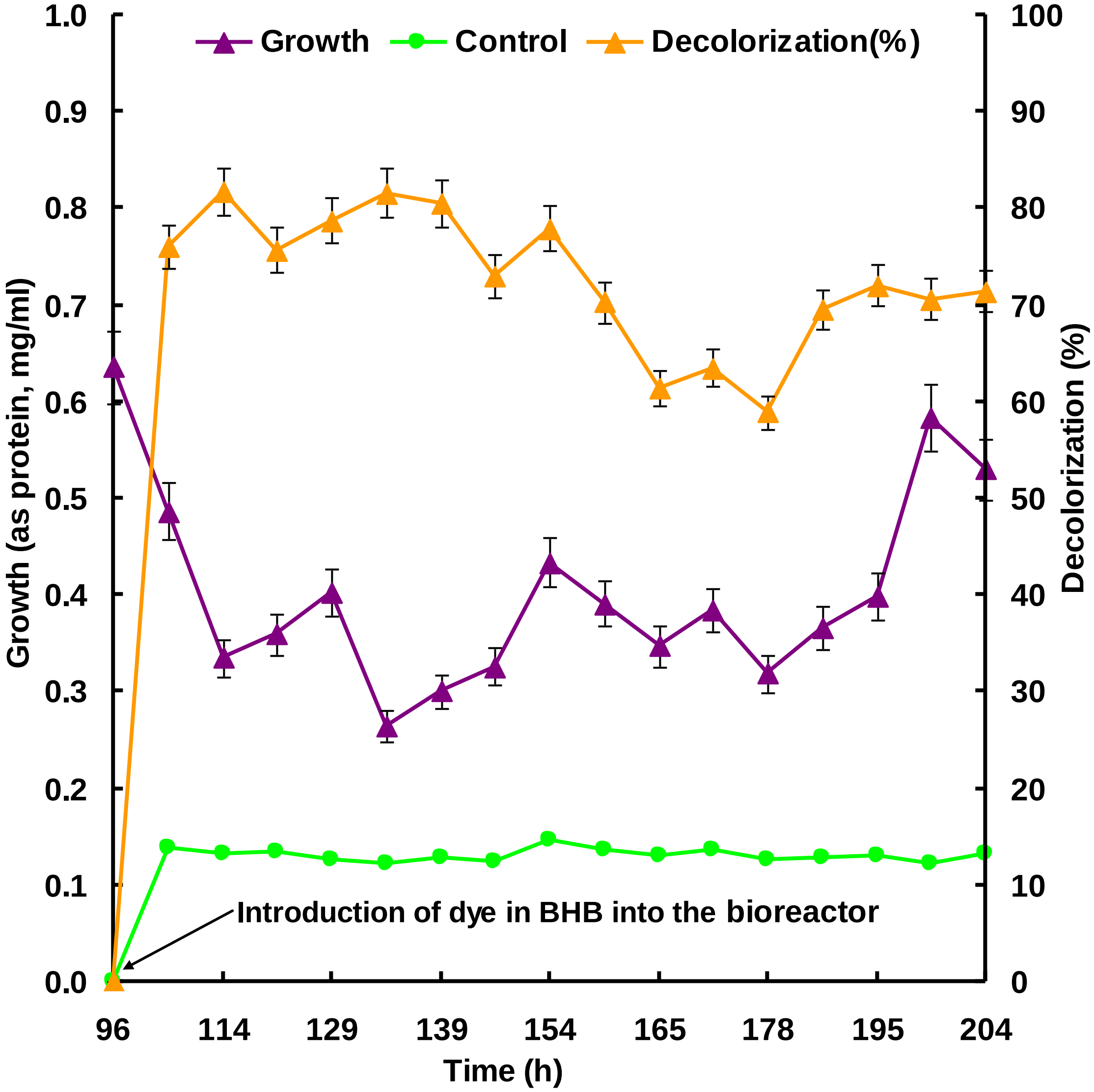


Fig.34b. Decolorization (%) of Direct Red 28 (10 mg/L) in a packed bed bioreactor with and without (control) mixed culture and growth (as protein) of mixed culture on marble chips at aeration rate of 0.4 mmoles/min. and hydraulic retention time of 4 hrs.

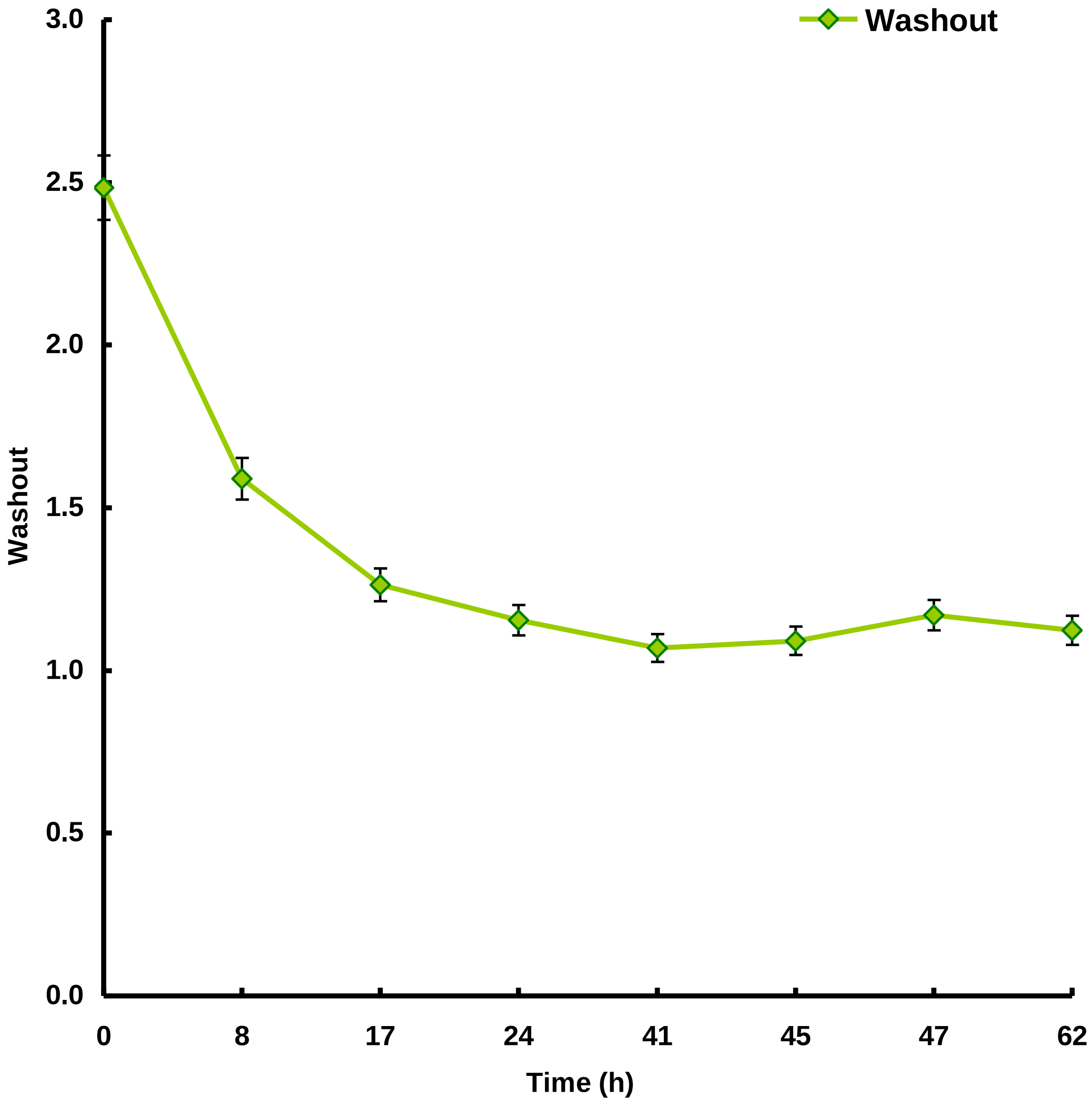


Fig.35a. Stability (washout at 550 nm) of the immobilized mixed culture on marble chips in a packed bed bioreactor at an aeration rate of 0.6 mmoles/min. and hydraulic retention time (HRT) of 8.16 hrs.

