Microbial Decolourization of Textile dye Compounds.

GIFT

A DISSERTATION SUBMITTED TO THE
DEPARTMENT OF BOTANY, UNIVERSITY OF DHAKA
BANGLADESH, FOR THE AWARD OF THE
DEGREE OF MASTER OF PHILOSOPHY

448756

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Session 2003-2004

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ACKNOWLEDGEMENTS

I am extremely grateful to almighty Allah for giving me the opportunity to carry out this project.

The work presented here was carried out in the Laboratory of Microbiology, Department of Botany, University of Dhaka and I am grateful to Professor Abul Hasan, Chairman, Department of Botany, University of Dhaka for providing with the laboratory facilities.

I express my profound gratitude to Professor Mihir Lal Saha, Department of Botany, University of Dhaka for suggesting me the problem and his supervision, valuable suggestions and tremendous help throughout the duration. I wish to express my sincerest gratitude to Professor Mahbubar Rahman Khan, Department of Botany, University of Dhaka, for his continuous guidance, and encouraging attitude in carrying out my research work.

I would like to extend my hearty thanks to all of my lab-mates and attendant who helped me in various ways.

Above all, I would like to express my deepest gratitude to almighty Allah for His mercy, blessing and guidance all through my career.

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Certificate

This is to certify that the thesis entitled '**Microbial decolourization of textile dye compounds**' has been prepared and submitted to the Department of Botany, University of Dhaka by Julia Yesmin under our supervision.

It is further to certify that the research work embodied in the thesis, is original and suitable for awarding an M.Phil. degree in Botany.

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LIST OF ABBREVIATIONS

%	Percent
&	And
α	Alpha
ß	Beta
µg	Microgram
µm	Micrometer
^{0}C	Degree celcius
cfu	Colony forming unit
cm	Centimeter
Co.	Company
Conc.	Concentration
Ed.	Editor
ed.	Edition
e.g.	For example
et al	and others
etc.	Etceteras
Fig.	Figure (s)
g	Gram
h	Hour (s)
i.e.	That is
Inc.	Incorporation
L or l	Liter
mg	Milligram
min	Minute
ml	Milliliter
M.R	Methyl Red
N	Normal Concentration

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NA	Nutrient agar
MNA	Modified Nutrient Agar
NaCl	Sodium Chloride
NH_4^+	Ammonium
No.	Number
NO_2^-	Nitrite
NO_3^-	Nitrate
O. D.	Optical Density
pH	Negative Logarithm of hydrogen ion concentration
P	Phosphorus
pp	Pages
M.R	Methyl Red
NA	Nutrient agar
NaCl	Sodium Chloride
Sec	Second
U.V	Ultraviolet
V.P	Voges-Proskauer
viz.	Namely
Vol.	Volume
w/v	Weight per volume

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Abstract

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Abstract

Textile industry is one of the important industries of Bangladesh. Textile dyes an essential component in the textile industries poses a real hazard to the environment and to the public health. Most of the industrial effluents are discharged into the river or in crop field without any treatment, which may cause severe pollution with carcinogenic and mutagenic substances. The potentiality of our indigenous and friendly microorganisms towards the decolouraization of textile dye compounds was investigated. In the present study textile dyeing effluents were collected as samples from Fatullah, Narayanganj. Dhaka, Bangladesh. Seven textile dyes viz. Red MEGBL, Orange ME2RL, Turquoise Blue BGF, Turquoise Blue RSPL, Violate S3RL, Yellow 4G and Green3R were used as test dye compounds.

The pH and temperature of the samples varied between 6.85 to 10.52 and 36°C to 38°C respectively. Two different media nutrient agar and modified nutrient agar were used for the isolating of aerobic bacteria present in the effluent samples.

85 strains were isolated from the textile dyeing effluents and finally 25 strains were selected.

Selected 25 isolates were studied in detail for identification. Out of 25 isolates 21 were Gram positive and 4 were Gram negative aerobic bacteria. Gram positive aerobic bacteria belonged to a single genus *Bacillus*. The different identified *Bacillus* were *B. badius, B. lentus, B. pumilus, B. mycoides, B. cereus, B. subtilis, B. globisporus, B. fastidiosus* and *B. megaterium, B.firmus, B. thuringiensis.*

Out of 21 Gram positive aerobic bacteria, two strains were found to resemble the reference strain of *B. badius*, four were similar to *B. firmus*, three strains were

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identified as *B. lentus*, three resembled *B. pumilus*, two strains were identified as *B. mycoides*.

Four Gram negative strains were found to be the members of the genus *Pseudomonas*. The different identified species of the genus *Pseudomonas* were *P. aeruginosa* , *P. alcaligenes* and two strains were under the species *P. pseudoalcaligenes*.

Out of 25 strains only 2 strains viz. *B. cereus* and *B. subtilis* were able to decolourize Red ME6BL and Orange ME2RL completely within 16 h. *B. lentus* and *B. globisporus* were also capable of decolourizing Tururquoise Blue BGF upto 50% to 60% in mono culture within 3-5 days.

In mixed culture selected three dyes were completely decolourized within 2-3 days. Waste samples used as inocula were found to decolourize dye compounds within 24 hrs.

Mixed culture was found to be better dye decolourizer than than the mono culture.

The present finding clearly indicated that in near future textile dyeing effluents can be treated biologically by proper designing of the traditional activated sludge system.

Under the humid tropical conditions of Bangladesh, residual dyes in textile dyeing effluents undergoes degradation and microbial consortia might play an important role towards this degradation and their potentiality can be exploited to clean up our environment.

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Chapter-1
Introduction

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Introduction

Textile is a running business in Bangladesh and growing rapidly. There are about 300 dyeing and printing industries, 63 textile mills and 32 composite textile mills are involved in this business (Bangladesh Textile Mill Corporation and BBS 1997-98). Most of the industrial effluents are discharged into the river or in crop field without any treatment, which may cause severe pollution with carcinogenic and mutagenic substances.Textile industry has a major impact not only on the nation's economy but also on the environmental quality of life in many communities.

Textile manufacturing consumes a considerable amount of water (Approx. 100 L of H_2O $kg^{-1}$of textile materials) its dyeing, finishing, and manufacturing processes. Considering both the volume generated and the effluent composition, the textile industry wastewater is rated as the most polluting among all industrial sectors.

In textile industry, production is carried on different processes using natural and synthetic raw materials such as organochlorine based pesticides, heavy metals, pigments and dyes. This cause complex and dynamic structure of environment impact of textile industry and it not only affect the characterization and quantity of wastewater but also make it being impossible to focus on a typical kind of wastewater and a standard purification technology(O'Neill *et al.* 1999).

Textile industry waste has been categorized into four types: dispersible wastes, hard-to- treat wastes, high-volume wastes, hazardous and toxic wastes. Of them, hard-to-treat textile wastes include synthetic dyes, metals, phenols, certain surfactants, toxic organic compounds, pesticides, and phosphates. All dyes used in the textile industry are designed to resist fading upon exposure to sweat, light, water, many chemicals including oxidizing agents, and microbial attack and contribute to aquatic or soil or air toxicity when released (Alexander, 1965; Vaidya and Datye, 1982).

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Dyes and pigments from printing and dyeing operations are the principal sources of color in textile effluent. They vary in structure and may contain one or more functional groups capable of forming a covalent bond between the carbon atoms of the dye and the hydroxyl groups in the cellulose (Laszlo, 1996). Dyes and pigments are highly colored materials used in relatively small quantities (a few percent or less of the weight of the substrate) to impart color to textile materials for aesthetic or functional purposes (AATCC, 1981), In typical dyeing and printing processes, *50* to 100 % of the color is fixed on the fiber, and the remainder is discarded in the form of spent dyebath or in wastewater from subsequent textile washing operations into wastewater streams (Wagner, 1993).

Synthetic dyes are widely used in textile and related industries. The presence of different chromophoric groups like azo, triphenyl methane and phthalocyanine are responsible for their structural diversity (Pagga and Brown, 1986; Bakshi *et al.* 1999), moreover, due to structural variation dyes fall into either the cationic or the anionic type. Anionic dyes are the direct, acid and reactive dyes (Mishra and Tripathy, 1993).

Brightly colored, water soluble reactive and acid dyes are the most problematic, as they tend to pass through conventional treatment systems unaffected (Willmott *et al.* 1998). Nonionic dyes refer to disperse dyes because they do not ionize in an aqueous medium (Weber and Wolfe, 1987). Baughman and Perenich (1988) demonstrated that azo- and nitro-compounds are reduced in sediments, and similarly, Chung *et al.* (1978) illustrated their reduction in the intestinal environment, resulting in the formation of toxic amines. Anthraquinone-based dyes are most resistant to degradation due to their fused aromatic ring structure.

Sulfonated and unsulfonated azo dyes not only have a negative aesthetic effect on the wastewater but some of these compounds and biodegradation products are toxic,

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carcinogenic and mutagenic (Grover *et al.* 1996). There exists a clear relationship between their chemical structure and their potential danger. All the azo dyes containing a nitro group were found to be mutagenic (Chung and Cerniglia, 1992), and a high toxicity of these azo dyes was observed for methanogenic granular sludge (Donlon *et al.* 1997). Furthermore, some azo dyes can produce toxic degradation products like 1, 4- phenylenediamine, 1-amino2-naphthol, benzidine and substituted benzidines like tolune (Chung *et al.* 1981; Reid *et al.* 1984; Rosenkranz and Klopman, 1990).

Water pollution and its effects

Water pollution is a state of deviation from pure conditions partially, wholly or largely as a by-product of human activity through direct or indirect effects of changes in energy patterns, chemical and physical composition in nature and abundance of organisms. The function and properties affecting the quality of water is of vital concern for humanity, since it is directly linked with human welfare. Water supply sources like ground water and surface water are related and interconnected by the hydrological cycles. The quality of life on earth is linked inextricably to the overall quality of the environment. Whether water is used as a habitat or to meet drinking and irrigation demands, the maintenance of quality of water is crucial for the survival of life (Khan, 1986).

Textile effluent

Textile industries consume large amount of water and chemicals for wet processing of textiles. The chemical reagents used are very diverse in composition, ranging from inorganic compounds to polymers and organic products (Banat *et al.* 1996). Textile effluents are mostly discharged into the environment after minimal pretreatment with a high amount of pollutants (Oxspring *et al.* 1993).

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In general, dye house effluent is grey (in pretreatment process) or colored (in colored treatment), high in temperature, chemical oxygen demand (COD), total dissolved solids (TDS), total suspended solid (TSS) and at times highly alkaline. Dye wastes and other effluents from textile processing can cause problems like foaming, color persistence, have abnormally high pH, temperature and heavy metal concentrations and variations in the hydraulic flow rates (Buckley, 1992).

Dyes are one major sources of metals like; Cd, Cr, Co, Cu, Hg, Ni, Mg, Fe and Mn. (Wagner, 1993). Sediments and suspended solids particles are important repositories for trace metals in wastewater (Chapman *et al.* 1982). The pollutants aggravated by the presence of free chlorine and toxic heavy metals, cause rapid depletion of dissolved oxygen leading to "oxygen sag' in the receiving water. These pollutants are known to destroy microorganisms that lead to a reduction in the self-purification capacity of the stream (Ademoroti *et al.* 1992).

Textile dyes are of environmental interest because of their wide spread use. Color, as contributed by textile industry due to the usage of various dyes, is another form of pollution. It not only gives an undesirable appearance to the receiving water bodies but it also interferes with the transmission of sunlight into streams, thereby decreasing photosynthetic activity. The color of the effluent is harmful for agricultural use and rearing of fish. Moreover, disposal of wastewater on an open land contaminates the subsoil water in the area so much that drinking water gives color as well as bad taste (GOP. 1983).

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Fig. 1.0 (A-B) Photographs showing different sampling sites.

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Dyes

Dyes contain chromophores, delocalized electron systems with conjugated double bonds, and auxochromes, electron-withdrawing or electron-donating substituents that cause or intensify the color of the chromophore by altering the overall energy of the electron system. Usual chromophores are -C=C-, -C=N, C=O, N=N-, $-NO_2$ and quinoid rings and auxochromes are $-NH_3$, -COOH, $-.SO_3H$ and —OH. (Van der Zee, 2002).

Brief history of dyes & their origin

Ever since primitive people could create, they have been endeavoring to add color to the world around them. They used natural matter to stain hides, decorate shells and feathers, and paint their stories on the walls of ancient caves. The dyes used were derived from natural sources and dyes like Tyrian purple, were particularly prized as an important part of the local economy and world trade at that time (Chippindale and Tacon 1998). Scientists have been able to date the black, white, yellow and reddish pigments made from ochre used by primitive man in cave paintings to over 15,000 BC. Earliest written record of the use of dyestuffs in China is 2600 BC. In 327 BC, Alexander the Great mentions 'beautiful printed cottons" in India. By 715 BC, wool dyeing was established as craft in Rome (Welham, 2000).

Organic natural colorants have also a timeless history of application, especially as textile dyes. These dyes are all aromatic compounds, originating usually from plants (e.g. the red dye alizarin from madder and indigo from woad) but also from insects (e.g. the scarlet dye kermes from the shield-louse *Kermes vermillo),* molluscs, fungi and lichens. Until the middle of the 19th century, all colorants applied were from natural origin. The use of natural dyes (mainly anthraquinone, indigoid, flavenol, flavone or chroman compounds that can be used as mordant, vat, direct, acid or

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solvent dyes) in textile- processing operations was/is very limited (Dyer, 1977; Severkow, 1988).

The first recorded synthetic dye was picric acid, which was produced in the 1770's from the interaction of indigo and nitric acid. Synthetic dye manufacturing started in 1856, when the English chemist WPFL 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 Mauveine. By the early 1880's, diazotization was a known reaction, that used to synthesize commercially useful dyes. In the beginning of the 20th century, synthetic dyestuffs had almost completely supplanted natural dyes (Welham, 2000).

During the ensuing century of dye development, thousands of synthetic colorants have been produced and used commercially. Traditionally, dye manufacturers' goals have been to produce low-cost dyes with high tinctorial value, brilliance, and good application and fastness properties; in particular, high resistance to washing, cracking, light, oxidation (ozone), reduction (gas fading), chlorine attack, acids and alkalis, etc. Dye research has been focused on dyes with improved stability, thus more resistant to treatment (Van der Zee 2002).

Classification of Textile dyes in Color index (C.I.)

The vast array of commercial colorants (mostly synthetic) divided into 15 classes in terms of color, structure and application method in the Color Index (C.I.) by the Society of Dyers and Colorists and the American Association of Textile Chemists and Colorists. The Color Index (3rd Edition, issue 2) lists about 28,000 commercial dyes names representing 10,500 different dyes, 45,000 of which are currently produced.

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Acid dyes

The largest class of dyes in the Color Index is referred to as Acid dyes (230 different acid dyes listed, ~40% of them are in current production). Acid dyes are anionic compounds that are mainly used for dyeing ions) like wool, polyamide, silk and modified acryl. Most acid dyes are azo (yellow to red, or a broader range colors in case of metal complex azo dyes) and anthraquinone or triarylmethane (blue and green) compounds.

Reactive dyes

The reactive group is often a heterocyclic aromatic ring substituted with chloride or fluoride, e.g. di-chloro-tri-amine. Another common reactive group is vinyl sulfur. In the Color Index, the reactive dyes form the second largest dye class with respect to the amount of active entries. About 600 of the 1050 different reactive dyes listed are in current production. The problem of coloured effluents is therefore mainly identified with the use of reactive dyes. Most (80%) reactive dyes are azo or metal complex azo compounds but also anthraquinone and naphthalocyanine reactive dyes are applied, especially for green and blue colours.