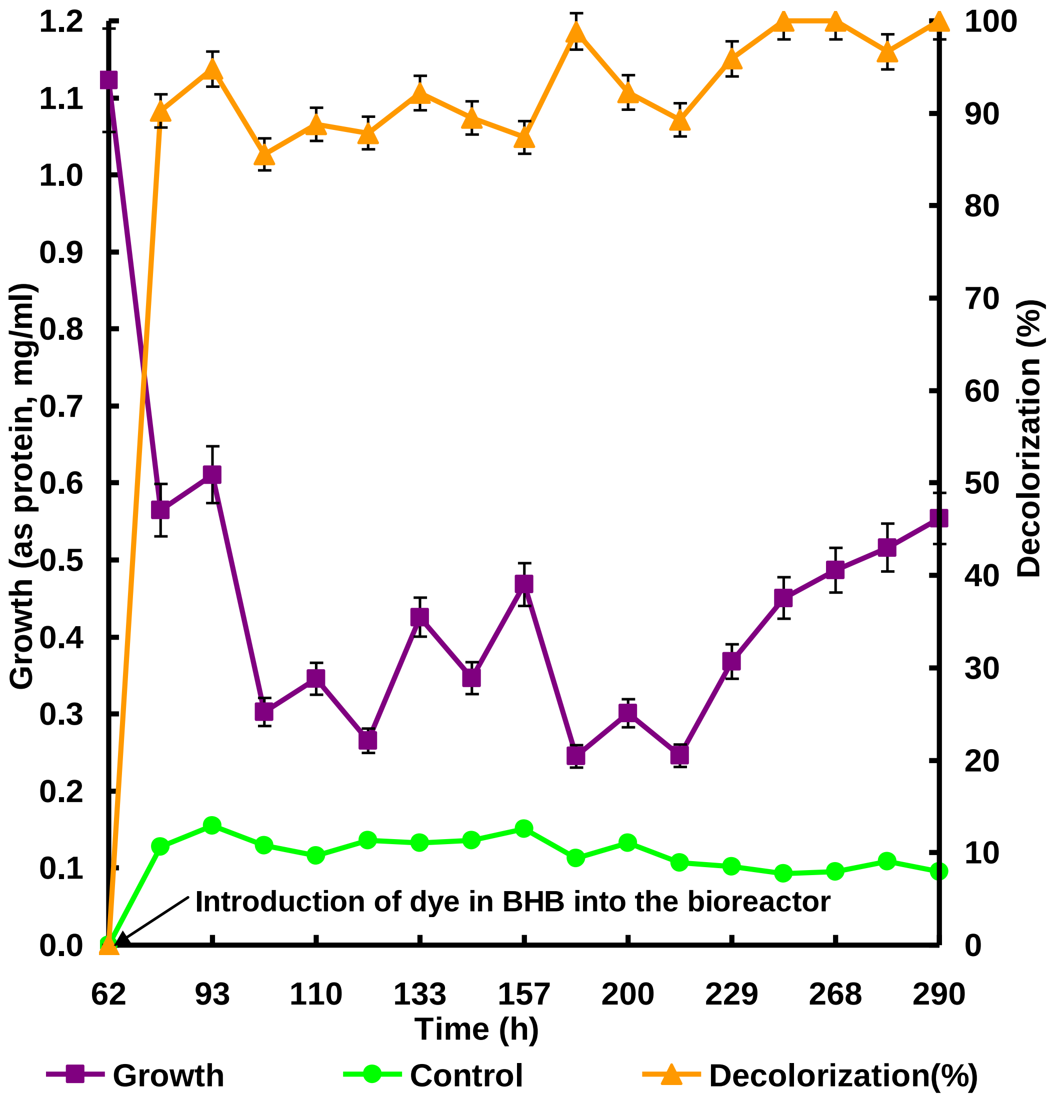


Fig.35b. Decolorization (%) of Direct Red 28 (10 mg/L) in a packed bed bioreactor with and without (control) mixed culture and growth (as protein) of mixed culture on marble chips at aeration rate of 0.6 mmoles/min. and hydraulic retention time of 8.16 hrs.

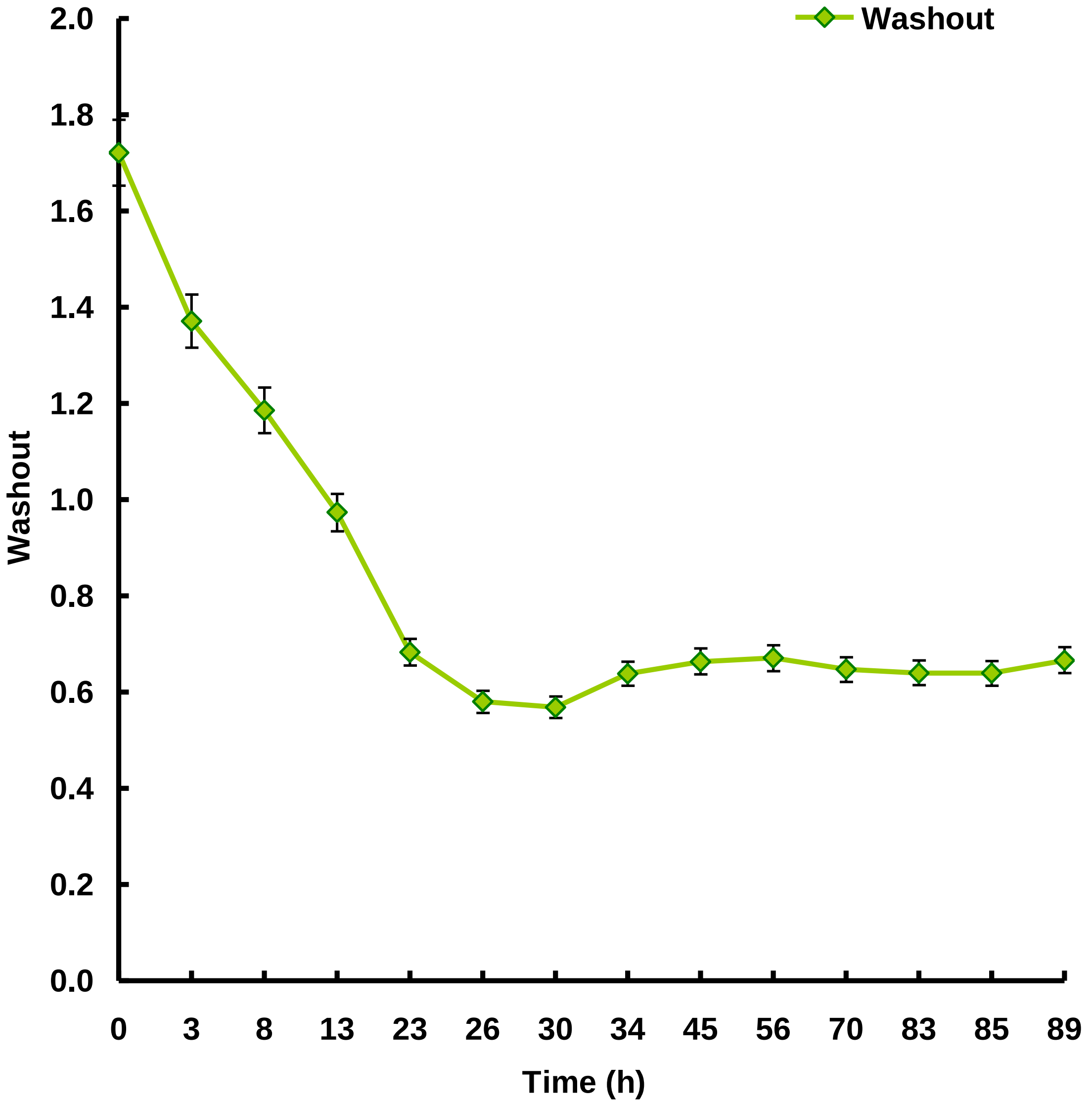


Fig.36a. Stability (washout at 550 nm) of the immobilized mixed culture on marble chips in a packed bed bioreactor at an aeration rate of 0.6 mmoles/min. and hydraulic retention time (HRT) of 5.4 hrs.

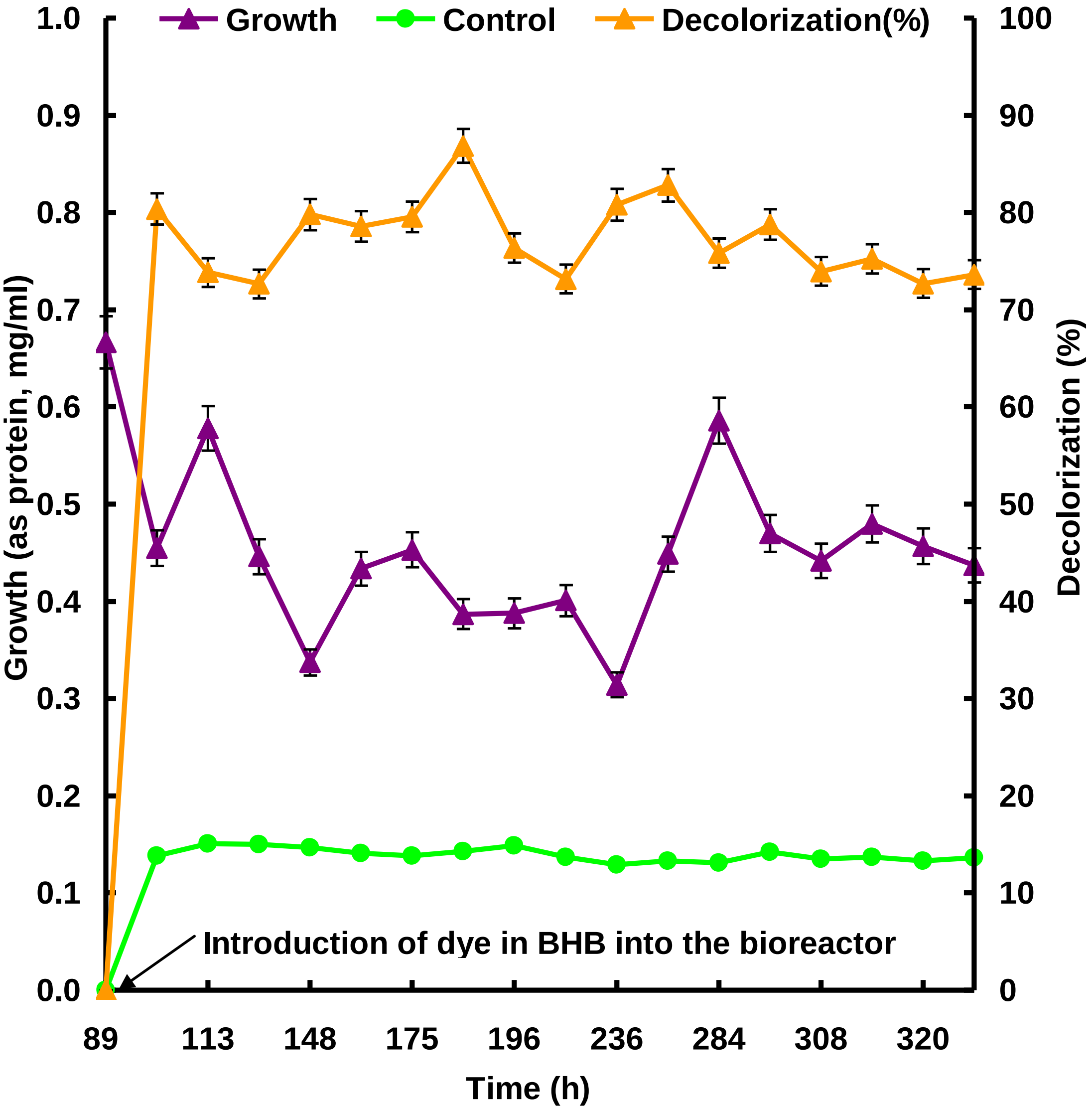


Fig.36b. Decolorization (%) of Direct Red 28 (10 mg/L) in a packed bed bioreactor with and without (control) mixed culture and growth (as protein) of mixed culture on marble chips at aeration rate of 0.6 mmoles/min. and hydraulic retention time of 5.4 hrs.

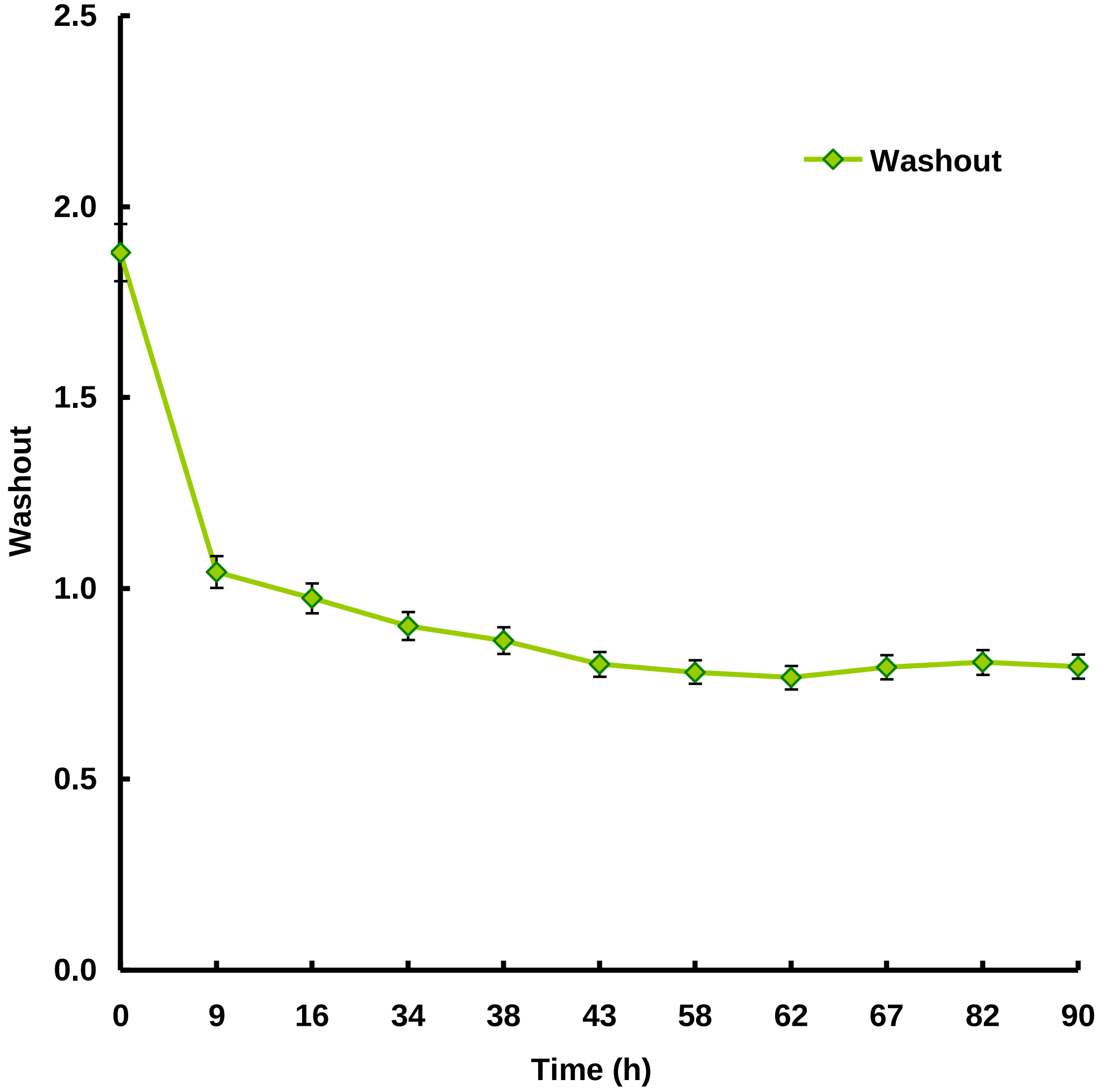


Fig.37a. Stability (washout at 550 nm) of the immobilized mixed culture on marble chips in a packed bed bioreactor at an aeration rate of 0.6 mmoles/min. and hydraulic retention time (HRT) of 4 hrs.

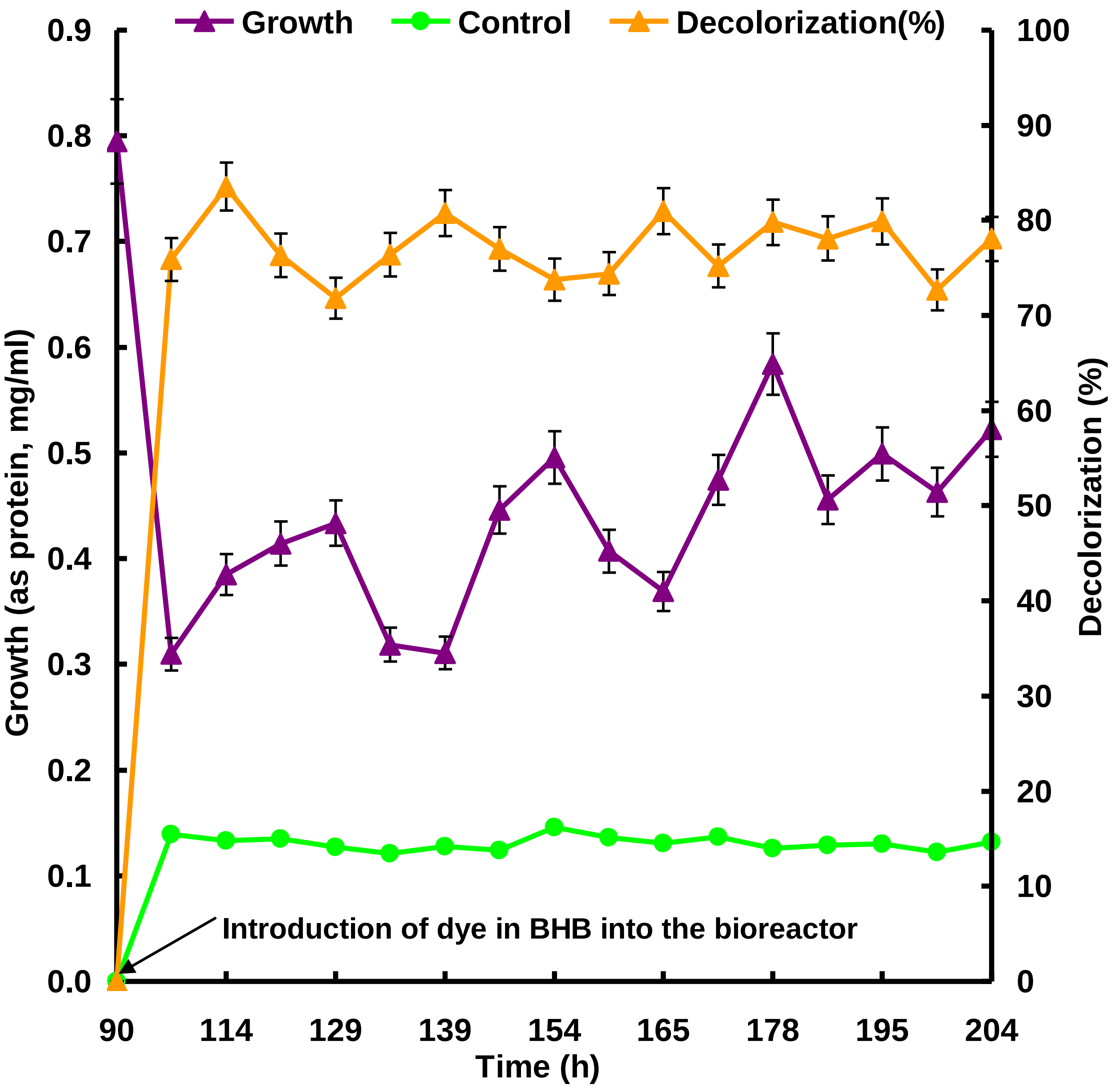


Fig.37b. Decolorization (%) of Direct Red 28 (10 mg/L) in a packed bed bioreactor with and without (control) mixed culture and growth (as protein) of mixed culture on marble chips at aeration rate of 0.6 mmoles/min. and hydraulic retention time of 4 hrs.

Maximum decolorization of Direct Red 28 dye using marble chips as matrix was observed at aeration rate of 0.6 mmoles/min., and hydraulic retention time of 8.16 hrs., by comparing the λ max. of the initial and final values of the treated dye before and after the treatment (Fig. 38)

Among all the parameters studied using two matrixes best decolorization efficiency by mixed culture was observed by using marble chips as matrix at aeration rate of 0.6 mmoles/min. and hydraulic retention time of 8.16hrs., hence the real raw untreated effluent was treated in the bioreactor at the same parameter. With the waste raw untreated effluent biofilm formation occurred at 63 hrs using marble chips as support matrix at aeration rate of 0.6 mmoles/min. and hydraulic retention time of 8.16hrs(Fig. 39a). Maximum decolorization of 88% was observed at 88 hrs which further reduced to 33% at the end of 154 hr experiment. Total solids, in the real raw effluent before treatment through the bioreactor was observed to be 3757mg/L which was reduced to 1014 mg/L after the effluent was treated through the bioreactor. The total dissolved solids before treatment through the bioreactor was 1464 mg/L which reuced to 436 mg/L after the raw effluent was treatment through the bioreactor. Hence, the total suspended solids before treatment was 2293 mg/L and 578 mg/L after the effluent was treated through the bioreactor. COD and BOD of the waste raw untreated effluent were 3600 and 30.25 before the treatment, which was reduced to 2400 followed by COD of 1920, 1600, 1200, 1120, 1040 and 880 at the end of 154 hrs respectively. BOD of the raw untreated effluent was 30.25 before treatment which reduced to 3.7 after 154 hours (Fig. 39b).