Metal complex dyes

Among acid and reactive dyes, many metal complex dyes can be found (not listed as a separate category in the Color Index). These are strong complexes of one metal atom (usually chromium, copper, cobalt or nickel) and one or two dye molecules respectively. Metal complex dyes are usually azo compounds. About 1/6 of all azo dye listed in the Color Index are metal complexes (Brown, 1987) but also naphthylamin metal complex dyes are applied.

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Direct dyes

Direct dyes are relatively large molecules with high affinity for especially cellulose fibers. Van dot Waals forces make them bind to the fiber. Direct dyes are mostly azo dyes with more than one azo bond. In the Color Index, the direct dyes form the second largest dye class with respect to the amount of different dyes.

Basic dyes

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

Mordant dyes

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

Disperse dyes

Disperse dyes are scarcely soluble dyes that penetrate synthetic fibers (cellulose acetate, polyester, polyamide, acryl, etc.). This diffusion requires swelling of the fiber, either due to high temperatures (>120 °C) or with the help of chemical softeners. Disperse dyes form the third largest group of dyes in the Color Index: about 1400 different compounds are listed, of which 40% is currently produced. They are

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usually small azo or nitro compounds (yellow to red), anthraquinones (blue and green) or metal complex azo compounds (all colours).

Pigment dyes

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

Vat dyes

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

Production and discharge statistics of dyes

The dye industry is characterized by a large number of producers (about 2000 worldwide), just four Western companies accounted for nearly half of the market in 2000 (Will *et al.* 2000). It is estimated that over 10,000, different dyes and pigments

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are in common use (McMullan *et al.* 2001). The total world organic colorant production by 1990 was about 950,000 tons/years and of them 54% textile dyes, 15% other substrate dyes e.g., leather, paper, 25% organic pigments and 6% organic fluorescent brightening agents and dyes for specific applications (Easton 1995). India, Russia, Eastern Europe, China, South Korea and Taiwan consume approximately 600 thousand tons of dyes per annum. Since 1995, China has been the leading producer of dyestuffs, exceeding 200 kilo ton per annum (Ishikawa *et al.* 2000). In 1999 the value of the global dyestuff market was estimated at 6.6 billion US$, North America accounting for 1.2 billion US$, Central and South America for 0.7 billion US$, Western Europe for 1.2 billion US$ and Asia for 2,7 billion US$. The distribution of global dyestuff market has changed during the last decade, with Asia being the largest dyestuff market (about 42% (Will *et al.* 2000).

To judge the relative share of the different dye classes in the wastewater of textile producing industries, dye consumption data should be considered together with the degree of fixation of the different dye classes (Table 1.1). It was estimated that about 10-15% of dyes are lost into the wastewater during the dyeing process (McMullan *et al.* 2001). Approximately 75% of the dyes discharged by Western-Europe textile processing industries belong to the classes of reactive (36%), acid (25%) and direct (15%) dyes, all of which are dye classes with mostly azodyes. So, it can be expected that azodyes make up the vast majority of the dyes discharged by textile processing industries. Anthraquinone dyes are second largest class (15% of the entries in the Color Index), followed by triarylmethanes (3%) and phthalocyanines (2%) (Easton, 1995).

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Table 1.1 Estimated degree of fixation for different dyes/fiber combination.

Dyes Class	Fiber	Degree of fixation %	Loss of effluent %
Acid	Polyamide	80-95	5-20
Basic	Acrylic	95-100	0-5
Direct	Cellulose	70-95	5-30
Disperse	Polyester	90-100	0-10
Metal complex	Wool	90-98	2-10
Reactive	Cellulose	5 0-90	10-50
Sulfur	Cellulose	60-90	10-40
Vat	Cellulose	80-95	5-20

Dyes as Environmental pollutant

Many dyes are visible in water at concentrations as low as 1 mg/l. Textile-processing wastewaters, typically with a dye content in the range 10-200 mg/l are therefore, usually highly colored. As dyes are designed to be chemically and photolytically stable, they are highly persistent in natural environments. The release of dyes may therefore present an ecotoxic hazard and introduces the potential danger of bioaccumulation (may eventually affect man by transport through the food chain.) Moreover, various dye are also one of the major sources of heavy metals (Wagner, 1993) in water and soil and at times, they can be phototoxic (Raskin *et al.* 1994) and due to their persistent nature cause a misbalancing in the ecosystem.

Dyestuff toxicity has been investigated in numerous researches. The toxicity (mortality, genotoxicity, mutagenicity and carcinogenicity) studies diverge from test with aquatic organisms (fish, algae, bacteria, etc.) to tests with mammals including human beings. Furthermore, research has been carried out to analyze the effects dye stuffs and dye containing effluents on the activity of both aerobic and anaerobic bacteria in wastewater treatment systems. The acute toxicity of dyestuffs is generally low (Little and Chillingworth, 1974; Greene and Baughman, 1996).

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Chronic effects of dye stuffs, especially of azo dyes, have been studied for several decades. Azo dyes in purified form are seldom directly mutagenic or carcinogenic, except for some azo dyes with free amino groups. However, reduction of azo dyes, leads to formation of aromatic amines and several aromatic amines are known mutagen and carcinogens to human beings (Table 1.2) (Brown and DeVito, 1993).

Table 1.2: Aromatic amines and potential dye metabolites that may be considered human carcinogens.

Aromatic Amine Group	**Human Carcinogen Evidence**
1-Napthylamine	Slight Mixed
2-Naphthylamine	Good
2,5-Diaminotolune	Slight
3,3-Dichlorobenzidine	Slight/Mixed
3,3-Dimethoxybenzidine	Slight/Mixed
3,3-Dimethylbenzidine	Slight
4-Biphenylamine	Good
4-4-Nifrobiphenyl	Slight Mixed
4,4-Methylenebis (2-chloroanilinc)	Slight
Auramine	Slight
Benzidine	Good
Magenta	Slight
N-Phenyl 1 -2-napthylamin	Some
N,N-Bis (2-chloroethyl)-napthylamine	Good

In mammals, metabolic activation (reduction) of azo dyes is mainly due to bacteria anaerobic parts of the lower gastrointestinal tract. Various other organs liver and the kidneys, can, however, also reduce azo dyes. After azo dye reduction in the intestinal tract, the released aromatic amines are absorbed by the intestine and are excreted in

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the urine. The acute toxic hazard of aromatic amines is carcinogenesis, especially bladder cancer. In 1975 and in 1982, the International Agency for Research on Cancer (IARC) summarized the literature on suspected azo dyes, mainly amino-substituted azo dyes, fat-soluble azo dyes and benzidine azo dyes, but also a few sulphonated azo dyes (IARC, 1975).

Generally stated, genotoxicity is associated with all aromatic amines with benzidine moieties, as well as with some aromatic amines with toluene, aniline and naphthalene moieties. The toxicity of aromatic amities depends strongly on the spatial structure of the molecule or in other words the location of the amino-group (s) (Cartwright, 1983). The toxicity of aromatic amines depends furthermore on the nature and location of other substituents. As an example, the substitution with nitro, methyl or methoxy groups or halogen atoms may increase the toxicity, whereas substitution with carboxyl or sulphonate groups generally lowers the toxicity (Chung and Cerniglia, 1992).

Techniques used in the removal of dyes

Various physical, chemical and biological pre-treatment, main treatment and post treatment techniques can be employed to remove color from dye containing wastewaters. Several factors that determine the technical and economic feasibility of each single dye removal technique include dye type and its concentration, wastewater composition, operation costs (energy and material), environmental fate and handling costs of generated waste products. In general, each technique has its limitations. The use of one individual process may often not be sufficient to achieve complete depolarization. Dye removal strategies consist therefore mostly of a combination of different techniques (Cooper, 1993; Grau, 1991; Vandevivere *et al.* 1998). In past years studies, dealing with treatment of textile wastewater, several high rate anaerobic/aerobic reactors (Biological and non- biological) including; baffled reactor

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(Bell *et al.* 2000), two stage up flow anaerobic sludge blanket (UASB) (Chinwekitvanich *et al.* 2000), GAC amended UASB + combined sequential anaerobic sludge reactor (SCAS) (Kuai *et al.* 1998), fluidized bed reactor (FBR) (Seshadri *et al.* 1994), up flow anaerobic filter (UAF) (Oxspring *et al.* 1996), Sequencing batch reactors (Lourenco *et al.* 2000) and filter reactors (Basib and Forster, 1997) were used. However, these different reactors showed different dye removal abilities for different dye due to their own limitations.

Biological techniques

Biological dye removal techniques are based on microbial biotransformation of dyes. Many researchers have demonstrated partial or complete biodegradation of dyes by pure and mixed cultures of bacteria, fungi and algae. In the 1950's, there was a strongly held view amongst microbiologists, often called the 'principle of microbial infallibility', that all chemicals were susceptible to microbial degradation if the right organisms am conditions could be provided. The metabolic capabilities of microbes were 'all powerful' The growing realization that many chemicals like dyes, which found their way into the environment, were not being degraded and were, persisting for many years, put an end to such thinking (Alexander, 1965).

Biotechnology, through the study of microorganisms, that are capable of resisting and surviving in polluted environment, provides the knowledge for bioremediation. Bioremediation is a pollution control technology that uses biological systems to catalyze the degradation or transformation of various toxic chemicals to less harmful forms. This natural process, bioremediation, includes bioengineering the capabilities of intrinsic microorganisms, to clean up the environment, is an effective alternative to conventional remediation methods (Vidali, 2001).

The general approaches to bioremediation are to enhance natural biodegradation b1

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native organisms (intrinsic bioremediation), to carry out environmental modification b applying nutrients or aeration (biostimulation) or through addition of microorganisms (bioaugmentation), Unlike conventional technologies, bioremediation can be carried out on-site moreover; it is cost-effective (Atlas and Unterman, 1999).

The different mechanism supposed to be involved in the bioremediation of textile dyes and related xenobiotic chemicals are; biodegradation by direct and indirect extracellular enzymes (Kulla, *et al.* 1983; Fewson, 1988), Bioaccumulation: in intracellular milieu am then their further assimilation in living beings specifically microbes (Kuhn and Pfister 1990), Biotransformation: which involves the detoxification of a chemical or metal by transforming its physical state (by enzymatically or by any chemical reaction) (Ishibashi *et al.* 1990), Bioadsorption: through ion exchange by living and dead microbial biomass (Zhau and Zimmerman 1993). Sometimes, a single biological mechanism of an organism may work out for the remediation of any chemical or at times, a combination of any of the aforementioned mechanisms in different organisms can go along together in a series.

Bacterial biodegradation

Investigations to bacterial dye biotransformation have so far mainly been focused to the most abundant chemical class of dyes i.e., azo. The electron withdrawing nature of the azo linkages obstructs the susceptibility of azo dye molecules to oxidative reactions (Fewson, 1988). Therefore, azo dyes generally resist aerobic bacterial biodegradation. Only bacteria with specialized azo dye reducing enzymes (azoreductase) were found to degrade azo dyes under fully aerobic conditions (Ganesh *et al.* 1994; Pagga & Taeger, 1994; Coughlin *et at.* 1999; Quezada *et al.* 2000). This anaerobic reduction implies decolorization as the azo dyes are converted to usually colorless but potentially harmful aromatic amines. Aromatic amines are generally not further degraded under anaerobic conditions. Anaerobic treatment must

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therefore be considered merely as the first stage of the complete degradation of azo dyes (Brown, 1981; Brown and Laboureur, 1983). The second stage involves conversion of the produced aromatic amines. For several aromatic amines, this can be achieved by biodegradation under aerobic conditions (Konopka, 1993; Russ *et al.* 1994).

The term azo dye reduction comprises different mechanisms (Brown and Devito, 1993). It occurs either by direct specific (e.g. azoreduses) and non-specific enzymes under anaerobic conditions by anaerobic and facultative aerobic bacteria (Kulla *et al.* 1983; Zimmermann *et al.* 1984) or by indirect means by different enzymatically produced redox mediators compounds like ($FADH_2$, $FMNH_2$, riboflavin (Gingell and Walker, 1971), NADH (Nassar and Magdy, 1997) and NADPH-generating system (Semde *et al.* 1998). Asides from these mechanisms, it is also possible that azo dyes are purely chemically reduced by biogenic bulk reductants like sulfide (Van der Zee, 2002). However, research has revealed that enzymatic anaerobic azo dye reduction is more or less a fortuitous reaction, catalyzed by enzymes (e.g. hydrogenases) which are usually used for other reaction (Rafii *et al.* 1990; Rafii and Cerniglia 1993).

Aims and objectives

- Isolation of aerobic heterotrophic bacteria from the selected Textile dyeing industrial areas.
- Characterization of the selected organisms for their possible identification.
- Decolourizing capability of the isolated bacteria.
- Screening of bacterial strains on the basis of their decoloureizing potentiality.

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Chapter - 2
Materials & Methods

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MATERIALS AND METHODS

2.1 Sampling Site

There are many textile dyeing industries are situated at Fatulla, Narayangonj. Textile industry is one of the important industries in Bangladesh. Fotullah was selected for the isolation of the bacteria associated with textile dyeing effluents.

2.2 Collection of Samples

Samples were collected from the selected sites of Fatulla textile dyeing areas. (Table 3.1). During sample collection, plastic bottles, markers, pen, field notebook, polythene bags and thermometer were taken to the sampling sites for the purpose of sampling. Samples were collected in plastic bottles. The temperatures of the samples were recorded carefully at the time of sampling. After collection, the samples were labelled and transferred to the laboratory as soon as possible. Collected samples were preserved in a refrigerator at 4°C.

2.3 Measurement of pH of the samples

The pH of each sample was measured in the laboratory with an electric pH meter (Jenway 3310 pH meter, U. K.) immediate after collection of samples.

2.4 Preservation of the samples

Collected samples were preserved in a refrigerator before and after the bacteriological and physico-chemical analysis.

2.5 Media and techniques for the enumeration and isolation of bacteria

2.5.1 Culture media used

Nutrient agar (NA) and modified nutrient agar (MNA) media were used for the enumeration and isolation of aerobic heterotrophic bacteria present in effluent.

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The pH of the medium was adjusted to 8.5. Since most of the samples were within the range of 8.0-9.5. The pH was adjusted before the addition of agar and sterilization.

Modified nutrient agar: Nutrient agar was modified with textile dyeing effluent instead of distilled water. Textile dyeing effluent was autoclaved at 121 ^{0}C then filtered before used.

2.5.2 Techniques employed

Two different techniques were used for the enumeration and isolation of bacteria. These techniques were as follows:

1. Dilution plate technique
2. Spread plate technique

2.5.2.1 Dilution plate technique:

For the enumeration and isolation of bacteria serial dilution plate technique was carried out (Greenberg *et al.* 1980). One ml of sample was diluted (1:100) with 99 ml sterile distilled water in a sterile conical flask and shaken well. This suspension was then used to prepare serial dilutions. One ml of this suspension was transferred to 9 ml of sterile water for ten-fold (1:10) dilution and further diluted up to 10^5 dilutions. Plating in duplicate plates were made for each diluted sample. One ml of each of the diluted sample was taken in a sterilized Petri plate by sterilized Pasteur pipette. Then molten agar medium was poured and mixed thoroughly by rotating the Petri plate, first in one direction and then in the opposite direction. After setting the medium the plates were inverted and incubated at 37°C for 24 hrs in an incubator (Memmert Gm b H + Co Kg 8540 Sehwabach).

2.5.2.2 Spread plate technique

Like dilution plate technique spread plate technique was also used for the isolation and enumeration of bacteria. The technique described by Sharp and Lyles (1969) was followed for this purpose. For this purpose 0.1 ml sample was directly poured

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on the centre of solidified and dried NA medium and spread over by a glass spreader backward and forward while rotating the plate. The plates were incubated in an incubator at 37°C for 24 h.

2.6 Enumeration of bacteria

After 24 h of incubation the plates having well spaced colonies were selected for counting. The selected plates were placed on a colony counter (Digital colony counter, DC-3, OSK 10086, Kayagaki, Japan) and the colonies were counted.

2.7 Isolation of bacteria

Isolation of selected well discrete aerobic heterotrophic bacterial colonies were carried out immediately after counting. On the basis of their colonial morphology, different colonies were randomly selected for isolation.

The selected colonies were marked and studied for various characters *viz.* color, form elevation, margin, surface, optical characters etc. (Eklund and Lankford 1967, Bryan 1950). Then the marked and observed bacterial colonies were transferred on nutrient agar slant for further studies.

2.8 Purification of the isolates

The selected isolates were purified through repeated plating (by streaking and dilution plate methods). When a plate yielded only one type of colonies the organisms were considered to be pure.

2.9 Maintenance and preservation of isolates

The purified isolates were then transferred on nutrient agar slant. The slants were kept in polythene bags and preserved as stock culture in a refrigerator at 4°C for further study. Periodical transfer of isolates on agar slants was done for maintaining viability of the organisms.

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2.10 Morphological observation of isolated strains

For the identification of selected isolated strains, following morphological characters were studied and recorded.

2.10.1 Colonial morphology

The bacterial colonies on plating medium were morphologically studied as their form, elevation, margin, surface, pigmentation, opacity, whether grown inside, at the bottom or on the surface of the medium and their rate of growth.

2.10.2 Appearance on NA & MNA slants

The selected bacteria were transferred to NA and MNA slants. The appearance of their growth on slants such as rhizoidal, spreading, adherent or slimy etc. were studied.

2.11 Preparation for microscopic examination of bacterial cells

Bacterial cell suspension was made by using fresh culture with physiological saline. The prepared suspension was used to make smear. A good quality glass slide was used for this purpose. Thin smear was prepared on the clean and oil free slide. The smear was allowed to dry in air and was fixed by passing the slide over the flame of a spirit lamp. The following two different staining methods were employed to stain the fixed smears.

1. Simple staining method and
2. Differential staining method.

2.11.1 Simple staining (Bryan 1950)

For this purpose different dyes such as crystal violet, basic fuchsin, mythelene blue, saffranin and mercurochrome were used. Each fixed smear was flooded with each dye solution for one minute. The flooded smear was washed off with water and dried in air.

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2.11.2 Differential staining

Staining procedures that make visible the differences between microbial cells or parts of cells were termed as differential staining (Pelczar *et al.*1993). For this purpose fixed smear was exposed to more than one dye solution. In our study two differential techniques were used *viz.* Gram staining and spore staining.

2.11.2.1 Gram staining

This is one of the most important and widely used differential staining techniques and which is also considered as one of the important steps in identifying an unknown bacterium. For Gram staining, the procedure as described by Claus (1995) was followed.

The fixed smear was treated with the following solutions and after application of each solution; slide was gently washed off with water.

Crystal violet 30 sec., Lugol's iodine solution 60 sec., 95% Ethyl alcohol 15-25 sec., Mercurochrome solution 60 sec. The slide was blot dried and observed under microscope. The results were recorded as Gram positive (blue-violet) and Gram negative (light red).

2.11.2.2 Spore staining

The method described by Claus (1995) was applied. In spore staining, 20 h old culture was used. The fixed smear was flooded with 5% aqueous solution of malachite green and heated on a hot brass plate for about 15 minutes but boiling was strictly avoided. The excess dye was washed off and basic fuchsin was used as a counter stain for 60 sec. The slide was washed gently, dried and examined under microscope. Morphology and position of the spores and sporangia were studied and properly recorded.

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2.11.3 Microscopic observation

The size and shape of vegetative cells of selected strains were observed. The arrangement of cells whether single or in chains or clusters were carefully recorded. Gram-reaction and spore formation of the isolates were also studied and recorded. Photomicrographs of the observed cells as well as stage micrometer of the same magnification were taken using microscope (Nikon Meirophot) with photographic attachment (Nikon, FX 35 WA, Japan).

2.11.4 Measurement of bacterial cells

Vegetative cells of the selected isolates were measured by comparing photomicrographs of bacterial cells with that of the scale of stage micrometer. The compared photographs of bacterial cells and stage micrometer were of same magnification (Objective 100X and Projection lense 5X). One division of the scale as found on the photograph was equal to 20 mm which is actually equivalent to 10 μm.

2.12 Physiological and biochemical studies of the isolates

Following Bergey's Manual (Sneath *et al.* 1986, 4th vol. 9th ed.) the physiological and biochemical tests of the isolated bacteria were carried out. Along with Bergey's Manual several other manuals such as Manual of Microbiological Methods (SAB 1957), Microbiological Methods (Collins and Lyne 1984) and Understanding Microbes (Claus 1995) were also consulted.

2.12.1 Acid production from carbohydrate (Gordon 1966)

The selected strains were tested for their ability to produce acid from different carbon source. For this purpose inorganic nitrogen base agar medium was used. Bromocresol purple as an indicator and 1% carbon source was added to this medium. Xylose, arabinose, mannitol, glucose were used as carbon source. All these were sterilized by membrane filter (pore size 0.45 *μm).* The medium was poured into the sterilized petri-plates and allowed to solidify. Inoculation was done by point inoculation method and incubated at 37°C for 5 days. Acid production

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from carbohydrate was determined by the acid color (yellow) of the indicator around the colony.

2.12.2 Catalase test (Claus 1995)

Catalase is an enzyme produced by and found in essentially all actively growing microorganisms capable of using oxygen for respiration. Catalase breaks apart the hydrogen peroxide into water and molecular oxygen.

$$2H_2O_2 \xrightarrow[\text{Catalase}]{} 2H_2O + O_2 \uparrow$$

To demonstrate catalase activity, test organisms were taken by a sterilized loop on microscope slide and a drop of hydrogen peroxide was added to each of them. Production of bubbles indicated the positive result i.e. production of catalase.

2.12.3 Deep glucose agar test (SAB 1957)

Microorganisms vary widely in their requirements for oxygen. The nature of microbial growth in agar deeps reflects the cells' relative need for oxygen or an oxygen free environment. In relation to free oxygen, organisms are generally classified as strict aerobes, microaerophiles, facultative anaerobes and strict anaerobes. A tube of deep glucose agar medium was inoculated in fluid condition approximately at 45°C. The tube was rotated to mix the inoculum with the medium and was allowed to solidify.

After incubation at 37°C for 7 days observation was made to find out whether the organisms grew on the surface and in the upper layer of the medium (strict aerobes), or the organisms grew just a few millimeters below the surface (microaerophiles), or the organisms grew throughout the medium (facultative anaerobes), or the organisms grew deeper in the medium (strict anaerobes).

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2.12.4 Degradation of tyrosine (Sneath *et al.* 1986)

Tyrosine agar plates were inoculated with a single streak and were incubated at 37°C for 7 days. Clearing of tyrosine crystals around and below the growth revealed degradation of tyrosine.

2.12.5 Egg-yolk lecithinase test (SAB 1957)

The yolk was diluted with equal volume of 0.85 percent NaCl and 1 ml of yolk suspension was added to each 9 ml of the medium which was sterilized before addition of egg. Egg-yolk agar plates were inoculated with a single streak and were incubated at 37°C for 24 hrs. The characteristic reactions obtained were due to principally to production of lecithinase.

2.12.6 Growth response at different concentrations of NaCl (Sneath *et al.* 1986)

For the salt tolerance test of the isolates, nutrient broth with different concentrations of NaCl i.e. 0, 2, 5, 7 and 10% were used. Test tubes were inoculated with 24 h bacterial culture and incubated at 37°C for 48 hrs. Turbidity of the inoculated broth indicated the growth of the isolates.

2.12.7 Growth response at different temperature (Sneath *et al.* 1986)

To test the growth response at different temperatures organisms of 24 h culture were inoculated in the nutrient broth and incubated at different temperatures *viz.* 5, 10, 30, 40 and 50°C. Growth of the organisms were recorded after 48hrs of incubation. Turbidity of the inoculated broth indicated the growth of the isolates.

2.12.8 Growth response at different pH (Sneath *et al.* 1986)

For testing the growth response of the isolates at different pH, buffered peptone water broth (in 4: 1 ratio) was adjusted to a wide range of pH i.e. 4.5, 6.5 and 8.5. Buffer solutions used were of the composition showed below (Williams *et al.*1971)

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pH of the buffer	Name and quantity of the chemical(s) used (g)	Volume of deionized water (ml)
4.5	KH_2PO_4- 5.45	200
6.5	KH_2PO_4- 2.72 K_2HPO_4- 1.74 Na_2HPO_4-1.39	200
8.5	K_2HPO_4- 3.48 Na_2HPO_4- 2.78	200

pH of the buffer solutions was checked after preparation. At each step, medium was adjusted with N/10 HCl or N/10 NaOH solution, as required. Growth was recorded after 72 hrs incubation.

2.12.9 Gas production from carbohydrates (SAB 1957)

Gas production from carbohydrates or fermentation test is of considerable significance in the identification and classification of bacteria. In the study of fermentation, D-glucose (monosaccharides) was used.

Fermentation tubes with the above carbohydrate and sugar alcohol were made using bromothymol blue as indicator. One Durham's tube was introduced in each of the test tubes. The tubes were then inoculated in duplicates with 24 hours old culture suspension with the help of sterilized pipette and incubated at 37°C for 48 h.

The change of colour of the indicator from blue-green to colourless or yellow showed the production of acid. No change in colour indicated negative reaction. If carbon dioxide was produced it would be collected in the Durham's tube.

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2.12.10 Hydrolysis of gelatin (Collins and Lyne 1984)

Many organisms possess the enzyme gelatinase which is capable of hydrolyzing gelatin resulting in its (gelatin) loss of property of gel formation. For this test, nutrient gelatin (5% gelatin) agar plates were inoculated and incubated at 37°C for 24 h.

After incubation, the inoculated plates were flooded with $HgCl_2$ which formed white opaque precipitate with the unchanged gelatin but liquefaction was demonstrated by clear zone around the bacterial growth.

2.12.11 Hydrolysis of casein (Collins and Lyne 1984)

Casein is a milk protein. Casein comprises about 85% of the total protein in milk. Many microorganisms have the capacity to hydrolyze casein. This test demonstrates the ability of microbes to degrade casein into soluble peptides and amino acids by the enzyme caseiase.

I ml of sterilized milk was taken in a sterilized petri-plate and then melted agar medium was poured and mixed thoroughly. After solidifying, the plates were inoculated and incubated at 37°C for 72 h. Formation of a clear, transparent zone around the growth indicated hydrolysis of casein.

2.12.12 Hydrolysis of starch (Claus 1995)

Organisms capable of hydrolyzing starch to form monosaccharide or disaccharide possess the enzyme amylase. This test revealed the presence or absence of the enzyme amylase in the organisms. For this test, starch-agar plates were inoculated with test organisms and the plates were incubated at 37°C for 48 h.

After incubation, the surface of these plates was flooded with iodine solution. Development of a clear zone around the growth indicated starch hydrolysis.

2.12.13 Levan test (Schaad 1988)

Levan is a poly-fructose which is an extra cellular capsular substance produced through the action of the enzyme levan sucrase. Levan formation is detected on

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nutrient agar plate to which 5% sucrose (w/v) was added. Inoculated plates were incubated at 37°C.

After 3-5 days incubation the presence of convex white mucoid colonies indicate levan formation.

2.12.14 Methyl red test (Bryan 1950)

Methyl red test is the test for mixed acid fermentation of glucose by microorganisms. Excreted acid contains large amount of formic, acetic, lactic and succinic acid and causes a major decrease in pH that can be detected by "Methyl Red" indicator.

For this test VP/ MR broth was inoculated and incubated for 5 days at 37°C.

After incubation 5-6 drops of methyl red indicator was added to the culture broth. Red color throughout the broth indicated positive reaction whereas yellow or any yellowish red indicated negative reaction.

2.12.15 Motility test

Motility test was carried out by two methods as described below:

(i) Wet mount method

(ii) Semi-solid medium method

2.12.15.1 Wet mount method

In this method, slide was prepared by placing bacterial suspension on a clean slide and covered by a cover glass. The edge of the cover glass was sealed with nail polish and the bacterial cells were examined under phase contrast microscope. Movement of the bacterial cells revealed the motility of bacteria and the result was recorded.

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2.12.15.2 Semi-solid medium method (Eklund and Lankford 1967)

In this method, tubes of semi- solid agar medium were inoculated by stabbing the medium to a depth of 5 mm with the help of a straight wire loop. The tubes were then incubated at 37°C for 24 h. Growth of the organism throughout the medium indicated the motility of the organism while non motile organism was confined to the stab region.

2.12.16 Nitrate reduction test (SAB 1957)

Nitrate reduction is evident by complete or partial disappearance of nitrate accompanied by appearance of nitrite, ammonia or free nitrogen.

This test was performed to observe the organisms capability on the reduction of nitrate to nitrite. The formation of nitrite indicated the presence of the enzyme nitrate reductase in the organisms. The following three reagents were required for this test:

Reagent A: Sulfanilic acid - acetic acid solution:

Sulfanilic acid	8.0 g
5N acetic acid	1000 ml

Sulfanilic acid was dissolved in acetic acid and stored in brown glass bottle.

Reagent B: Dimethyl-a-napthalamine solution:

Dimethyl-α-napthalamine	6.0 ml
5N acetic acid	1000 ml
Stored in brown glass bottle.	

Reagent C: Zinc dusts

The tubes of nitrate broth in duplicates were inoculated with test organisms and then incubated at 37°C for 72 hrs. After incubation, 1 ml of reagent A was added

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to the incubated tube and shaken. Then 1 ml of reagent B was also added to each tube and shaken well.

Formation of a distinct red or pink color indicated the reduction of nitrate to nitrite. Absence of nitrite may be due to complete conversion of nitrate as well as no reduction at all. A pinch of zinc dust was then added to the tube showing absence of nitrite and it was allowed to stand for a few minutes. Any remaining nitrate (in case) would be reduced to nitrite by zinc and the characteristic pink or red color would appear and no color indicated complete reduction.

2.12.17 Oxidase test (Claus 1995)

To perform this test filter papers were soaked in 1% aqueous tetramethyl-phenylenediamine dihydrochloride. Fresh young culture was rubbed on the filter paper with a clean glass rod. Results were recorded within 10 seconds.
Blue colour indicated a positive result.

2.12.18 Phenylalanine deaminase test (Sneath *et al.* 1986)

For this test, phenylalanine agar slant tube was inoculated with 24 h culture and incubated at 37°C for 3 days. After incubation a few drops of 10% ferric chloride solution was added to slopes. A green colour indicated positive reaction.

2.12.19 Production of indole (Sneath *et al.* 1986)

For this test, Kovac's (1928) method was followed. In this method 1% tryptone broth medium was used. The inoculated tubes were incubated at 37°C for 4 days. After incubation 2 ml of the test reagent (Kovac's reagent) was added and shaken vigorously. A rose pink colour indicated formation of indole.