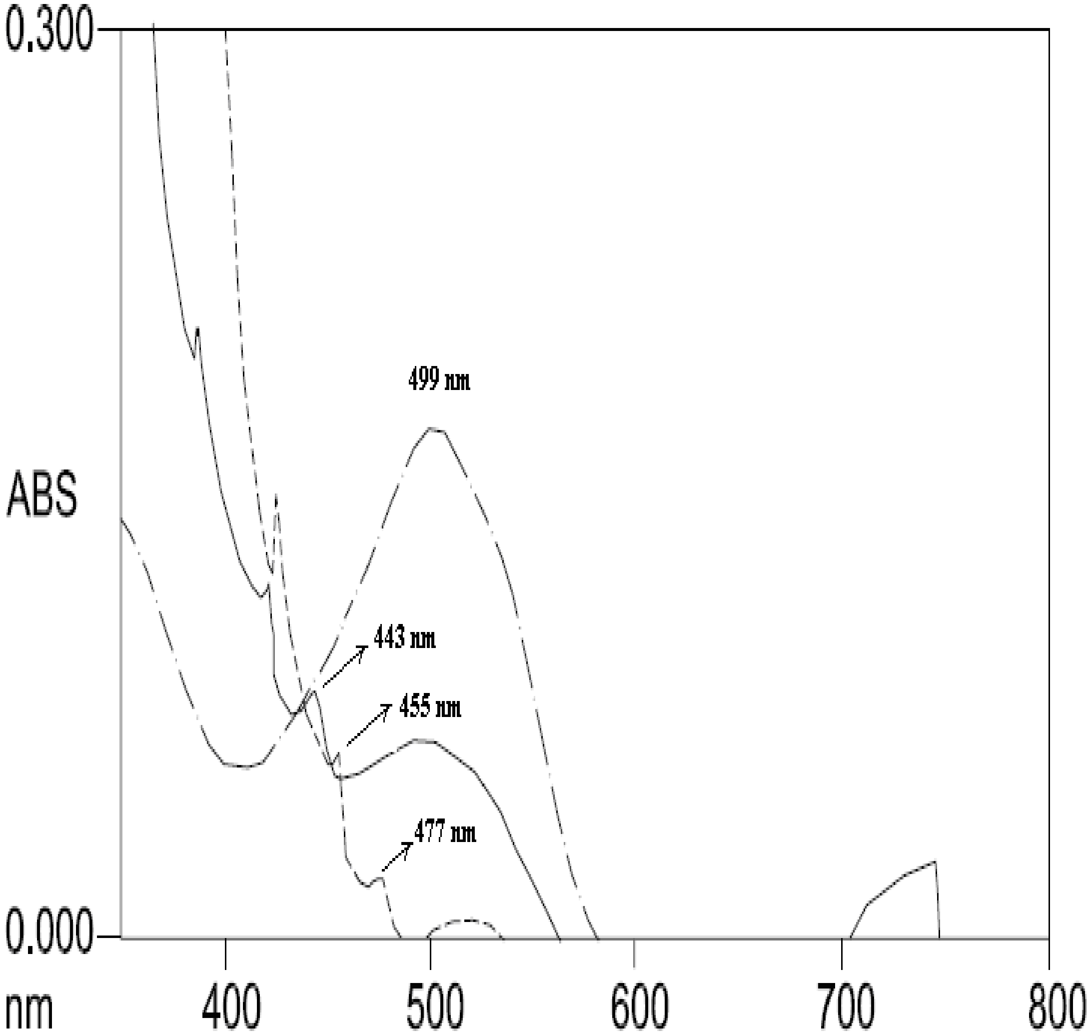


Fig.38. Absorption spectra of Direct Red 28 dye treated in the bioreactor using marble chips as matrix at an aeration rate of 0.6 mmoles/min. and hydraulic retention time of 8.16 hrs.

Input feed of the bioreactor 499 nm kmax of the dye (---), output feed of the bioreactor (___),output feed of the duplicate bioreactor(…..).

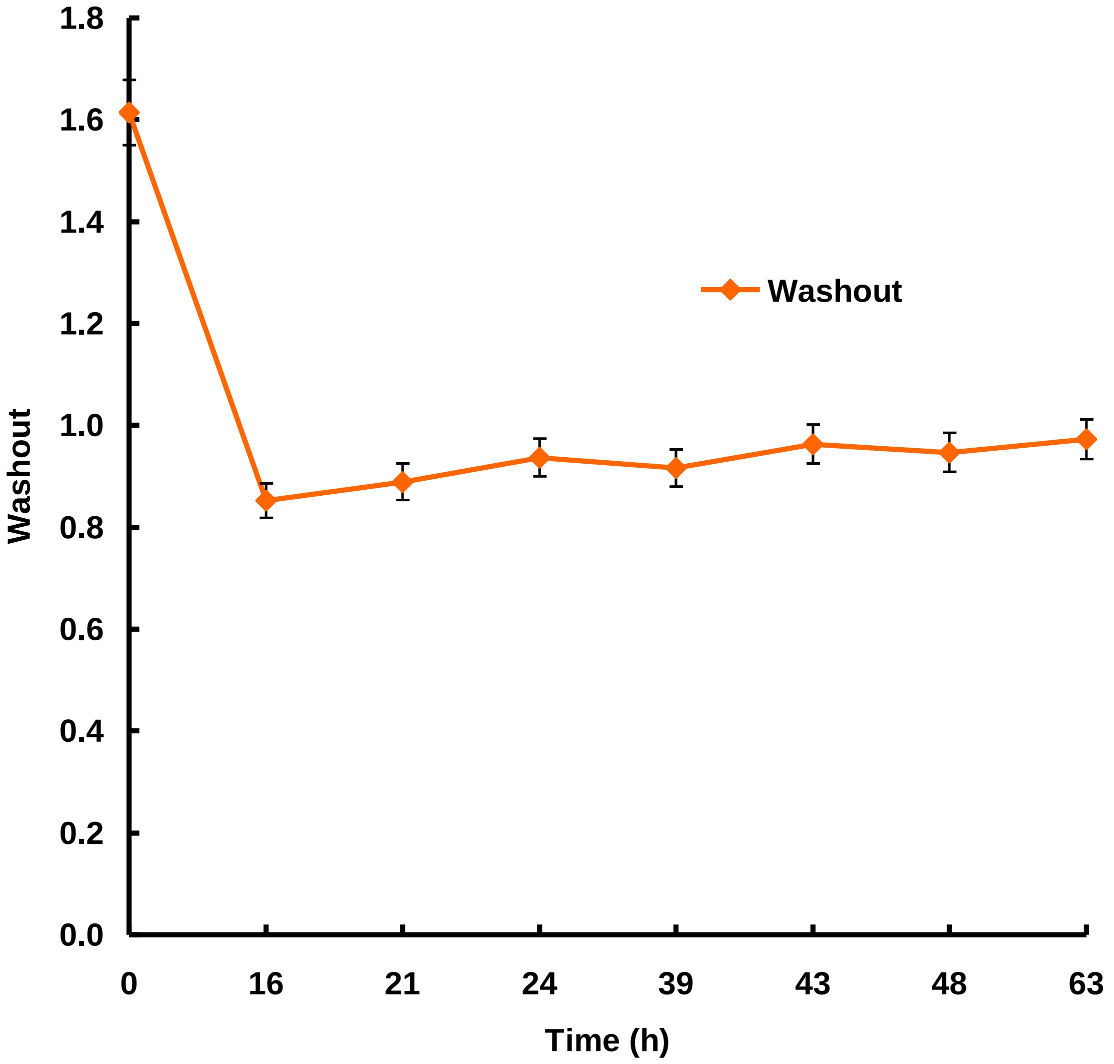


Fig.39a. Stability (washout at 550 nm) of the immobilized mixed culture on marble chips in a packed bed bioreactor at an aeration rate of 0.6 mmoles/min. and hydraulic retention time (HRT) of 8.16 hrs.