2.12.20 Utilization of citrate (Claus 1995)

For this test, tubes of citrate (Simmon's citrate agar) medium in duplicates were inoculated with 24 h bacterial culture by stabbing the butt and then streak the

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slant. Incubated at 37^{0}C for 2-3 days. The positive test was indicated by changing the colour from green to blue.

2.12.21 Utilization of propionate (Sneath *et al.* 1986)

Propionate agar slants were inoculated with 24 h old culture and incubated at 37°C for 3-5 days.

Production of a pink colour indicates the utilization of propionate by bacteria.

2.12.22 Voges Proskauer test (Bryan 1950)

Voges-Proskauer (VP) test is a color reaction test for the production of a neutral product during glucose fermentation by microorganisms. Acetoin or acetyl-methyl carbinol oxidases to diacetyl, which reacts with creatine and form a red complex.

For this test VP broth tubes were inoculated and incubated for 5 days at 37°C. When sufficient growth was observed, 3 ml of 5% alcoholic α-napthol solution was added to each tube followed by 1 ml of 40% potassium hydroxide and 0.3% creatine solution. The tubes were then shaken vigorously and allowed to stand.

Development of crimson to ruby red color indicates a positive reaction that is the production of acetyl-methyl carbinol.

2.13 Identification of the isolates

Following Bergey's Manual of Systematic Bacteriology Vol. 2 (Sneath *et al.* 1986) Gram positive aerobic heterotrophic bacteria were identified and following Vol. 1 of Bergey's Manual of Systematic Bacteriology (Kreig and Holt. 1984) Gram negative aerobic bacteria were identified.

2.14 Decolourization test

Decolourization of the selected textile dyes were tested by three methods – (1) by using waste samples as inocula, (2) by using test organism isolated from the waste samples and (3) by using mixed culture of test organisms.

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2.14.1 Dye decolourization test using waste samples

For this test four different dyes viz. Red ME6BL, OrangeME2RL, Turquoise Blue BGF were mixed with nutrient broth at concentration of 0.1% (w/v). These mixed broths were directly inoculated with the samples (15ml/100ml) and incubated at 37°C for 7 days. The cultures were observed periodically.

2.14.2.1 Dye decolourization test by using single isolates (different condition)

For this test six selected dyes viz. Red ME6BL, Orange ME2RL, Tarquish Blue BGF, Tarquish Blue RSPL, Green3R , Yellow4G was mixed with nutrient broth separately at a concentration of 0.01% (w/v).These modified broths were inoculated with the individual single organism isolated from effluents and incubated at three different conditions viz. static, continuous shaking and regular filtered aeration at room temperature (28-34^{0}C) for 7 days. The decolourization was observed periodically.

2.14.2.2 Dye decolourization test by using single organism

For this test three different dyes viz. Red ME6BL, Orange ME2RL and Turquoise Blue BGF was mixed with modified nutrient broth separately at a concentration 0.01% (w/v). These modified nutrient broths were inoculated with the individual single test organism isolated from waste samples and incubated at 37C for 7 days. The decolourization was observed periodically.

2.14.2.3 Test of decolourization potentialities by using single organism (different concentration of dye)

For this test two different dyes viz. Red ME6BL and Orange ME2RL at different concentrations were used. Each dye compound at different concentration viz.0.005%, 0.01%, 0.015% (w/v) was mixed with nutrient broth. These modified broths were inoculated with the individual single test organism isolated from the waste samples and incubated at 37°C for 7 days. The decolourization was observed periodically.

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2.14.2.4 Dye decolourization test using impregnated disks.

0.1 g dye was dissolved in 2 ml of distilled water and 20 filter paper discs (Whatman No. 1, 11.00 cm) were soaked for 5 min.

To test the decolourization activities the test organisms were inoculated in petriplates containing sloppy NA (pH 8.5), then the prepared disks were placed on the inoculated plate and was incubated in an incubator at 37°C .On the other hand the soaked discs were placed on the sloppy NA plate. After 24h selected strains were inoculated surround the disk by streak method. Decolourization activities were visually observed after 24 hrs of incubation and the complete decolourization was observed periodically.

2.14.3 Dye decolourization test by using mixed culture

For this test three different dyes viz. Red ME6BL, Orange ME2RL and Tarquish Blue BGF were mixed with nutrient broth at a concentration of 0.01% (w/v). This broth was inoculated with the mixed culture for different test organisms and incubated at 37°C.

2.14.4 Spectrophotometric analysis of the dye test samples

In all the experiments of decolourization after incubation all the inoculated broth cultures were centrifused (H-200 nR, KOKUSAN ENSINKI CO. LTD.) at -4C for 15 min. then filtered.

Percentage of selected dyes decolourization of pure culture and mixed culture were measured by UV – visible spectrophotometer (UV-1601PC, Shimadzu).In case of pure culture percentage of decolourization of the dye *viz.* Red ME6BL, Orange ME2RL and Turquoise blue BGF was measured at lambda max 450 nm. Percentage of dye decolourization was calculated by the following formula.

$$\% \text{ of decolourization} = \frac{\text{Sample concentration}}{\text{Standard concentration}} \times 100$$

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2.14.5 Effects of pH on dye decolourization

The selected bacterial isolates were grown individually in modified nutrient broth (MNB) having dye Red ME6BL, Orange ME2RL and Turquoise Blue BGF at a concentration 0.01% (w/v) as a component and over a pH range of 4.5 – 11.5. The incubation temperature was kept at 37°C. After 24 h, decolourization was observed and recorded. The result was recorded in term of decolourization and was estimated individually.

2.14.6 Effects of temperature on dye decolourization

The isolated bacterial strains which were active Red ME6BL, Orange ME2RL and Turquoise Blue BGF dye decolourizers, were inoculated individually into modified nutrient broth (MNB) having dye 0.01% (w/v) and were incubated at different temperatures (4, 10, 30, 40, 50, 55 and 65°C). After 24h the results were observed and recorded. The result was recorded in term of decolourization and estimated individually.

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Chapter - 3
Results

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RESULTS

3.1 Temperature and pH of the collected samples

The temperature and pH of the collected samples were shown in Table 3.1. The temperature ranged in between 36 to38^{0}C. The maximum temperature (38°C) was found in the sample S-4 and the minimum temperature (36°C) was found in the sample S-8. Colour of the collected samples varied sample to sample.

pH of the samples ranged between 6.85 – 10.52. Most of the samples were found to be alkaline in nature.

3.2 Enumeration of aerobic heterotrophic bacteria

The aerobic heterotrophic bacterial count of textile dyeing effluent samples was shown in Table 3.2. The used two different isolating media were found to be satisfactory for the isolation of bacteria from textile dyeing effluents. The average bacterial count varied in between $4.6x10^5$ to $3.85x10^7$ cfu/ml. The maximum count was noticed in the sample S-4.

3.3 Isolation and selection of the isolates

During this study a total of 85 discrete colonies were primarily selected from different samples. Finally 25 isolates were selected and purified for detailed study towards identification.

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Table: 3.1 Temperature and pH of textile effluent samples.

Sample Nos.	Date of Collection	Sample Colour	Temperature (°C)	pH
S-1	11.07.06	Blue	37	8.27
S-2	15.07.06	Purple	36	8.66
S-3	15.07.06	Dark Purple	36	8.79
S-4	15.07.06	Maroon	38	10.52
S-5	20.07.06	Dark Maroon	36	8.75
S-6	20.07.06	Dark Purple	36	6.85
S-7	10.08.06	Light Maroon	36	8.47
S-8	10.08.06	Dark Blue	36	9.00
S-9	15.08.06	Light Coffee	36	8.19
S-10	15.08.06	Purple	37	9.04

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Table 3.2 Aerobic bacterial count (cfu/ml) of textile dyeing effluent samples on two isolating media.

Sample	Nutrient Agar (cfu/ml)	Modified Nutrient Agar (cfu/ml)	Average (cfu/ml)
S-1	6×10^5	2.9×10^6	1.75×10^6
S-2	8×10^5	0.12×10^6	0.46×10^6
S-3	5.5×10^6	6.7×10^6	6.10×10^6
S-4	7×10^6	7.00×10^7	3.85×10^7
S-5	4×10^6	2.6×10^7	1.50×10^7
S-6	5×10^6	2.2×10^7	1.35×10^7
S-7	9×10^6	1.9×10^7	1.40×10^7
S-8	6×10^6	1.7×10^7	1.15×10^7
S-9	1.5×10^6	9.6×10^6	5.50×10^6
S-10	2.0×10^6	9.2×10^6	5.60×10^6

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3.4 Colonial morphology of the selected isolates

Colonies of the selected isolates were found to be different in their form, elevation, margin, surface, colour and optical characteristics. The colonial morphology were observed on Nutrient Agar (NA) was presented in Tables 3.4. Most of the colonies were raised, circular, smooth and whitish in colour. The diameter of the bacterial colonies ranged in between 0.5 to 18mm.(Fig.3.1)

3.5 Microscopic observation of the selected isolates

All aerobic heterotrophic bacterial isolates were rod shaped. On the basis of Gram reaction 21 isolates were Gram positive and the remaining 4 isolates were Gram negative (Table 3.5). Fig. 3.2(A-D) & 3.3(A-F) representative vegetative cells, sporangia and spore of the isolated strains. Fig. 3.3- staining, wet mount.

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Table 3.4 Colony morphology of the selected isolates on Nutrient Agar medium.

Strain No.	Form	Elevation	Margin	Surface	Colour	Optical characteristics	Diameter (mm)
T-1	Circular	Convex	Entire	Smooth	Brown	Moist, translucent	3.5
T-2	Circular	Umbonate	Entire	Smooth	Off White	Moist, translucent	9.0
T-3	Circular	Umbonate	Undulate	Rough	White	Moist, opaque	4.0
T-4	Circular	Convex	Entire	Smooth	Off White	Moist, translucent	2.5
T-5	Circular	Umbonate	Undulate	Smooth	White	Moist, translucent	2.0
T-6	Circular	Umbonate	Entire	Smooth	Off White	Moist, opaque	6.5
T-7	Circular	Convex	Undulate	Smooth	Off White	Moist, translucent	4.0
T-8	Circular	Effuse	Entire	Smooth	Brown	Moist, translucent	4.0
T-9	Circular	Effuse	Erose	Smooth	Orange	Moist, opaque	9.5
T-10	Circular	Effuse	Undulate	Smooth	White	Moist, opaque	18.0
T-11	Circular	Convex	Undulate	Smooth	Off White	Moist, opaque	7.0
T-12	Circular	Convex	Erose	Concentric	White	Moist, opaque	4.5
T-13	Circular	Convex	Entire	Smooth	Light Pink	Moist, opaque	1.5
T-14	Circular	Convex	Undulate	Smooth	White	Moist, opaque	0.5
T-15	Circular	Effuse	Erose	Smooth	White	Moist, opaque	4.0
T-16	Circular	Effuse	Undulate	Smooth	Brown	Moist, translucent	6.0
T-17	Circular	Effuse	Entire	Concentric	Brown	Moist, translucent	3.0
T-18	Circular	Effuse	Erose	Smooth	Orange	Moist, opaque	11.0
T-19	Circular	Convex	Entire	Smooth	Orange	Moist, opaque	5.5
T-20	Circular	Convex	Entire	Smooth	Orange	Moist, opaque	4.0
T-21	Circular	Convex	Entire	Smooth	Brown	Moist, translucent	5.0
T-22	Circular	Effuse	Entire	Rough	Off White	Moist, opaque	7.5
T-23	Circular	Effuse	Undulate	Smooth	White	Moist, opaque	4.5
T-24	Circular	Effuse	Entire	Smooth	White	Moist, opaque	8.0
T-25	Circular	Convex	Entire	Smooth	Light sky	Moist, translucent	5.5

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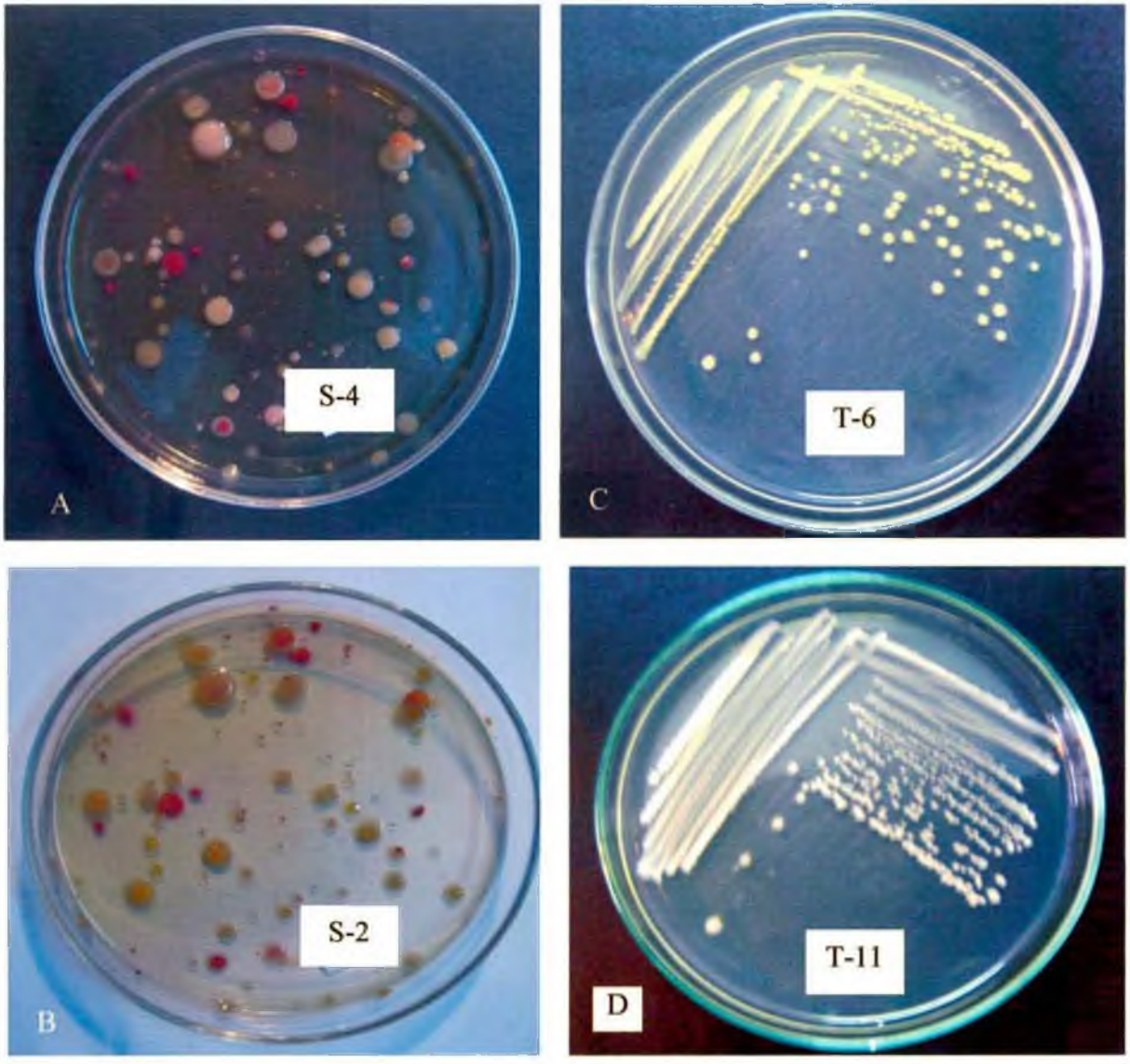

Fig. 3.1 (A-D) Photographs showing bacterial colony morphology A-B. Dilution plate and C-D. Streak method

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Table 3.5 Microscopic observation of the strains

Strain No	Vegetative cell	Shape and position of spore	Sporangia	Gram reaction
T-1	Rods, rounded end, occur in chain	Ellipsoidal, Terminal	Not swollen	+
T -2	Rods, rounded end, occur in chain	Ellipsoidal, Terminal	Not swollen	+
T -3	Rods, rounded end, occur in chain	Ellipsoidal, Terminal	Not Swollen	+
T-4	Rods, rounded end, occur in chain	Ellipsoidal, Terminal	Not swollen	+
T-5	Rods, rounded end, occur in chain	Ellipsoidal, Terminal	Not swollen	+
T-6	Rods, rounded end, occur in chain	Ellipsoidal, Terminal	Not Swollen	+
T-7	Rods, rounded end, occur in chain	Ellipsoidal, Terminal	Not swollen	+
T-8	Rods, rounded end, occur in chain	Ellipsoidal, Terminal	Not swollen	+
T-9	Rods, rounded end, occur in chain	Ellipsoidal, Terminal	Not swollen	+
T-10	Rods, rounded end, occur in chain	Ellipsoidal, Terminal	Not swollen	+
T-11	Rods, rounded end, occur in chain	Ellipsoidal, Terminal	Not swollen	+
T-12	Rods, rounded end, occur in chain	Ellipsoidal, Terminal	Not swollen	+
T-13	Rods, rounded end, occur in chain	Ellipsoidal, Terminal	Not swollen	+
T-14	Rods, rounded end, occur in chain	Ellipsoidal, Terminal	Not swollen	+
T-16	Rods, rounded end, occur in chain	Ellipsoidal, Terminal	Not swollen	+
T-15	Short rod, rounded end, occur in singly	Absent	-	-
T-17	Rod, rounded end, occur in chain	Ellipsoidal, Central	Not swollen	+
T-18	Short rod, rounded end, occur in singly	Ellipsoidal Central	Not swollen	+
T-19	Short rod, rounded end, occur in singly	Ellipsoidal Terminal	Not swollen	+
T-20	Short rod, rounded end, occur in singly	Absent	-	-
T-21	Short rod, rounded end, occur in singly	Absent	-	-
T-22	Short rod, rounded end, occur in singly	Absent	-	-
T-23	Short rod, rounded end, occur in singly	Ellipsoidal, central	Not swollen	+
T-24	Rod, rounded end, occur in singly	Ellipsoidal, central	Not swollen	+
T-25	Rod, rounded end, occur in chain	Ellipsoidal Terminal	Not swollen	+

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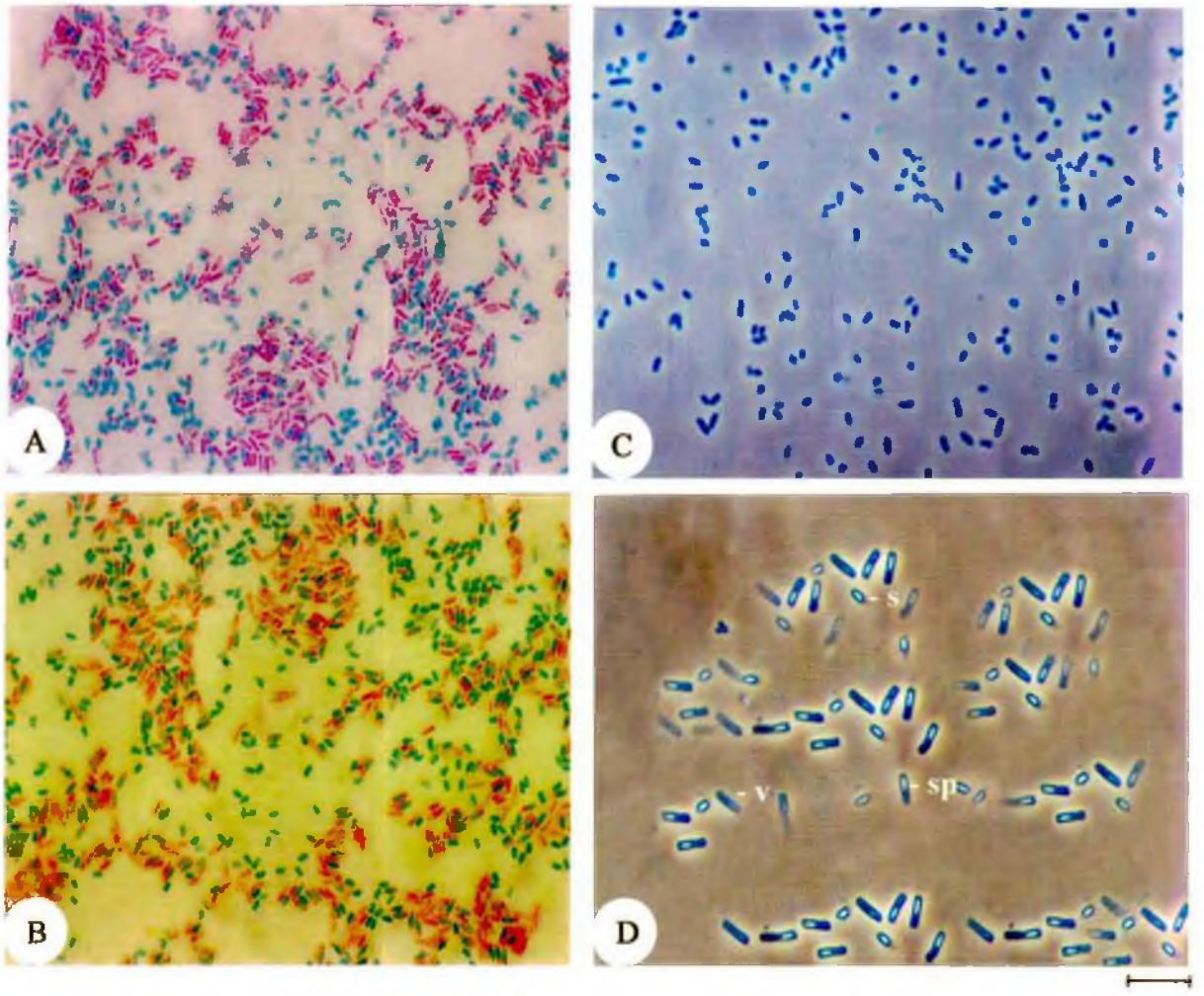

Fig. 3.2 (A-D) Photomicrographs showing vegetative cells (v), spore (s) and sporangia (sp) some bacteria isolated. A and B spore stain (T-12 and T-13), C and D bacteria under phase contrast microscope (T-18 and T-10). (Bar=5μm)

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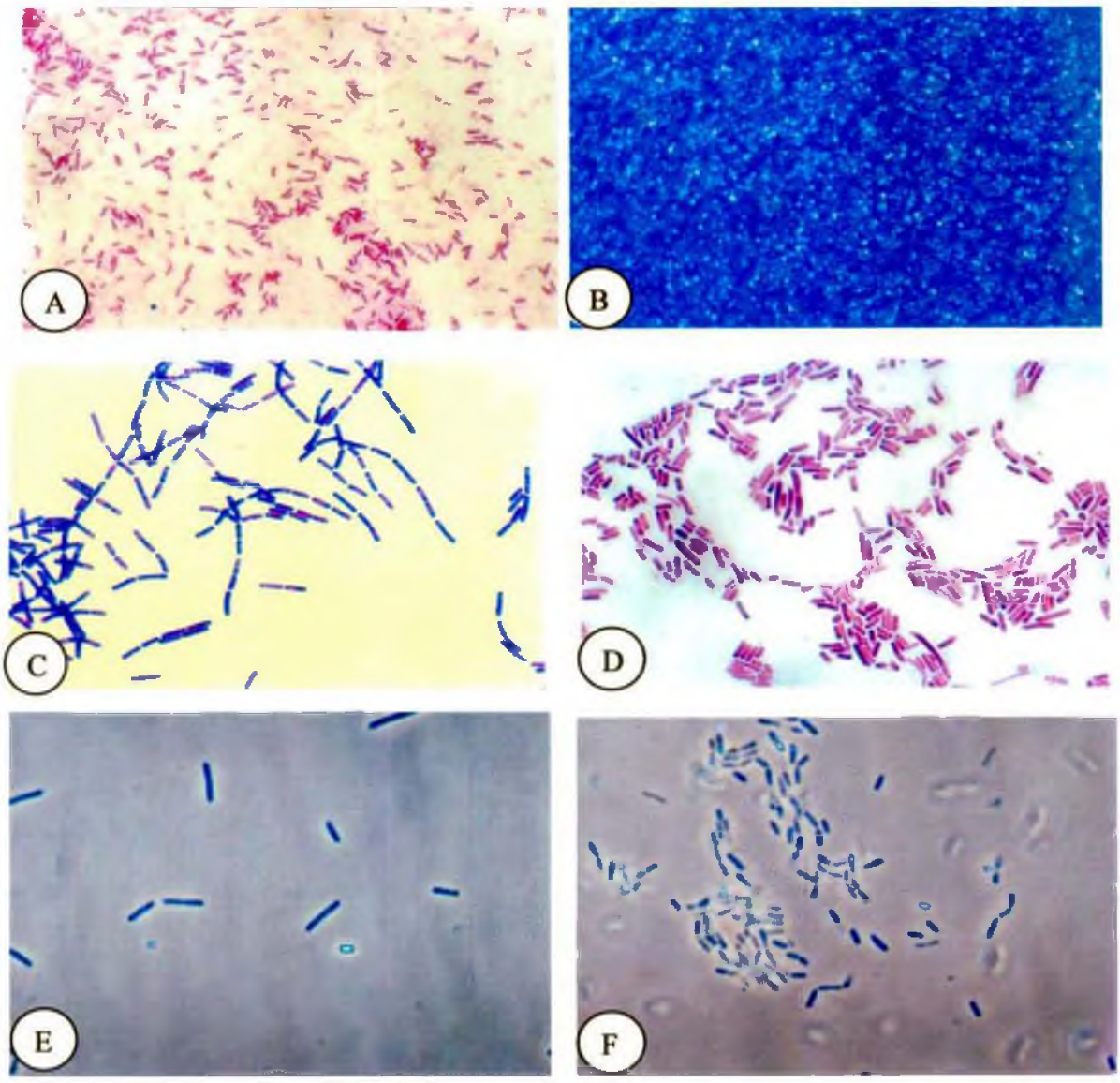

Fig. 3.3 (A-F) Photomicrographs showing cells of aerobic heterotrophic bacteria using different stains and wet mount. A. simple staining (Basic fucshin, strain T-15), B. Negative staining (strain T-13), C-D. Gram staining (strain T-10 and T-20). E-F. Wet mount of strain T-7 and T-9.

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3.6 Physiological and biochemical characteristics of the selected isolates

Important physiological and biochemical tests(e.g. catalase, oxidase, oxygen requirement, hydrolysis of starch, casein, gelatin, VP, MR, egg yolk lecithinase, nitrate reduction, indole formation, deamination of phenylalanine, degradation of tyrosin, utilization of citrate and propionate, acid production from carbohydrates and fermentation) of the isolated strains were grouped into two categories. (i) aerobic Gram positive heterotrophic bacteria, (ii) aerobic Gram negative heterotrophic bacteria.

3.6.1 Catalase, oxidase, motility and oxygen requirement test

All the test isolates were found positive for catalase. Out of 25 isolates15 were able to produce oxidase. On the basis of their oxygen requirements 10 strains were found to be aerobic and 8 strains were facultative anaerobes (Table 3.6.1). Out of 25 isolates 11 were motile and remaining were non-motile.

3.6.2 Hydrolysis of starch, casein and gelatin by the selected isolates

Out of 25 isolates only two strains were amylase positive i.e. capable of hydrolyzing starch. Ten out of 25 isolates were able to hydrolyze casein. All strains were capable of hydrolyzing gelatin except one (T-23). (Table 3.6.2 and Fig.3.5).

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Table 3.6.1 Oxidase, motility and oxygen requirement test of selected isolates.

Isolate no.	Oxidase	Motility	Oxygen requirement
T-1	+	-	Strictly aerobes
T-2	+	-	Not done
T-3	-	+	Facultative anaerobes
T-4	+	-	Strictly aerobes
T-5	-	+	Strictly aerobes
T-6	-	-	Facultative anaerobes
T-7	+	-	Facultative anaerobes
T-8	+	-	Facultative anaerobes
T-9	-	-	Facultative anaerobes
T-10	-	+	Facultative anaerobes
T-11	-	-	Strictly aerobes
T-12	+	+	Facultative anaerobes
T-13	+	+	Facultative anaerobes
T-14	-	-	Not done
T-15	-	+	Not done
T-16	+	+	Strictly aerobes
T-17	+	-	Not done
T-18	+	+	Not done
T-19	-	-	Strictly aerobes
T-20	+	+	Not done
T-21	+	-	Strictly aerobes
T-22	+	-	Strictly aerobes
T-23	+	+	Strictly aerobes
T-24	-	-	Facultative anaerobes
T-25	+	+	Not done

"+" sign indicates positive result and "-" sign indicates negative result

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Table 3.6.2 Hydrolysis of Starch, casein and gelatin

Isolate no.	Starch	Casein	Gelatin
T-1	-	-	+
T-2	-	-	+
T-3	-	-	+
T-4	-	+	+
T-5	-	-	+
T-6	-	ND	+
T-7	-	+	+
T-8	+	+	+
T-9	-	+	+
T-10	-	+	+
T-11	-	+	+
T-12	-	+	+
T-13	-	+	+
T-14	+	-	+
T-15	-	+	+
T-16	-	-	+
T-17	-	ND	+
T-18	-	-	+
T-19	-	-	+
T-20	-	-	+
T-21	-	-	+
T-22	-	-	+
T-23	-	-	-
T-24	-	-	+
T-25	-	+	+

" + " indicate hydrolysis of Starch, casein and gelatin.
" – " indicate casein and gelatin not hydrolyzed.
"ND" indicate Not done.

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3.6.3 Tests for VP, MR, egg yolk lecithinase, nitrate reduction and indole formation by the selected isolates:

All strains showed negative result in the indole formation. isolate viz. T-10 showed. In case of VP all strains were also negative except one (T-10). Out of 25 strains only 8 strains showed positive result in methyl red reaction test. Most of the strains were negative in case of egg yolk lecithinase enzyme. All the selected isolates except T-20 possessed nitrate reductase enzyme i.e. capable of reducing nitrate to nitrite (Table 3.6.3 & Fig.3.4).

3.6.4 Tests for deamination of phenylalanine, degradation of tyrosine and utilization of citrate and propionate by the selected isolates:

Table 3.6.4 and Fig.3.4 & 3.5 showed results of above-mentioned tests. All strains showed negative result for the deamination of phenylalanine test. From a total of 25 strains 6 were able to degrade tyrosine. 10 isolates out of 25 could utilize citrate and 6 isolates (viz. T-3, T-5, T-7, T-9, T-10 and T-11) could utilize propionate.

3.6.5 Acid production test of the selected carbohydrates by the isolated strains:

Acid production test was done by isolated strains. Most of the strains showed positive result in acid production from glucose except T-1, T-2, T-6, T-17 and T-22. Result was shown in Table 3.6.5 & Fig.3.5.