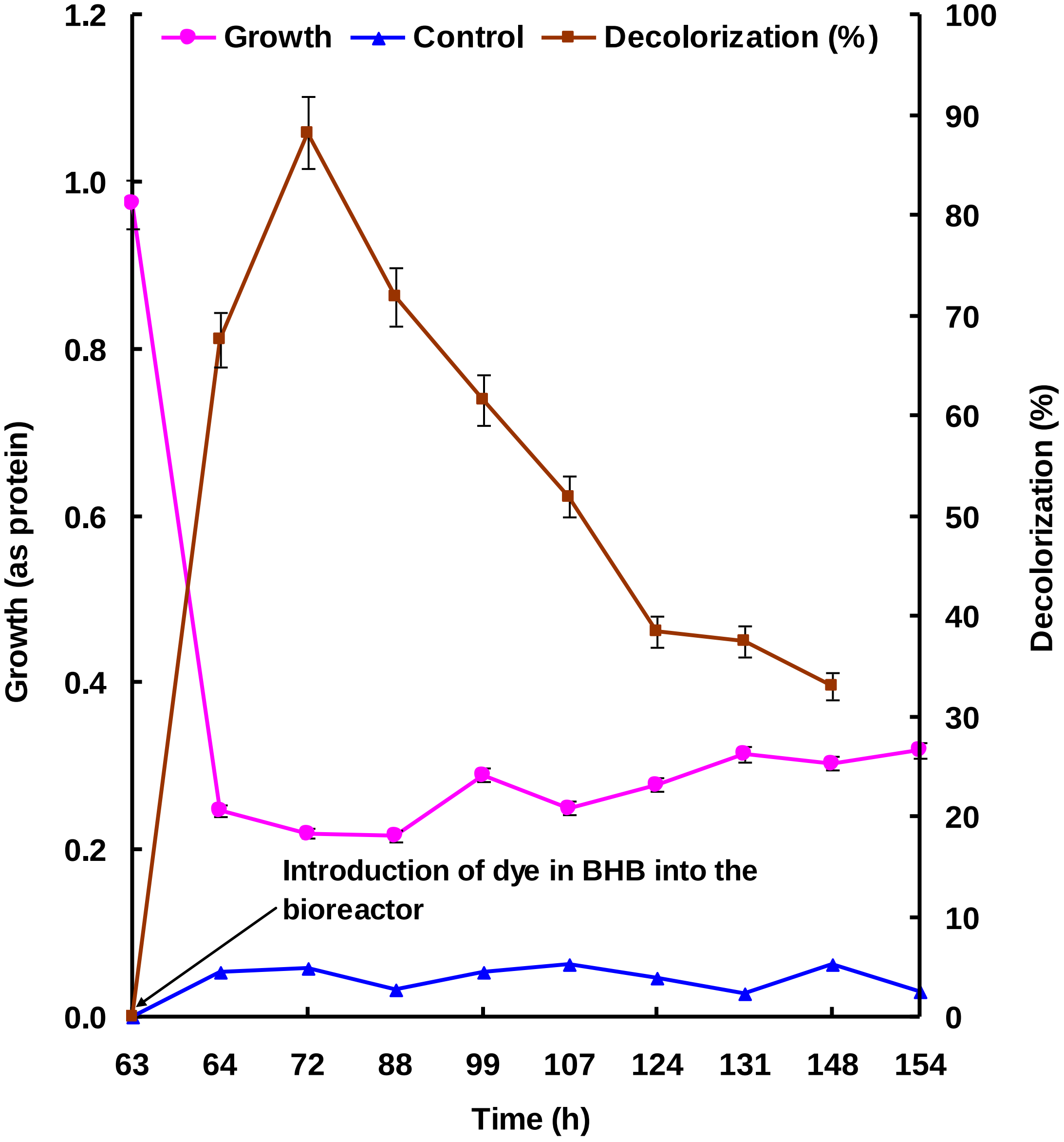


Fig.39b. Decolorization (%) of 5% real raw untreated effluent in a packed bed bioreactor with and without (control) mixed culture and growth (as protein) of mixed culture on marble chips at aeration rate of 0.6 mmoles/min. and hydraulic retention time of 8.16 hrs.

5. DISCUSSION

Azo dyes account for majority of the synthetic dyes used in textile industries and the release of untreated effluents of these industries into the environment is of utmost concern due to the recalcitrant nature of these dyes and/or their reduced products/intermediates and also as it is aesthetically displeasing (Khehra *et* al 2005). A microbial system was developed for decolorizing dyes since various physical and/or chemical methods used for removal of dyes from wastewater, are cost intensive and generate toxic sludge that need special disposal protocols. It has been reported that anaerobic microbial degradation of azo dyes leads to formation of toxic, carcinogenic and mutagenic aromatic amines (Robinson *et al* 2001) therefore, this study was initiated to develop a microbial population, from the aerobic sludge samples of effluent treatment plant (ETP) of Nahar Group of Textile & Dyeing Industry, Punjab, India for the treatment of certain azo dyes. Aerobic treatment is thought to be a reliable and safe method for the biodegradation of textile dyes by the action of azoreductase, which reductively cleaves the azo group (Stolz 2001; Senan and Abraham 2004).

➢ Development of microbial systems for the decolorization of dyes in wastewater from the textile printing and dyeing industry

Although literature reports the capability of pure cultures to decolorize dyes (Chang *et al* 2001, Hu 2001) but they do not find much application in treatment system for industrial effluent because of heterogeneity of the components in effluents. A microbial consortium consisting of many microbes would treat the effluent effectively due to the inherent quality of the individual strains present in the microbial consortium to attack the different dye molecule at different positions or may use decomposition products produced by another strain (Banat 1996). And therefore, in this research microbial consortium was chosen for decolorization studies as they can collectively carry out biodegradation that cannot be achieved by pure culture (Learoyd *et al* 1992; Knapp and Newby 1995; Nigam *et al* 1996a, Sharma *et al* 2004 d; Senan and Abraham 2004). Further it has been observed that mixed cultures developed from sites contaminated with organic compounds of similar nature are generally more efficient than those developed from any other site. Thus similar strategy was adopted to isolate mixed cultures from a domestic wastewater treatment plant (Vijaya and Sandhya 2003) and from activated sludge of the municipal wastewater treatment (Adedayo *et al* 2004) to efficiently decolorize toxic azo dye Methyl red. Four different aerobic mixed consortia

(CT, ST, NF and CF) were developed from wastewater of dyeing plants to decolorize Drimarene Orange K-GL, Drimarene Brilliant Red K-4BL, Foron Yellow SE4G and Foron Blue RDGLN dyes by Asgher *et al* (2006). Besides using wastewater from dyeing industries, microbial population capable of decolorizing dyes have also been reportedly isolated from soil samples. A consortium RVM 11.1 was isolated by Moosvi *et al* (2005) from soil contaminated with dyes wastewater and decolorized 94% of Reactive violet 5. Another, consortium from effluent contaminated soils in the vicinities of dyestuff manufacturing units decolorized 90% Direct Red 81 dye (Junarrkar *et al* 2006). While consortium JW-2, isolated from soil samples of dye contaminated sites by Moosvi *et al* (2007) completely decolorized Reactive violet 5R and also efficiently decolorized nine out of 10 dyes (Ponceau Red GR, Procion Golden Yellow HR, Reactive Navy Blue HER, Reactive Blue MR (RBMR), Reactive Black B, Reactive Violet 5R, Red HE8B, Procion Red H7B, Reactive Yellow FG and Reactive Golden Yellow).

In our study we observed that among the three consortium/mixed cultures capable of decolorizing dyes two consortiums namely SKB-I and SBK-II which were developed from the sludge samples of an effluent treatment plant of dyeing industry were most efficient in decolorization of Direct Red 28, Direct Red 7, Acid Blue 113, Direct Blue 53, Reactive Orange 107 & Reactive Red 120 than the third consortium; Con-3 which was developed from a site contaminated with organic compounds but not dyes. Interestingly, in contrast to usual reports of usage of contaminated sites (wastewater and soil) for the development of mixed cultures, cattle dung was also used by Sarnaik and Kanekar (1995) to isolate bacterial strains for dye decolorization. Apart from activated sludge and soil, natural Asphalt was used by Khalid *et al* (2008) to isolate azo-dye degrading bacteria capable to decolorize azo dyes: Acid Red 88, Reactive Black 5, Direct Red 81 and Disperse Orange 3. Therefore it appears that microbial populations capable of decolorizing azo dyes could be developed from various natural sources although the possibility for isolating efficient azo dye decolorizers would be high from sources contaminated with aromatic compounds.

- *Decolorization of dyes by pure bacterial isolates*

Apart from consortiums, individual bacterial strains isolated from contaminated sites for dye decolorization have also been reported. Nigam *et al* (1996a) obtained aerobic bacterial cultures from aerated columns inoculated with textile and soil samples of effluent sites and anaerobic cultures from the dye-effluent samples. Bacterial cultures were isolated from domestic sewage treatment plant, paper mill effluent treatment plant and tannery effluent treatment plant by Padmavathy *et al* (2003) to decolorize dyes Red RB, Remazol Red, Remazol Blue, Remazol Violet, Remazol Yellow, Golden Yellow, Remazol Orange and Remazol Black. Sharma *et al* (2004 b) isolated bacterial strains from effluent disposal sites of textile processing industries capable of decolorizing 75% of Acid Violet-17 dye (30 mg/L) while some other isolated bacterial strains capable of decolorizing azo dyes present in soil/sludge of waste disposal sites of local textile industries, completely decolorized 20 mg/L of Acid Red 88 (Khehra *et al* 2005). Halotolerant and halophilic bacteria were isolated by Asad *et al* (2007) to decolorize high concentrations (5000 and 10000 ppm) of dyes: Remazol Black B, Maxilon Blue, Sulphonyl Scarlet BNLE, Sulphonyl Blue TLE, Sulphonyl Green BLE, Remazol Black N, and Entrazol Blue IBC while bacterial strain (SL186) showed 90% decolorization of textile azo dyes Reactive Red 3B-A, Reactive Black 5, and Reactive Yellow 3G-P (Joe *et al*; 2008). Similarly bacterial strains capable of completely decolorizing Direct Red 5 B (Jadhav *et al*; 2008) and reactive textile dye Red BLI (50 mg/L) (Kalyani *et al*; 2008) have been reported.

Microbial diversity in mixed cultures has been observed by many workers. Predominantly gram negative bacteria have been implicated and shown to be involved in the decolorization of the dyes. Among the most predominant are *Pseudomonas sp.* (Sarnaik and Kanekar 1995, Senan and Abraham 2004), *Alcaligenes sp.* (Nigam *et al* 1996a; Sharma *et al* 2004 b; Mutafov *et al* 2006), *Commamonas sp.* (Nigam *et al* 1996a; Jadhav *et al* 2008), *Klebsiella pneumoniae* (Wong and Yun 1998), *Sphingomonas xenophaga* (Coughlin *et al* 1999), *Proteus mirabilis* (Chen *et al* 1999; Dafale *et al* 2008a), *Burkholderia cepacia* (Laszlo 2000), *E.coli* (Chang and Lin 2001), *Xenophilus azovorans* (Blumel *et al* 2002), *Citrobactor sp.* (An *et al* 2002), *Aeromonas sp.* (Chen *et al* 2003; Sharma *et al* 2004 b; Ren *et al* 2006; Khalid *et al* 2008), *Vibrio logei* and *Pseudomonas nitroreducens (*Adedayo *et al* 2004), *Pseudomonas sp.* (Khehra et al 2005; Dafale *et al* 2008a; Khalid *et al* 2008; Kalyani *et al* 2008), *Stenotrophomonas acidaminiphila* (Khehra *et al* 2005), *Shewanella decolorationis* (Xu *et al* 2006)*, Halomonas sp.* (Guo *et al* 2007), *Rhodobacter sphaeroides* (Dafale *et al* 2008a) and *Massillia sp.* (Khalid *et al* 2008).

The bacterial consortium SKB-II developed in this study comprised of *Bacillus sp.* (*Bacillus megaterium, Bacillus vallismortis, Bacillus pumilus*, *Bacillus cereus,* and *Bacillus subtilis)* presumably due to the fact that the sludge sample of the effluent treatment plant had a pH between 9-13. Similarly *Bacillus sp.* has been isolated for dye decolorizations by Whurmann *et al* 1980, Zissi *et al* 1997, Sharma *et al* 2004 b; Khehra *et al* 2005, Pourbabee *et al* 2005; Khalid *et al* 2008 and Dafale *et al* 2008 a&b. Other gram positive population like *Kurthia sp.* (Sani and Banerjee 1999 a), *Nocardia corallina* (Azmi *et al* 1998) *Clostridium sp.* (Kim *et al* 2002; Joe *et al* 2008), *Paenibacillus sp.* (Meehan *et al* 2001; Moosvi *et al* 2007; Ramya *et al* 2007), *Rhodococcus erythropolis* (Mutafov *et al* 2006), *Micrococcus sp.* (Moosvi *et al* 2007), *Enterococcus faecalis* (Handayani *et al* 2007), *Exiguobacterium sp*. (Dhanve *et al* 2008) have also been isolated for dye decolorization.

- *Decolorization of different types of azo dyes with different chemical structures at varying concentrations by mixed bacterial culture*

The wastewater streams from dyeing industries carry differential loads of different types of dyes based upon the schedule and process in the industry. Upon obtaining the mixed bacterial culture for decolorization, the ability of the same was evaluated to decolorize different types of azo dyes at varying concentrations as the waste water streams carries differential loads of different types of dyes from dyeing industries which varies from day to day and even hour to hour based on the schedule and process in the industry is therefore difficult to characterize. The developed mixed bacterial culture comprising of five *Bacillus sp.* decolorized four out of the six selected azo dyes (concentration of 10 to 100 mg/L) efficiently. Moosvi *et al* (2005) working with dye Reactive Violet 5 at concentration of 50 to 400 ppm observed increase in decolorization with increase in initial dye concentration upto 200 ppm (3.55 mg/l/h) by bacterial consortium RVM 11.1 (consisting of three gram-negative rod-shaped bacteria and four gram-negative coccobacilli) beyond which there was reduction in decolorization rates. Junarrkar *et al* (2006) also showed increase in decolorization with increase in initial dye concentration up to 200 ppm (2.29 mg/L/h) with dye Direct Red 81 (50 to 400 mg/L concentration) using bacterial consortium NBNJ6 however further increase in dye concentration resulted in reduction in decolorization rates. Moosvi *et al* (2007) showed decolorization of various dyes: Ponceau Red GR (90%), Procion Golden Yellow HR (69.2), Reactive Navy Blue HER (90%), Reactive Blue MR (5%), Reactive Black B (88.5%), Reactive Violet 5R (92%), Red HE8B (87.2%), Procion Red H7B (93.3%), Reactive Yellow FG (88.8%), Reactive Golden Yellow R (66.3%) by consortium JW2 (comprising of three bacterial isolates *Paenibacillus polymyxa, Micrococcus luteus and*

Micrococcus sp.) which increased proportionately with increase in initial dye concentration up to 100 ppm but further increase in dye concentration resulted in reduction in decolorization rates.

Results of this study showed that the mixed bacterial culture decolorized dyes with different chemical structures however, contrary to the above reports increasing dye concentrations led to decrease in decolorization efficiency of our mixed bacterial culture. Similar experiences have been reported by Sani and Banerjee (1999a) who showed that dyes were easily decolorized at concentration of 10 mM by *Kurthia sp.*, but color removal was reduced when dye concentration was increased to 30 mM. The decrease in decolorization has been attributed to the color reduction capability of the cells due to three reasons (1) reduction in the transfer of the dye through the cell membrane and the its accumulation on the cell surface (Chen *et al* 1999) (2) blocking of the active sites of the enzymes involved in the decolorization and degradation of the dyes (Isik & Sponza 2004) and (3) decrease in cell growth, due to the dye toxicity causing the inhibition of metabolic activities such as DNA synthesis or cell growth (Wuhrmann *et al* 1980; Chen *et al* 2003a; Asad *et al* 2007; Silveira *et al* 2008).

Furthermore, the ability of the mixed bacterial culture to decolorize different dyes with different chemical structures was studied since the effluent treatment plants whether at the individual factory or at the community level would always have streams of various dyes being mixed and so the final effluent being received for treatment would always have a mixture of dyes present. Thus, the microbial population to be used as inoculum in the treatment process for removing color from these effluents must have the capability to decolorize/degrade dyes of different colors and different chemical structures. Mixed bacterial culture comprising of five *Bacillus sp.* (*Bacillus megaterium, Bacillus vallismortis*, *Bacillus pumilus*, *Bacillus cereus* and *Bacillus subtilis*) decolorized mixture of four structurally diverse azo dyes to a lesser extent in combination as compared to the decolorization of individual dye compounds. The variation in decolorization by the microbial culture for the different dyes may be attributed to the structural diversity of the dyes as has been postulated by Zimmermann *et al* (1982), Paszcezynski *et al* (1992), Zhang *et al* (1995) and Moosvi *et al* (2005) and/or due to the lack of enzyme system or redox mediators required for metabolism of the dyes and their metabolites (Ambrosio and Campos-Takaki 2004). Junarrkar *et al* (2006) showed range of decolorizing efficiency of bacterial consortium NBNJ6 to decolorize different dyes with different chemical structures: Reactive Black B, Reactive Blue 172, Reactive Violet 5R, Reactive

Red 5B and Reactive Black RL from 85 to 89% and dyes: Ponceau 4R (500 mg/L), Raspberry Red (1000 mg/L), Tartrazine (300 mg/L) and Sunset Yellow FCF (300 mg/L) from 84–95%. Similarly, consortium JW-2 (comprising of *Paenibacillus polymyxa, Micrococcus luteus* and *Micrococcus sp.*) developed by Moosvi *et al* (2007) showed rapid complete decolorization within 24 hrs for dyes: Reactive Navy Blue HER, Reactive Violet 5R, Red HE8B, Procion Red H7B, Reactive Yellow FG, Reactive Black B (89 to 92%) but dyes: Procion Golden Yellow, Reactive Golden Yellow were decolorized between 60 to 65% while RBMR showed only 5% decolorization.

In this research, among the four azo dyes Direct Red 28 had an amino group and Direct Red 7 had a hydroxyl group while Acid Blue 113 had sulphonic group whereas Direct Blue 53 had both amino as well as hydroxyl group in the structure. It has been shown that azo dyes with hydroxy or amino group are degraded better than with a methyl, methoxy, sulpho or nitro group (Zimmermann *et al* 1982 and Hu 1994). Our results also show that Acid Blue 113 due to the presence of sulphonic group was decolorized less efficiently than Direct Red 28, Direct Red 7 and Direct Blue 53 and is in agreement with Wuhrmann *et al* (1980) and Chen *et al* (2003a) who also showed that azo dyes with sulphonic acid groups, affect the DNA synthesis of the microbial population and thus show less decolorization. Nigam *et al* (1996a) also showed differential rates of decolorization among the various dyes mixture used: two dyes, Disperse Navy D2GR (slow decolorization of 80% within 5 days) and Remazol Turquoise Blue G 133 dye were decolorized by only 20% while Cibacron Red C-2G, Remazol Red RB and Remazol Golden Yellow RNL were completely decolorized within 24-30 hrs along with dyes Remazol Navy Blue GG and Cibacron Orange CG which required 48 hrs and Remazol Blue B, 54 hrs respectively for decolorization.

Contrary to these reports, Hsueh and Chen (2007) showed that sulfonic acid group located at appropriate position (e.g., para to azo bond) could enhance azo dye decolorization by *P.luteola* (>95% color removal efficiency of methyl orange for 400 mg/L at 20 hrs) and therefore concluded that biodegradability or recalcitrance is due to combined effects of chemical nature of dyes and biochemical characteristics towards the dyes. Six strains isolated by Khalid *et al* (2008) efficiently decolorized a mixture of four dyes azo dyes: Acid Red 88, Reactive Black 5, Direct Red 81 and Disperse Orange 3 (25 mg/L each) but at a differential rate. Complete decolorization of the dye mixture was achieved with *Shewanella putrefaciens* within 4 hrs while *Aeromonas punctata* decolorized after 8 hrs and the other four strains *Bacillus cereus*, *Bacillus thuringeinsis*, *Pseudomonas nitroreducens* and *Massillia timonae* partially decolorized the mixture between 69 to 89% after 16 hrs. Furthermore, strain *Shewanella putrefaciens* completely decolorized the mixture of dyes: Acid Red 88 and Direct Red 81 (100 mg/L), Reactive Black 5 and Disperse Orange 3 (100 mg/L), Acid Red 88, Reactive Black 5 and Direct Red 81 (300 mg/L) and mixture of Reactive Black 5 and Direct Red 81 (500 mg/L). *Clostridium bifermentans* decolorized Reactive Red 3B-A efficiently followed by Reactive Black 5 and after 36 hrs both these dyes were decolorized completely. However dye Yellow 3 G-P showed 80% decolorization after 36 hrs. Sani *et al* (1999a) reported that dyes with simple structures and low molecular weights exhibit higher decolorization rates, whereas color removal is more difficult with highly substituted, high molecular weight dyes.