3.6.6 Determination of carbon source utilization by selected isolates:

Selected isolates were tested for their carbon utilization properties. Seven different carbohydrates viz. Cellobiose, Galactose, Mannose, Mellibiose, Rhamnose, Sorbose, and Sucrose were used for this test (Table 3.6.6)

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Table 3.6.3 VP, MR, egg yolk lecithinase and nitrate reduction by the selected isolates

Isolate no.	Voges-Proskauer (VP)	Methyl Red (MR)	Egg yolk lecithinase	Nitrate reduction
T-1	-	-	-	+
T-2	-	-	-	+
T-3	-	-	+	+
T-4	-	-	-	+
T-5	-	-	-	+
T-6	-	-	-	+
T-7	-	+	-	+
T-8	-	-	-	+
T-9	-	+	-	+
T-10	+	+	-	+
T-11	-	-	-	+
T-12	-	+	-	+
T-13	-	+	-	+
T-14	-	-	-	+
T-15	-	-	-	+
T-16	-	+	-	+
T-17	-	-	-	+
T-18	-	+	+	+
T-19	-	-	-	+
T-20	-	-	-	-
T-21	-	-	-	+
T-22	-	-	-	+
T-23	-	+	+	+
T-24	-	-	-	+
T-25	-	-	+	+

"+" sign indicates positive result and "-" sign indicates negative result

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Table 3.6.4 Degradation of tyrosine and utilization of citrate and propionate by the selected isolates

Isolate no.	Degradation of Tyrosine	Utilization of Citrate	Utilization of Propionate
T-1	-	-	-
T-2	-	-	-
T-3	-	+	+
T-4	-	-	-
T-5	-	+	+
T-6	-	-	-
T-7	+	-	+
T-8	-	+	-
T-9	+	-	+
T-10	+	-	+
T-11	-	+	+
T-12	+	-	-
T-13	+	+	-
T-14	+	-	-
T-15	-	+	-
T-16	-	-	-
T-17	-	+	-
T-18	-	-	-
T-19	-	-	-
T-20	-	-	-
T-21	-	-	-
T-22	-	+	-
T-23	-	-	-
T-24	-	+	-
T-25	-	+	-

"+" sign indicates positive result and "-" sign indicates negative result

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Table 3.6.5. Acid production tests of the selected carbohydrates by the isolated strains.

Strain No	Name of carbohydrates			
	D-Glucose	L-Arabinose	D-Xylose	D-Mannitol
T-1	-	-	-	-
T-2	-	-	-	-
T-3	+	+		-
T-4	+	-	-	-
T-5	+	-	-	+
T-6	-	-	-	-
T-7	+	-	-	-
T-8	+	-	-	-
T-9	+	-	-	-
T-10	+	-	-	-
T-11	+	+	-	+
T-12	+	-	-	-
T-13	+	-	-	-
T-14	+	-	-	-
T-15	+	+	-	+
T-16	+	-	-	-
T-17	+	-	-	-
T-18	+	-	-	-
T-19	+	-	-	-
T-20	+	-	-	-
T-21	+	-	-	-
T-22	-	-	-	+
T-23	+	-	-	-
T-24	+	+	-	+
T-25	+	-	-	-

"+" sign indicates positive result and "-" sign indicates negative result

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Table 3.6.6 Carbon source utilization by selected isolates

Isolate no.	Cellobiose	Galactose	Mannose	Mellibiose	Rhamnose	Sorbose	Sucrose
T-3	-	+	+	+	+	-	+
T-5	-	+	-	-	+	-	+
T-7	+	+	+	+	+	-	-
T-9	+	+	+	+	+	-	+
T-10	+	+	+	+	+	-	-
T-12	-	-	+	+	+	-	-
T-13	+	+	+	+	+	-	-
T-14	+	+	-	+	+	-	-
T-15	+	+	-	+	+	-	+
T-20	-	-	+	-	+	-	+
T-23	-	+	-	+	+	-	+
T-24	-	-	+	-	-	-	+
T-25	-	+	-	+	+	-	+

"+" sign indicates carbohydrate utilized and "-" sign indicates carbohydrate not utilized.

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3.6.7 Fermentation test of the selected carbohydrates by the isolated strains:

Fermentation was done by the selected isolates. Out of 25 isolates 20 were able to produce acid from glucose but none was able to produce gas from glucose. In case of fructose, 4 isolates produce acid and only two isolates viz. T-1 and T-19 produced both acid and gas (Table 3.6.7 and Fig. 3.4).

3.6.8 Proteolysis test of the selected strains

Organisms varied in their ability of disintegration of egg albumin, which was indicative of their proteolytic activity. Number of plus (+) signs indicated the degree of proteolysis. Result was shown in Table 3.6.8 and Fig. 3.6.

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Table 3.6.7 Fermentation test of the selected carbohydrates by the isolated strains.

Isolate no.	Name of Carbohydrates							
	Glucose		Fructose		Sucrose		Maltose	
	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas
T-1	-	-	+	+	-	-	+	-
T-2	-	-	-	-	+	-	+	-
T-3	+	-	-	-	+	-	+	-
T-4	+	-	-	-	+	-	+	-
T-5	+	-	-	-	+	-	+	-
T-6	-	-	-	-	-	-	+	-
T-7	+	-	-	-	-	-	+	-
T-8	+	-	+	-	+	-	-	-
T-9	+	-	-	-	+	-	+	-
T-10	+	-	-	-	-	-	+	+
T-11	+	-	-	-	+	-	+	-
T-12	+	-	-	-	-	-	-	-
T-13	+	-	-	-	-	-	+	-
T-14	+	-	-	-	-	-	-	-
T-15	+	-	+	-	+	-	+	-
T-16	+	-	-	-	+	-	+	-
T-17	-	-	-	-	-	-	+	-
T-18	+	-	-	-	+	-	+	-
T-19	+	-	+	+	+	-	+	-
T-20	+	-	-	-	+	-	Not done	
T-21	+	-	-	-	+	-	-	-
T-22	-	-	-	-	-	-	+	-
T-23	+	-	-	-	+	+	-	-
T-24	+	-	+	-	+	-	+	-
T-25	+	-	-	-	+	-	+	-

"+" sign indicates positive result and "-" sign indicates negative result.

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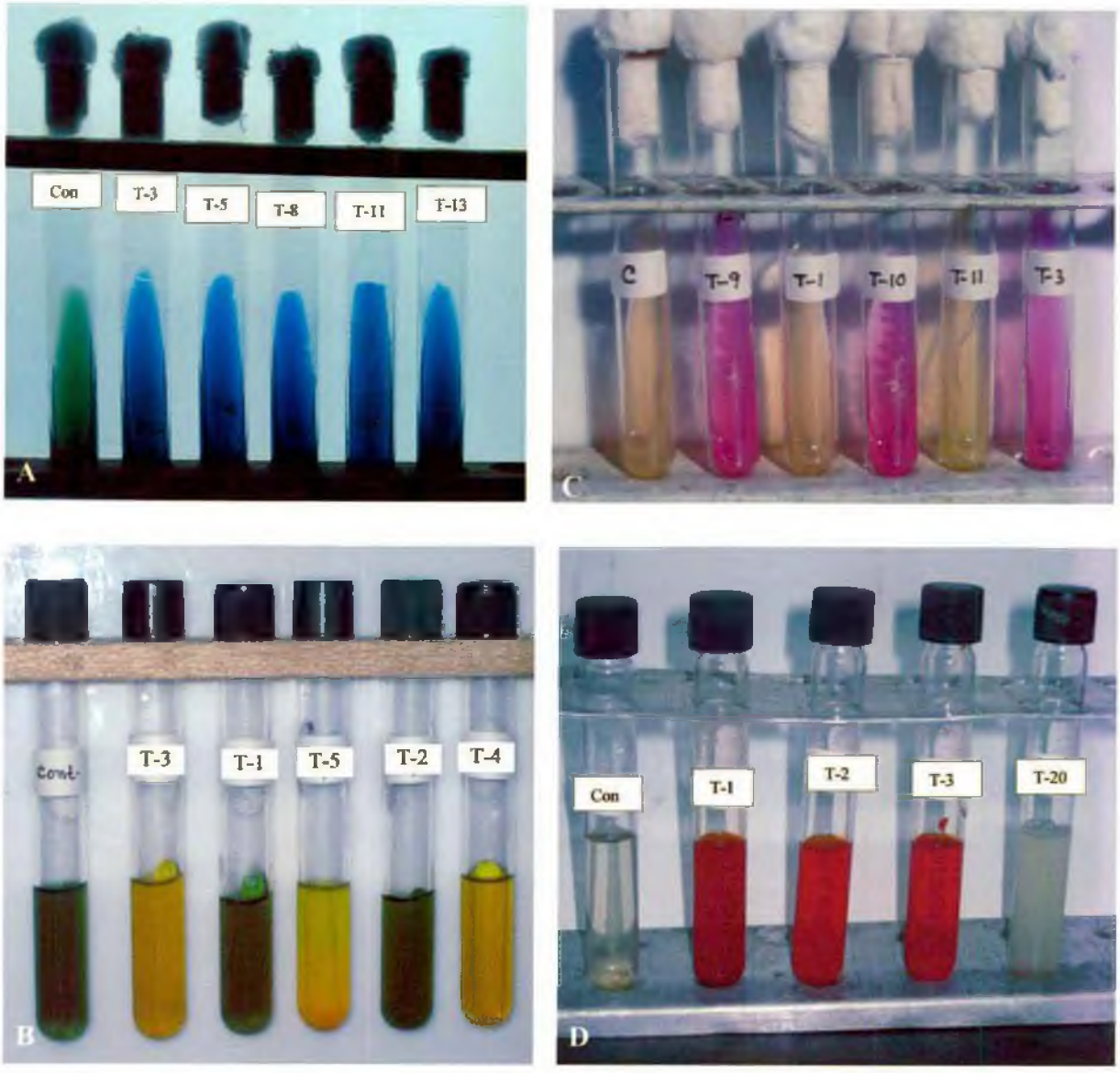

Fig. 3.4 (A-D) Photographs showing biochemical tests for A. Simmon's citrate (blue positive), B. Acid and gas production from glucose (yellow-acid production), C. Propionate test (pink positive), D. Nitrate test (red positive).
Con. - Control

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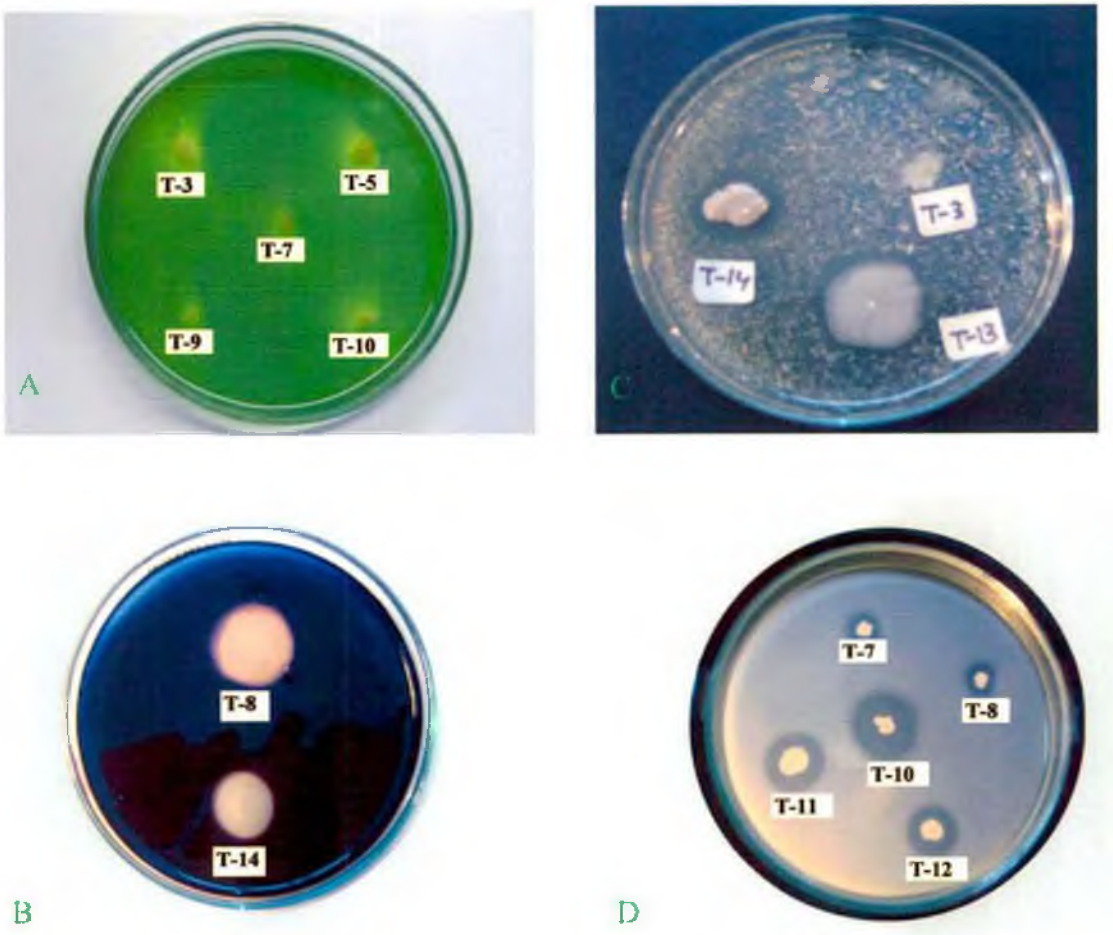

Fig. 3.5 (A-D) Photographs showing tests for: A. Acid production from Glucose (Yellow-positive); Hydrolysis of B. Starch C. Tyrosine, D. Casein, respectively-clear zone positive)

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Table 3.6.8 Proteolytic activity of the selected strains.

Strain no.	**Proteolytic activity** (Visual estimation)
T-10, T-14, T-18	++++
T-7, T-9, T-12, T-13	+++
T-3, T-20, T-21, T-23	++
T-22, T-1, T-2	+

Number of plus (+) signs shows the degree of proteolysis.