- *Decolorization of real raw untreated effluent by mixed bacterial culture*

The potential of the our mixed bacterial culture to decolorize real raw untreated effluent showed that higher decolorization was attained with low effluent concentration in the basal medium and as the raw untreated effluent concentration was increased from 1 to 10% the decolorization rate decreased drastically hence, the results of our study is in corrobation with the reports of O'Neill (1999); Moosvi *et al* (2005) and Junarrkar *et al* (2006) that rate of decolorization is inversely proportional to the initial dye concentration and the structure of various dyes present in the industrial effluent can also be accounted for the lower decolorization efficiency of the microbial culture. Chung and Stevens (1993) reported that the concentration of dye influences the decolorization efficiency of the microbial culture as the dyes and their degradation products may be toxic at higher concentrations.

- **Techno economic evaluation of nutritional and process parameters for the decolorization of the wastewater from the textile printing and dyeing industry using packed bed column**

Textile industry releases gallons of wastewater that needs treatment before disposal and treatment of such huge volumes in batch scale is not practical. For continuous aerobic degradation of dye effluents bioreactors having organisms in attached or suspended biological systems are required. Bioreactors such as rotating biological contactors, fluidized bed bioreactor, packed bed bioreactors, flocculated cell reactors, bubble column reactors, stirred tank reactors and air lift reactors have been reported by many researchers.

A commonly applied technique is the use of immobilized biofilm (Hutchins *et al* 1986; Bryers *et al* 1990) which capitalizes on the ability of mixed culture of microorganisms to adhere to inert support and form biofilm on the support materials (matrix) as the physical attachment provides high loading rate and prevent biomass washout. Other advantages of immobilized biofilm are lower land use, less capital outlay, recycling of sludge, greater stability and potential to degrade toxic chemicals faster than conventional wastewater treatment systems as immobilized cultures are more resistant to environmental disturbances than the free cells (Rajaguru *et al* 2000; Panswad and Luangdilok 2000, Kapdan *et al* 2003; Buitron *et al* 2004). Bishop *et al* (1986) reported that fixed biofilm treatment processes may be more amenable to removal of xenobiotic organics than suspended growth systems as the biofilm can support different populations which degrade different substrates at different points within the biofilm. Tall columnar packed bed bioreactors containing stationary bed of media which provides a large surface area for biological growth (Lee *et al* 2004) have been used for the decolorization of Direct Red 28 and real raw untreated effluent in the research. Similarly, Senan *et al* (2003) also reportedly used aerobic packed bed reactors for continuous aerobic decolorization of seven azo dyes: Direct yellow 86, Basic Orange, Reactive Green 19, Direct Blue 54, Reactive Blue 171, Reactive Red 141 and Acid Red 260 by microbial consortium comprising of two isolated strains (RRL & TVM) and one known strain of *Pseudomonas putida*.

- *Immobilization of mixed bacterial culture on various matrices*

For biofilm formation by immobilized microbial cultures variety of matrixes have been studied by many researchers. The criteria for the selection of matrixes were sturdiness, inertness (should not absorb the dyes), and resistance to biodegradation. Nigam and Marchant (1995) used abundantly and cheaply available substrates *viz.* mineral kissiris, α-biofixation material, polyethylene pieces, nylon-web, polyurethane foam, vermiculite, thermocol chips, gravels and calcium alginate beads for the development of biofilm for decolorizing textile effluent by bacterial consortium PDW, in an up-flow biofilm reactor. Similarly, Kapdan *et al* (2000) reported that metal mesh particles supported better immobilization and decolorization efficiency of *Coriolus versicolor* MUCL as compared to foam, wood ash, wood chip, stone and polyurethane foam. Rajaguru *et al* (2000) used plastic beads of 2 mm diameter as support material to immobilize bacteria for the development of up-flow fixed bed column reactor for the decolorization of azo dyes Orange G, Amido Black 10B, Direct Red and Congo Red. Ability of *Trametes versicolor*, by surface immobilization on wheat straw, jute, hemp, maple, woodchips and nylon and polyethylene tetraphthalate fibers out of which jute, straw and hemp proved to be best support materials for decolorization of Amaranth by Shin *et al* (2002). Various cheap and sturdy matrixes: coir, jute, laterite pebbles, polyurethane foam, polystyrene foam, coconut pith, straw, wood, plastic, foam rubber, glass, charcoal, polyethylene, plastic were screened for bacterial immobilization by Senan *et al* (2003) for aerobic decolorization of azo dyes (Direct yellow 86, Basic Orange, Reactive Green 19, Direct Blue 54, Reactive Blue 171, Reactive Red 141 and Acid Red 260). Sharma *et al* (2004 a & b) used seashells and brick pieces for treating Acid Violet 15 in up-flow immobilized reactors. Moutaouakkil *et al* (2004) immobilized cells of *E. agglomerans* in calcium alginate, polyacylamide, cooper beech and vermiculite to decolorize 100 mg/L of Methyl Red from synthetic water by using a fluidized bed bioreactor. In 2006, Khehra *et al* used polyurethane foam as immobilization support to decolorize azo dye Acid Red 88.

In our work, low cost and easily available matrixes *viz.* plastic clips, thermocol, marble chips and foam were tried and tested for immobilization of the mixed bacterial culture SKB-II comprising of five *Bacillus sp.* It was observed that thermocol proved to be a poor matrix for biofilm formation while in case of foam, although the mixed culture was able to form biofilm on this matrix however during the dye treatment through the bioreactor the dye solution was adsorbed to this matrix inspite of being saturated with the dye solution prior to filling in the bioreactor. Among the four matrixes studied, plastic clips and marble chips proved better and equally good in sustaining microbial growth and were also nontoxic, non-biodegradable and mechanically more stable than thermocol and foam.

- *Decolorization of Direct Red 28 dye at various operational parameters (aeration rate and hydraulic retention time) by the stable biofilm formed on matrices plastic clips / marble chips*

Immobilization of mixed bacterial culture was checked by washout stabilization (the outflow of suspended biomass was noted by Biuret method). In continuous operation, washout stabilization at high hydraulic retention time was achieved in shorter time period as compared to washout stabilization achieved at low hydraulic retention time with both the matrixes (plastic clips and marble chips) as low hydraulic retention time increases the microbial cell washout that come along in the sample collected from the outlet of the bioreactor. During decolorization process, initial hours of dye decolorization was not accounted due to the mixing of effluent with the contents of the reactor and also to avoid the accounting of the decolorization contributed by matrix adsorption of dye which is negligible in a continuous flow fixed bed process as has reported by Chen *et al* (2005). Among the various operational parameters with marble chips as matrix, maximum decolorization of 93 to 91% was observed at HRT of 8.16 hrs along with aeration rates of 0.4 and 0.6 mmoles/min. And with plastic clips as matrix, at HRT of 11 hrs with aeration rates of 0.4 and 0.6 mmoles/min., 69 to 73% decolorization was observed. In both cases (plastic clips and marble chips) decolorization declined at HRT of 7.33 and 5.5 hrs for plastic clips and HRT of 5.44 and 4.08 hrs for marble chips as low HRT (i.e.rapid flow) affects the anchoring of the biofilm on the support particles and the resultant turbulence limits the growth of the biofilm and increases the detachment of biofilm, resulting in cell wash out and also the time for contact between the dyes and the organism at lower hydraulic retention time is less thereby resulting in comparatively lesser decolorization efficiency of the process (Bishop *et al* 1995).

Our results that low hydraulic retention time affects the time required for washout stabilization and overall decolorization efficiency of the reactor to decolorize the respective dyes is in agreement with Senan *et al* (2004) who using Laterite pebbles in packed bed bioreactor, showed maximum degradation of seven azo dyes mixture (concentrations: 25, 50, and 100 µg) at a HRT of 6.23 hrs (flow rate of 34 ml/h) as compared to a HRT of 1.41 & 0.78 hrs (flow rates of 150 & 270 ml/h). Decolorization when compared across the two aeration rates (0.4 and 0.6 mmoles/min) was observed to be higher at aeration rate of 0.6 mmoles /min. (93% with marble chips and 73% with plastic clips) due to the aerobic nature of the mixed bacterial culture. These results are in agreement with Yang *et al* (2004) who showed that in a rotating biological contactor (RBC) higher rotational speed of 40 rpm provided better aeration resulting in increased decolorization efficiency of phthalocyanine dye Basic Blue 22 (C.I. 61512), by the white-rot fungus *Phanerochaete sordida* ATCC90872 and also of Faison and Kirk (1985) who reported that the increase in rotational speed from 10 to 40 rpm increased the decolorization from 35% to 77%. Coughlin *et al* (2002) showed higher rotational speeds provided better aeration resulting in increased decolorization efficiency in treating Acid

Orange 7, in an aerobic biofilm reactor and complete biodegradation of the dye's breakdown products including biomineralization of the aromatic amine: 1-amino-2-naphthol. In our work, during the decolorization process it was found that there was simultaneous adsorption and desorption of the dyes onto the microbial biomass and onto the starch present in the medium from the random increase and decrease in the percentage of decolorization which is in corrobation with the observations of Senan and Abraham (2004).