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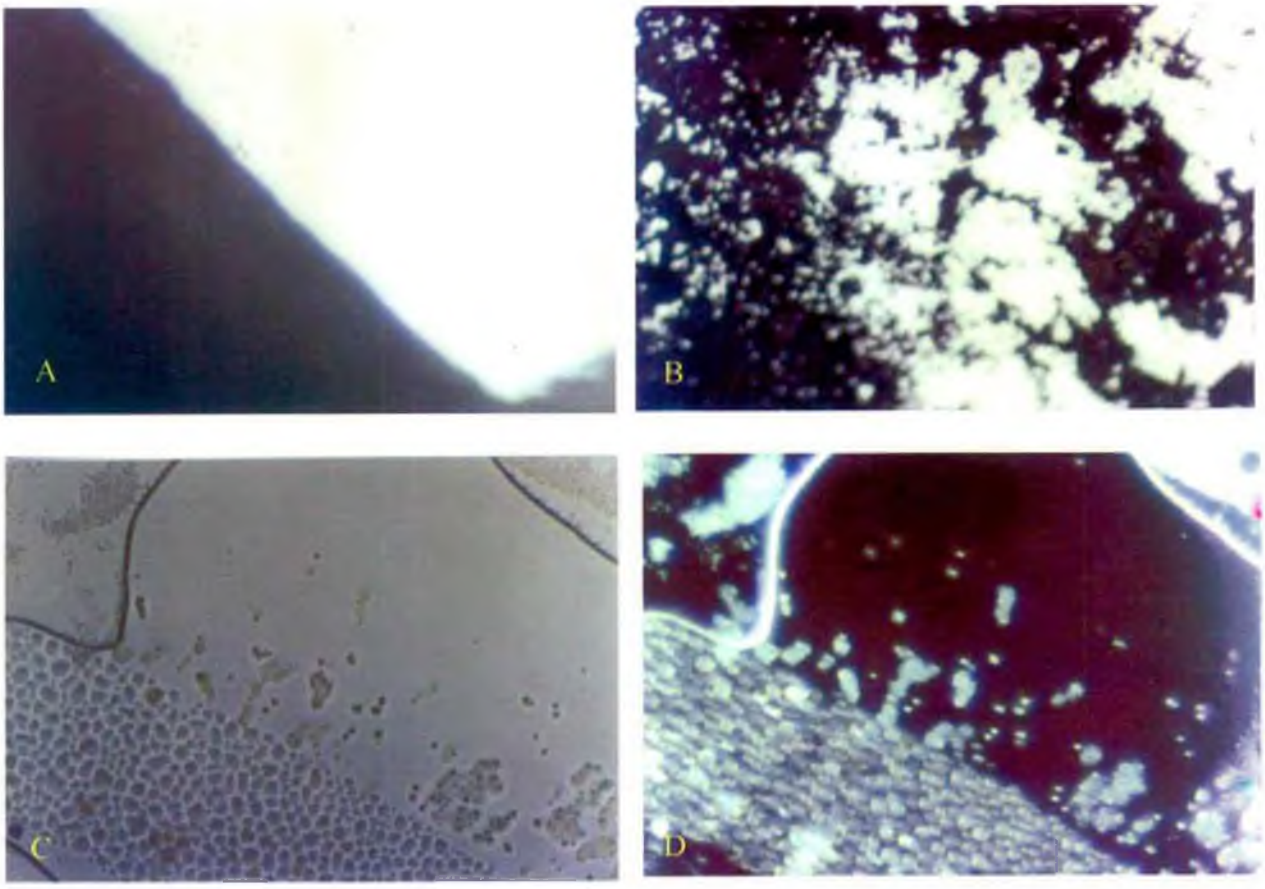

Fig. 3.6 (A-D) Microphotographs showing proteolytic activity A. control (a piece of coagulated egg albumin remaining unchanged after incubation, without bacteria), B-D. Disintegrated egg albumin by the strain T-10, T-14 and T-18 respectively.

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3.7 Growth response of the selected isolates at different concentration of NaCl

Growth responses of the selected isolates at different salt concentration were shown in Table 3.7. The results showed that the organisms had a wide range of tolerance towards NaCl concentration. The maximum growth of the organisms were in between 0 – 2 % of NaCl.

3.8 Growth response of the selected isolates at different temperatures

The effect of temperature on the isolated bacteria was shown in Table 3.8. The results showed that the organism could grow well at a wide range of temperature. The maximum growth was observed in between 30-40°C and thus the isolated organisms were found to be mesophilic.

3.9 Growth response of the selected isolates at different pH

The effect of pH on the isolated bacteria was shown in Table 3.9. The results showed that the organisms had a wide range of tolerance towards pH. Most of the isolated organism grew well at pH 8.5 and the organisms were found to be alkalophilic.

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Table 3.7 Growth response of selected isolates at different salt concentrations.

Strain	Concentration of NaCl (%)				
	0	2	5	7	10
T-1	ND	Pellicle	Pellicle	Pellicle	Pellicle
T-2	ND	0.013	0.063	ND	ND
T-3	1.387	0.493	Pellicle	Pellicle	ND
T-4	ND	0.00	0.228	ND	Pellicle
T-5	1.580	Pellicle	Pellicle	Pellicle	ND
T-6	ND	0.00	0.029	0.00	Pellicle
T-7	1.673	1.156	0.255	1.249	ND
T-8	Pellicle	Pellicle	Pellicle	Pellicle	Pellicle
T-9	1.419	1.195	0.627	1.223	0.392
T-10	1.236	0.745	0.361	1.025	0.514
T-11	Pellicle	Pellicle	Pellicle	Pellicle	Pellicle
T-12	1.473	1.66	0.523	0.949	0.515
T-13	1.158	1.00	0.329	0.940	0.416
T-14	1.402	1.25	0.253	0.170	0.233
T-15	1.393	Pellicle	Pellicle	0.720	0.686
T-16	ND	0.085	0.048	ND	ND
T-17	ND	0.00	0.022	ND	ND
T-18	ND	1.40	0.019	ND	ND
T-19	1.015	0.90	0.719	0.780	0.556
T-20	ND	0.00	0.036	ND	ND
T-21	0.976	0.25	0.088	0.219	0.227
T-22	ND	0.00	0.045	ND	ND
T-23	ND	0.00	0.022	ND	ND
T-24	1.096	Pellicle	Pellicle	0.901	0.488
T-25	0.714	0.097	0.062	0.215	0.233

(ND- Note done)

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Table. 3.8 Growth response of the isolates at different temperature (Absorbance at 450nm).

Strain	Temperature °C						
	4	10	30	40	50	55	65
T-3	0.010	0.021	0.081	0.481	0.401	0.037	0.00
T-5	0.011	0.008	0.151	0.770	0.092	0.035	0.109
T-7	0.050	0.421	0.630	0.871	0.562	0.092	0.071
T-9	0.080	0.391	0.932	0.821	0.041	0.188	0.172
T-10	0.231	0.182	0.752	0.550	0.871	0.278	0.095
T-12	0.131	0.212	0.571	1.093	0.060	0.113	0.099
T-13	0.082	0.431	0.611	0.842	0.041	0.130	0.148
T-14	0.041	0.012	0.732	0.101	0.071	0.157	0.058
T-18	0.441	0.432	0.493	0.780	0.00	0.155	0.121
T-20	0.08	0.31	0.92	0.57	0.00	0.071	0.078
T-23	0.16	0.10	0.22	1.00	0.00	0.094	0.178
T-24	0.25	0.20	0.49	0.49	0.01	0.044	0.045
T-25	0.06	0.60	0.45	0.93	0.75	0.00	0.00

(ND- Not done)

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Table 3.9 Growth responses of isolates at different pH (Absorbance at 450 nm).

Strain	pH		
	4.5	6.5	8.5
T-1	0.22	1.62	1.32
T-2	0.03	0.31	0.75
T-3	1.74	2.16	1.82
T-4	2.10	1.78	1.53
T-5	1.50	1.85	2.06
T-6	0.23	0.18	1.44
T-7	2.10	1.93	1.86
T-8	ND	ND	ND
T-9	1.58	1.84	1.43
T-10	1.38	1.92	1.68
T-11	0.80	1.81	1.32
T-12	ND	ND	ND
T-13	ND	ND	ND
T-14	2.26	ND	1.64
T-15	1.45	1.68	1.85
T-16	0.18	1.31	1.00
T-17	0.11	0.10	0.38
T-18	0.14	0.18	1.41
T-19	0.21	1.27	0.160
T-20	0.19	1.83	0.63
T-21	0.05	0.02	1.29
T-22	0.02	0.13	2.08
T-23	0.08	0.40	1.15
T-24	1.62	1.96	1.69
T-25	1.87	1.90	1.38

(ND- Not done)

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3.10 Identification of the selected strains

Considering all observed characters of the isolated organisms, identification was attempted. Table 3.10.1 and 3.10.2.

3.11 Decolourization test by using waste water samples

Using waste water sample as inocula the decolourization test of the selected seven dyes viz. Red ME6BL, Orange ME2RL, Turquoise Blue BGF, Turquoise Blue RSPL, Yellow4G, Violate S3RL and Green3R was done. Within 24h of incubation most of the dyes were found to be decolourized. The result clearly revealed that the used waste water sample could provide a good source of useful microbial consortia for dye decolourization. Results were shown in Table 3.11 and Fig. 3.7 (A-C).

3.12.1 Decolourization test by using single organism (different conditions)

In three different conditions viz. static, shake and aerated. Most of the isolates showed better result towards the dyes viz Red ME6BL, Orange ME2RL,Turquoise Blue BGF, Turquoise Blue RSPL, Yellow4G and Green3R degradation in static condition. (Table 3.12.1, 3.12.2 and 3.12.3 and Fig. 3.8)

3.12.2.1 Decolourization test by single organisms

In this test only ten strains showed their capability to degrade dyes viz. Red ME6BL, Orange ME2RL and Turquoise Blue BGF. Result was shown in Table 3.12.2 & Fig.3.9.

3.12.2.2 Time requirements to decolourize the dye by single organism.

In this test only two strains showed their capability to degrade dyes viz. Red ME6Bland Orange ME2RL within 16h.

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Table 3.10.1 Provisional identification of the Gram positive strains.

Strain Nos.	Name of isolates	
T-1	*Bacillus badius*	Batchelor 1919
T-2	*B. firmus*	Bredemann and Werner 1933
T-3	*B. thuringiensis*	Berliner 1915
T-4	*B. badius*	Batchelor 1919
T-5	*B. firmus*	Bredemann and Werner 1933
T-6	*B. lentus*	Gibson 1935
T-7	*B. firmus*	Bredemann and Werner 1933
T-8	*B. pumilus*	Meyer and Gottheil 1901
T-9	*B. mycoides*	Fligge 1886
T-10	*B. cereus*	Frunkland and Frankland 1887
T-11	*B. pumilus*	Meyer and Gottheil 1901
T-12	*B. lentus*	Gibson 1935
T-13	*B. subtilis*	Ehrenberg 1835
T-14	*B. badius*	Batchelor 1919
T-16	*B. lentus*	Gibson 1935
T-17	*B. pumilus*	Meyer and Gottheil 1901
T-18	*B. glabisporus*	Larkin and Stokes 1967
T-19	*B. mycoides*	Fligge 1886
T-23	*B. fastidiosus*	den Dooren de Jong 1929
T-24	*B. megaterium*	de Bary 1884
T-25	*B. firmus*	Bredemann and Werner 1933

Table 3.10.2 Provisional identification of the Gram Negative strains

Strain No.	Provision identification names
T-15	*Pseudomanas aeruginosa*
T-20	*P. alcaligenes*
T-21	*P. pseudoalcaligenes*
T-22	*P. pseudoalcaligenes*

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Table 3.11: Dye decolorization test by textile waste water sample.

Waste Sample Nos.	Decolourization activity						
	Red ME6BL	Orange ME2RL	TB BGF	TB Blue RSPL	Violate S3RL	Yellow 4G	Green 3R
S-1	+(1)	+(1)	+(1)	+(2)	+(2)	+(2)	+(2)
S-2	+(1)	+(1)	+(1)	+(2)	+(2)	+(3)	+(3)
S-3	+(1)	+(1)	+(1)	+(3)	+(1)	+(2)	+(3)
S-4	+(1)	+(1)	+(1)	+(2)	+(1)	+(1)	+(4)
S-5	+(1)	+(1)	+(1)	+(3)	+(2)	+(1)	+(4)
S-6	+(1)	+(1)	+(1)	+(3)	+(2)	+(2)	+(3)
S-7	+(1)	+(1)	+(1)	+(1)	+(2)	+(2)	+(3)
S-8	+(1)	+(1)	+(1)	+(4)	+(2)	+(1)	+(3)
S-9	+(1)	+(1)	+(1)	+(2)	+ (2)	+(1)	+(4)
S-10	+(1)	+(1)	+(1)	+(2)	+(1)	+(1)	+(4)

+ sign indicates positive decolourization. The numbers with the parentheses indicate the time (days) required for complete decolourization (visual estimation).

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Fig. 3.7 (A-C) Photographs showing decolourization of selected dyes by using waste water sample. A, B. Violate S3RL, Turquoise Blue BGF and Yellow 4G and the waste water samples S-3 and S-9 respectively. C. Orange ME2RL, Red ME6BL and Turquoise Blue RSPL and the waste water sample S-4.
(Con.- Control)

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Table: 3.12.1 Dye decolourization test by using single organism (Static condition).

Strain	Red ME6BL	Orange ME2RL	Turquoise Blue BGF	TB. RSPL	Yellow 4G	Green 3R
T-2	++++	++++	++	+	+++	++
T-7	++++	+++	++	+	+++	++
T-10	++++	++++	+++	+	++	+++
T-13	+++	+++	+++	+	+++	++
T-14	++++	++++	+++	++	++	+++
T-15	++++	+++	++	++	+++	++
T-20	+++	+++	++	+	++	++
T-22	++	+++	++	++	+	+
T-12	++	+++	+	+	++	++
T-9	++	++	+	++	+	+

Table: 3.12.2 Dye decolourization test by using single organism (Shake culture).

Strain	RedME6BL	O. ME2RL	TB-BGF	TB-RSPL	Yellow 4G	Green 3R
T-2	+++	+++	+	+	++	+
T-7	+++	++	+	+	++	+
T-9	+	+	++	+	+	++
T-10	+++	+++	++	++	++	++
T-12	+	++	++	+	+	+++
T-13	++	++	+	+	++	++
T-14	+++	+++	+	++	+	++
T-15	+++	++	+	-	-	+
T-20	++	++	-	+	-	+
T-22	+	++	-	-	-	+

Visual estimation:
(-) sign indicates 0% decolourization.
(+) sign indicates 25% decolourization.
(+++) sign indicates 50% decolourization.
(++++) sign indicates 100% decolourization.

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Table: 3.12.3 Dye decolorization by using single organism (Regular filtered aeration condition).

Strain	R-ME6BL	O-ME2RL	TB-BGF	TB-RSPL	Yellow 4G	Green 3R
T-2	++	++	+	+	+	+
T-7	++	++	+	+	+	+
T-9	+	++	+	+	+	-
T-10	+++	+++	++	++	++	+++
T-12	+	+	+	-	+	+
T-13	++	++	+	-	-	-
T-14	+++	+++	++	++	+	++
T-15	++	++	+	-	+	-
T-20	++	+	+	+	-	+
T-22	+	+	-	-	+	-

Visual estimation:

(-) sign shows 0% decolourization.

(+) sign shows 25% decolourization.

(++) sign shows 50% decolourization.

(+++) sign shows 75% decolourization.

(++++) sign shows 100% decolourization.