Towards the end of the experiment decrease in color removal efficiency was observed which can be attributed to the blockage of active sites of azoreductase enzymes leading to inhibition of enzymatic reduction of azo bonds due to continuous flow of the dye into the bioreactor (Isik and Sponza 2004). Another factor responsible for the decreased decolorization rate might be increased toxic potential of azo dyes and their reduced aromatic metabolites at higher dye concentrations (Gottlieb *et al* 2003). Decrease in color removal efficiency was followed by cell lysis of the mixed culture as continuous addition of the azo dyes will shift more biofilm from thicker to thinner, and more toxic the dye, the more obvious is the shift as toxins can slow down the microorganism's respiration rate which influences the substrate conversion rate. Prolonged exposure to toxic compounds can slow down microorganism growth rates and even lead to changes in the composition of the microbial population (Lewandsowski 1987).

The UV–VIS spectra of the input feed stream and the output feed stream showed a shift from the maximum of absorption to shorter wavelengths upon mixed bacterial culture treatment which indicates that the decolorization of dye solution Direct Red 28 occurred by degradation in addition to the visual observation of the biosorption process. Spectral shifts towards shorter wavelengths after the treatment with mixed bacterial culture were probably produced by the biodegradation of the dye molecule that led to a decrease of the conjugation effects between the aromatic rings. Moreover, no dye was found adsorbed, on the support material in the abiotic control bioreactor, suggesting that dye decolorization was mainly due to the biological activity of the microbial consortium immobilized in the bioreactor.

- **Characterization of wastewater to quantify the reduction in the color as well as the total COD and BOD load of the wastewater**

Decolorization of real untreated effluent procured from local dyeing house at operational parameters (0.6 mmoles/min of aeration and hydraulic retention time of 21 hrs.) by the stable biofilm formed on matrices marble chips was studied.

Bioreactor based on plastic clips as immobilization support showed lower decolorization efficiency as compared to bioreactor based on marble chips due to the lack of rough surfaces as observed on the SEM of sterile un-inoculated marble chips. Overall, the bioreactor based on marble chips as immobilization support proved to be cost effective and an efficient pilot-scale treatment system. In light of these observations, marble chips was selected as immobilization support for mixed bacterial culture SKB-II for treating real raw untreated effluent at an optimized parameter of 0.6 mmoles/min of aeration and hydraulic retention time of 8.16 hrs. and which showed 34% decolorization of 5% real raw untreated effluent. The analysis of the pH, TDS and BOD/COD of the real raw untreated effluent before and after the treatment through the bioreactor showed that pH was reduced to 6.8 from 9.56 as initial value; TDS was reduced to 578 mg/L from a high initial value of 2293 mg/L, a total of 78.4% reduction in BOD (Initial BOD of 1860mg/L was reduced to 402mg/L) load was observed. And in case of COD, 75.5% reduction load (initial value of 3600 mg/L was reduced to 880 mg/L) was observed after the treatment of real raw untreated effluent through the bioreactor this may be due to the presence of another group of dyes and dissolved solids in the textile and dye-stuff effluent as was also observed by Sani and Banerjee 1999

CONCLUSION

1. Among the three mixed bacterial cultures tried and tested, SKB-II proved to be the most efficient. Out of the six tested dyes (at concentration of 10 mg/L) efficient decolorization was observed with four dyes. Dyes' being deficient in carbon source, starch was supplemented in the basal medium along with NaCl for salinity conditions. Results with low starch (0.65 g/L) and NaCl concentration (0, 2%) showed decrease in decolorization rates thereby proving that 1.3 g/L starch and 4% NaCl were essential to achieve higher decolorization rates by the culture.
2. The five bacterial constituents *B. pumilus, B. megaterium, B.cereus, B. vallismortis* and *B. subtilis* isolated from consortium SKB-II could efficiently decolorized azo dye Direct Red 28 only and least decolorization was observed with other dyes even on extended incubation. Therefore, further research work was carried out with mixed culture rather than the pure bacterial isolates.
3. The mixed bacterial culture comprising of five *Bacillus sp.* efficiently decolorized four out of the six individual azo dyes (concentration of 10 to 100 mg/L). However, with mixture of four structurally diverse azo dyes, decolorization was lesser in combination as compared to the decolorization of individual dye compounds. Apart from decolorization by live cultures, low or negligible rates of decolorization were observed by the heat killed cells kept as control thereby indicating that microbial decolorization was preceded primarily by biological degradation.
4. Among the two (real raw untreated effluent procured from local dyeing house and textile dyeing industry), mixed culture was able to decolorize effluent procured from local dyeing house and showed higher decolorization with low effluent concentration in the basal medium and as effluent concentration was increased the decolorization rate decreased drastically.
5. In bioreactor studies, marble chips in contrast to plastic clips efficiently decolorized azo dye Direct Red 28 dye and real raw untreated effluent procured from local dyeing house at higher at aeration rate of 0.6 mmoles /min. and low hydraulic retention time. However, even after several trials real raw untreated effluent from textile industry was not decolorized by the mixed culture.
6. The treatment of the wastewater through the bioreactor reduced the BOD/COD load of real raw untreated effluent to 78.4% and 75.5% respectively.

APPLICATION

As is known; that Azo dyes constitute the largest (60–70% of all textile dyes produced) and most important class of commercial dyes being used, the mixed bacterial culture SKB-II (comprising of five bacterial isolates viz. *Bacillus sp.*) developed in this research efficiently decolorization of four out of six structurally different azo dyes (individually and as well as mixture and real raw untreated effluent from local dyeing house) under aerobic conditions. Normally, dye concentration in the effluent varies within a narrow range of 0.1–0.2 g/L (O'Neill *et al* 1999) and SKB-II could decolorize dyes much above the reported dye concentration and therefore, is highly promising for applications involving bio-decolorization of azo dyes. The ability of the culture to utilize starch as cheap co-substrate and NaCl for salinity conditions for dye decolorization gives it an advantage for treatment of textile industry wastewaters.

Microbial immobilization on biofilm system, using packed bed bioreactors with economically/abundantly available support materials such as plastic clips and marble chips resulted in high-efficiency decolorization of azo dye Direct Red 28 and real raw untreated effluent procured from local dyeing house. The high specific decolorization rate obtained and the simplicity of the immobilization method suggest that this technique would be suitable for using the developed aerobic mixed bacterial culture SKB-II to remove azo dyes from wastewater on an industrial scale

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ANNEXURE

Bushnell Haas Broth (g/L)

$MgSO_4$	0.20 gm
$CaCl_2$	0.02 gm
KH_2PO_4	1.00 gm
K_2HPO_4	1.00 gm
Soluble Starch	1.3 gm
Sodium chloride	40 gm
pH	7

The total volume was made upto one litre. pH was set with 10 N NaOH

Solutions used for Gram Staining

Aqueous Crystal Violet (1%)	1 g Crystal violet in 100 ml distill water
Gram's Iodine	2 g potassium iodide in 100 ml distill water
Decolorizer	Acetone (50%) and alcohol (50%)
Aqueous Safranin (2%)	2 g Safranin in 100 ml distill water

Reagents used for COD

1) Standard potassium dichromate solution (0.0417 M or 0.25 N, dissolved 12.259 g primary standard grade K2Cr2O7, previously dried for 2h at 103ºC in 1l distilled water)
2) Sulphuric acid reagent (5.5 g Ag2SO4 /kg H2SO4 was dissolved. 1-2 days was required for complete dissolution of Ag2SO4)

3) 0.25 N Ferrous ammonium sulphate standard reagent (dissolved 98 gm Fe(NH4)2(SO4)2 in water and added 20 ml H2SO4. Final volume was made up to 1L. This solution is standardized daily against 0.25 N K2Cr2O7 solution)
4) Ferrion indicator (dissolved 1.48 g 1,10-orthophenanthroline and 0.7 g FeSO4.7H2O in 100 ml-distilled water).
5) Mercuric sulphate (HgSO4, crystal or powder is used).
6) Potassium hydrogen phthalate (KHP) standard (Dissolve 425 mg KHP in distilled water and dilute to 1000 ml. KHP has theoretical COD of 1.176 mg O2/mg).

<u>Reagents used for BOD</u>

1) Phosphate buffer solution: Dissolved 8.5 g KH2PO4, 21.75 g K2HPO4, 33.4 g Na2HPO4.7H2O, and 1.7 g NH4Cl in 500 ml distilled water and diluted to 1 L (pH-7.2).
2) Magnesium sulfate solution: Dissolved 22.5 g MgSO4.7H2O in distilled water and diluted to 1L.
3) Calcium chloride solution: Dissolved 22.5 g CaCl2 in distilled water and diluted to 1L.
4) Ferric chloride solution: Dissolved 0.25 g FeCl3.6H2O in distilled water and diluted to 1L.
5) Acid and alkali solutions: 1N H2SO4 and 1N NaOH, for neutralization of caustic or acidic waste samples.
6) Glucose-glutamic acid solution: Reagent-grade glucose and reagent-grade glutamic acid was dried at 103ºC for 1h. Added 150-mg glucose and 150 mg glutamic acid to distilled water and diluted to 1L.
7) Manganous sulfate solution: Dissolved 364 g MnSO4.H2O in distilled water, filter and diluted to 1L.
8) Alkali-iodide-azide reagent: Dissolved 500 g NaOH and 135 g NaI in distilled water and diluted to 1L. Added 10 g NaN3 and dissolved in 40 ml-distilled water.
9) Concentrated H2SO4
10) Starch: Dissolved 2g soluble starch in 100-ml hot distilled water.
11) Standard sodium thiosulfate titrant: Dissolved 6.205 g Na2S2O3.5H2O in distilled water. Added 1.5 ml 6N NaOH or 0.4-g solid NaOH and diluted to 1000 ml.

Standardize with bi-iodate solution.

12) Standard potassium bi-iodate solution 0.0021 M: Dissolved 812.4 mg KH (IO3) in distilled water and diluted to 1000 ml.

Biochemical reagents

Biuret reagent

NaOH	150g
$CuSO_4.5H_2O$	1.05g
MQ water	500ml

Dissolve NaOH in ice-bath and add Cupric Sulphate solution slowly with continuous stirring. Make up the final volume. Reagent discarded if black or reddish precipitation persisted.