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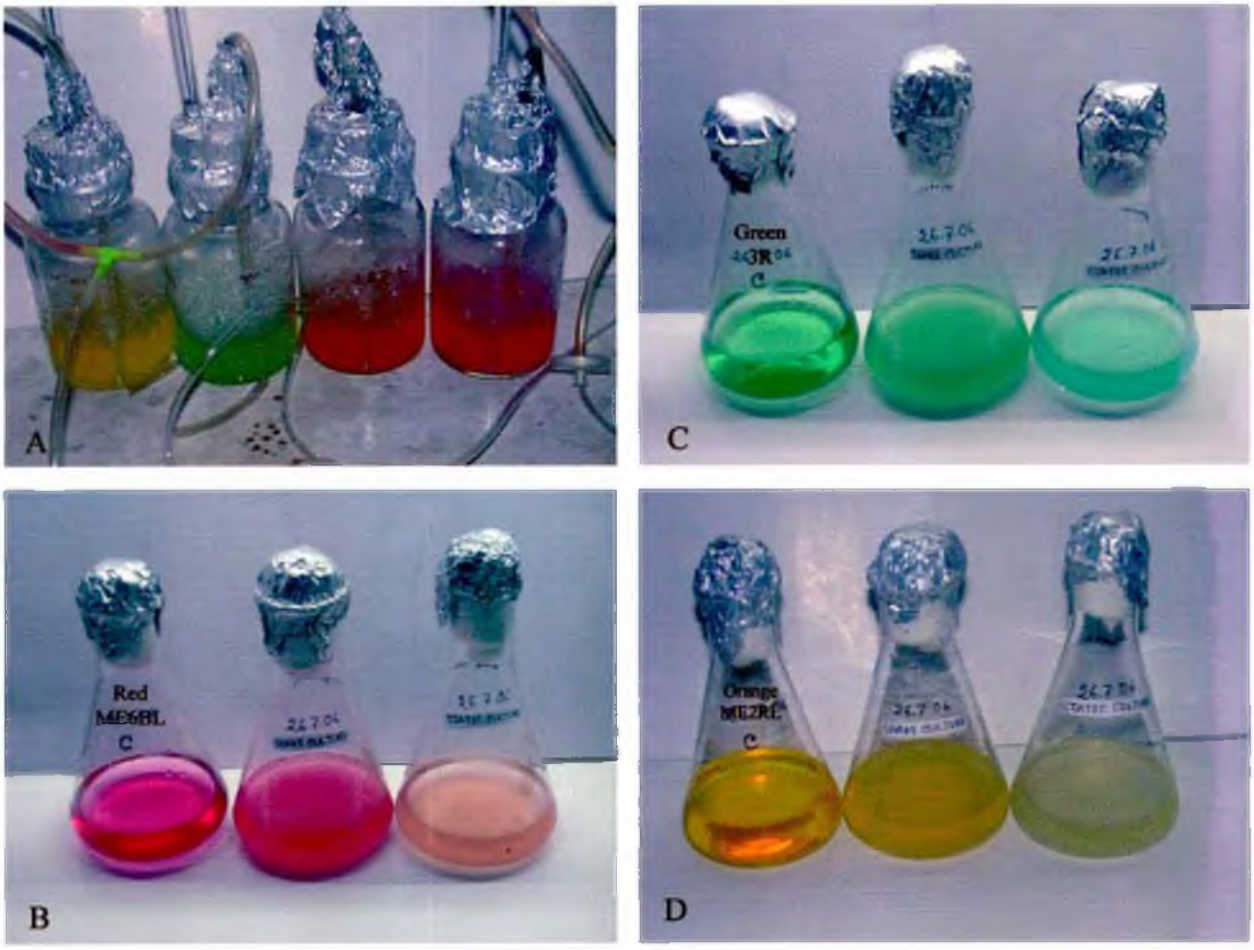

Fig. 3.8 (A-D) Photographs showing dye decolourization in three different conditions viz. static, shake and regular filtered aeration.
C-Control.

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Table: 3.12.4 Dye decolourization rate by the selected bacterial strains.

Strain Nos.	Name of Bacteria	Decolourization rate (%)		
		Red ME6BL	Orange ME2RL	Turquish Blue BGF
T-3	*Bacillus thuringiensis*	05	33	06
T-5	*B. firmus*	70	54	60
T-7	*Bacillus firmus*	60	80	60
T-9	*Bacillus mycoides*	62	80	50
T-10	*B. cereus*	91	90	60
T-12	*B. lentus*	58	89	55
T-13	*B. subtilis*	91	90	63
T-14	*B. badius*	91	80	52
T-18	*Bacillus globisporus*	60	91	00
T-20	*P. alcaligenes*	25	81	60

Table: 3.12.5 Time requirements to decolourize the dye Red ME6BL and Orange ME2RL by single organism.

Dye: Red ME6BL

Strain	4h	8h	12h	16h
T-10	-	+	+++	++++
T-14	-	+	+++	++++

Dye: Orange ME2RL

Strain	4h	8h	12h	16h
T-10	-	+	+++	++++
T-14	-	+	+++	++++

Visual estimation:
(-) sign indicates 0% decolourization.
(+) sign indicates 25% decolourization.
(++) sign indicates 50% decolourization.
(+++) sign indicates 75% decolourization.
(+++) sign indicates 100% decolourization.

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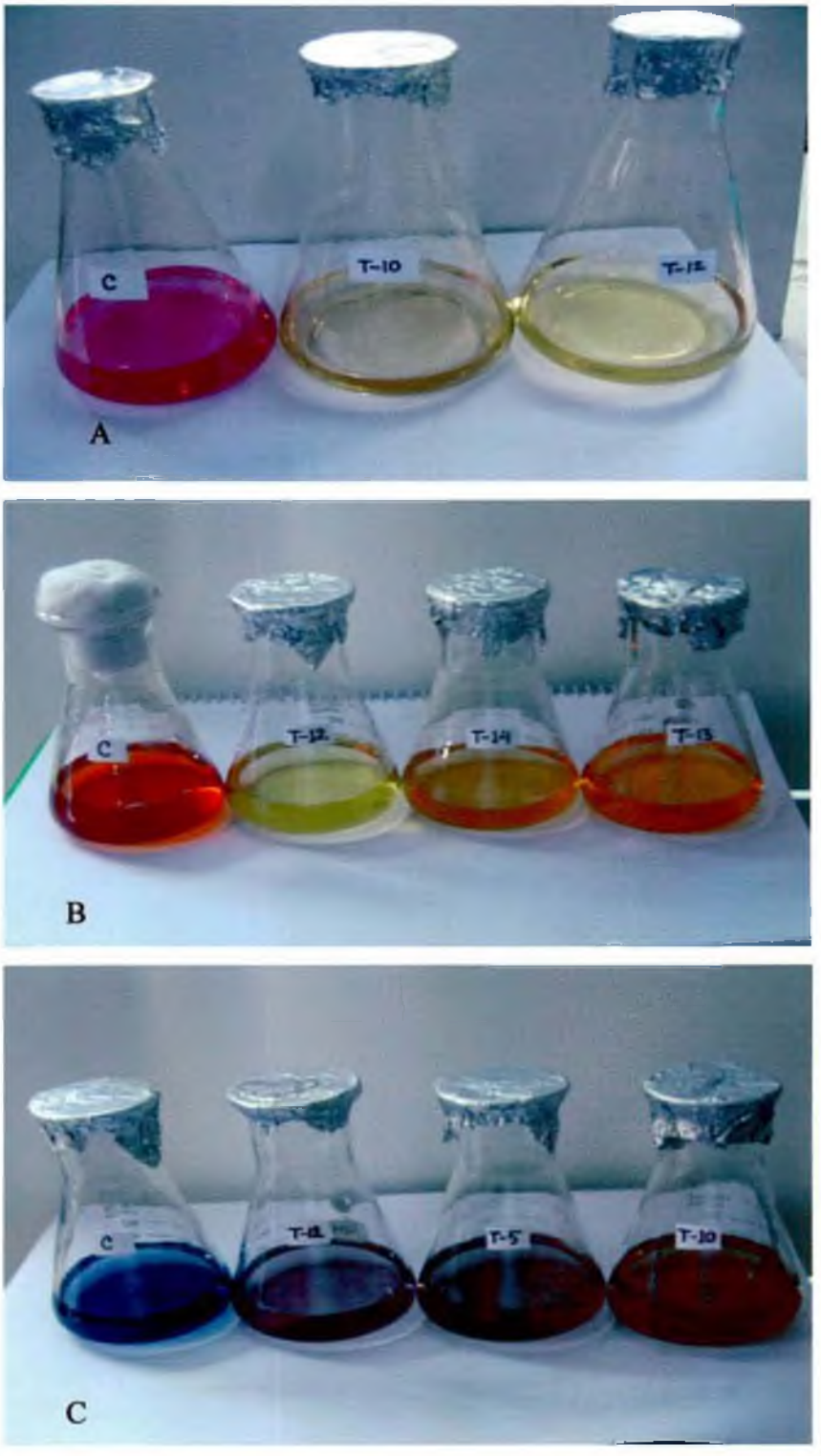

Fig. 3.9 Decolourization test by single organism. (A. Red ME6BL, B. Orange ME2RL, C. Turquoise Blue BGF.

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3.12.3 Decolourization test by using single organisms (different concentrations of dye)

In static condition out of 25 isolates (both Gram positive and Gram negative) only ten were found to decolourize the dyes Red ME6BL and Orange ME2RL, Results were shown in Table 3.12.6.1 and Fig. 3.10, 3.11.

3.12.4 Dye degradation test by bacteria using dye impregnated discs

In this test strains T-3, T-5, T-9, T-10, T-12, T-14 and T-18 showed positive result towards the dyes viz. Red ME6BL, Orange ME2RL, Turquoise Blue BGF, Turquoise Blue RSPL diffused from impregnated discs. Result was shown in Table 3.12.7 and Fig.3.12.

3.13 Decolourization test by mixed bacterial culture

Mixed cultures of the isolates showed better performance towards the dye decolourization. The three tested dyes viz. Red ME6BL, Orange ME2RL and Turquoise Blue BGF were decolourized within 48h. Result was shown in Table 3.13 and Fig. 3.13. Red ME6BL was found easier to decolourize. About 95% decolourization was achieved in case of Red ME6BL by the mixture of T-10, T-13, T-17 and T-20.

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Table 3.12.6.1 Rate of decolourization of Red ME6BL dye at different concentrations by selected bacterial isolates.

Isolate no.	Decolourization rate (%)		
	0.005 (%)	0.01(%)	0.015 (%)
T-7	80	72	70
T-9	82	77	69
T-10	81	79	75
T-12	88	77	76
T-13	76	63	67
T-14	91	93	81
T-18	91	94	90
T-20	25	40	37

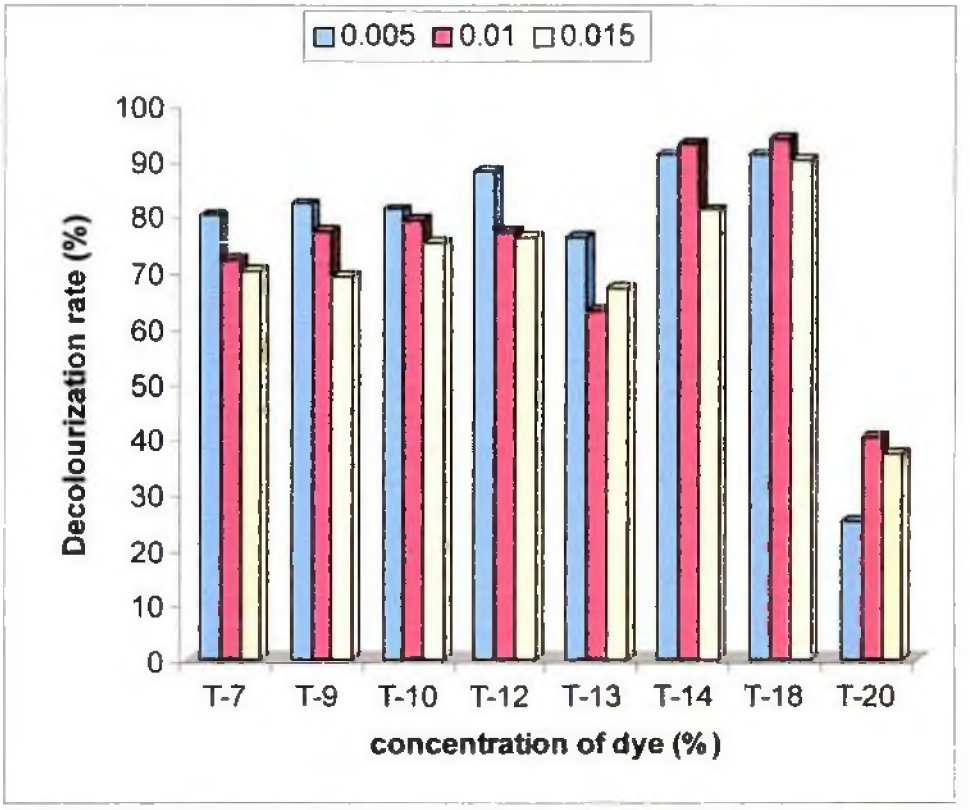

Fig.3.10 Rate of decolourization of textile dye Red ME2RL by the strains T-7, T-9, T-10, T-12, T-13, T-14, T-18 and T-20

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Table 3.12.6.2 Rate of decolourization of Orange ME2RL dye at different concentrations by selected bacterial isolates.

Isolate no.	Decolourization rate (%)		
	0.005 (%)	0.01(%)	0.015 (%)
T-7	79	74	76
T-9	94	75	79
T-10	79	72	81
T-12	87	80	78
T-13	79	76	77
T-14	90	82	81
T-18	87	80	81
T-20	10	12	07

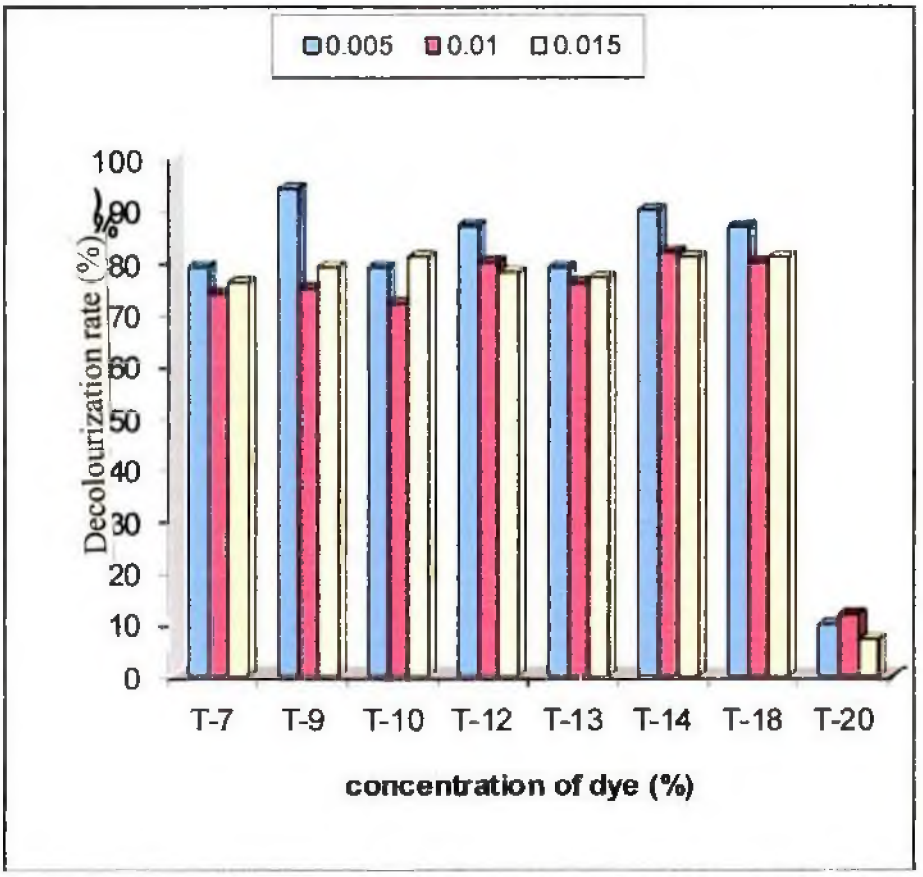

Fig.3.11 Rate of decolourization of textile dye Orannge ME2RL by the strains T-7, T-9, T-10, T-12, T-13, T-14, T-18 and T-20.

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Table: 3.12.7 Dye decolourizatio test by bacteria using impregnated discs.

Strain	Red ME6BL	Orange ME2RL	Turquoise Blue BGF
T-3	+	+	-
T-5	-	-	+
T-7	-	+	+
T-9	++	+	++
T-10	+++	+++	+++
T-12	++	++	++
T-13	+++	+++	+++
T-14	+++	+++	+++
T-18	++	++	++
T-20	+	+	-

Visual estimation:
(-) sign indicates 0% decolourization.
(+) sign indicates 25% decolourization.
(++) sign indicates 50% decolourization.
(+++) sign indicates 75% decolourization.
(++++) sign indicates 100% decolourization.

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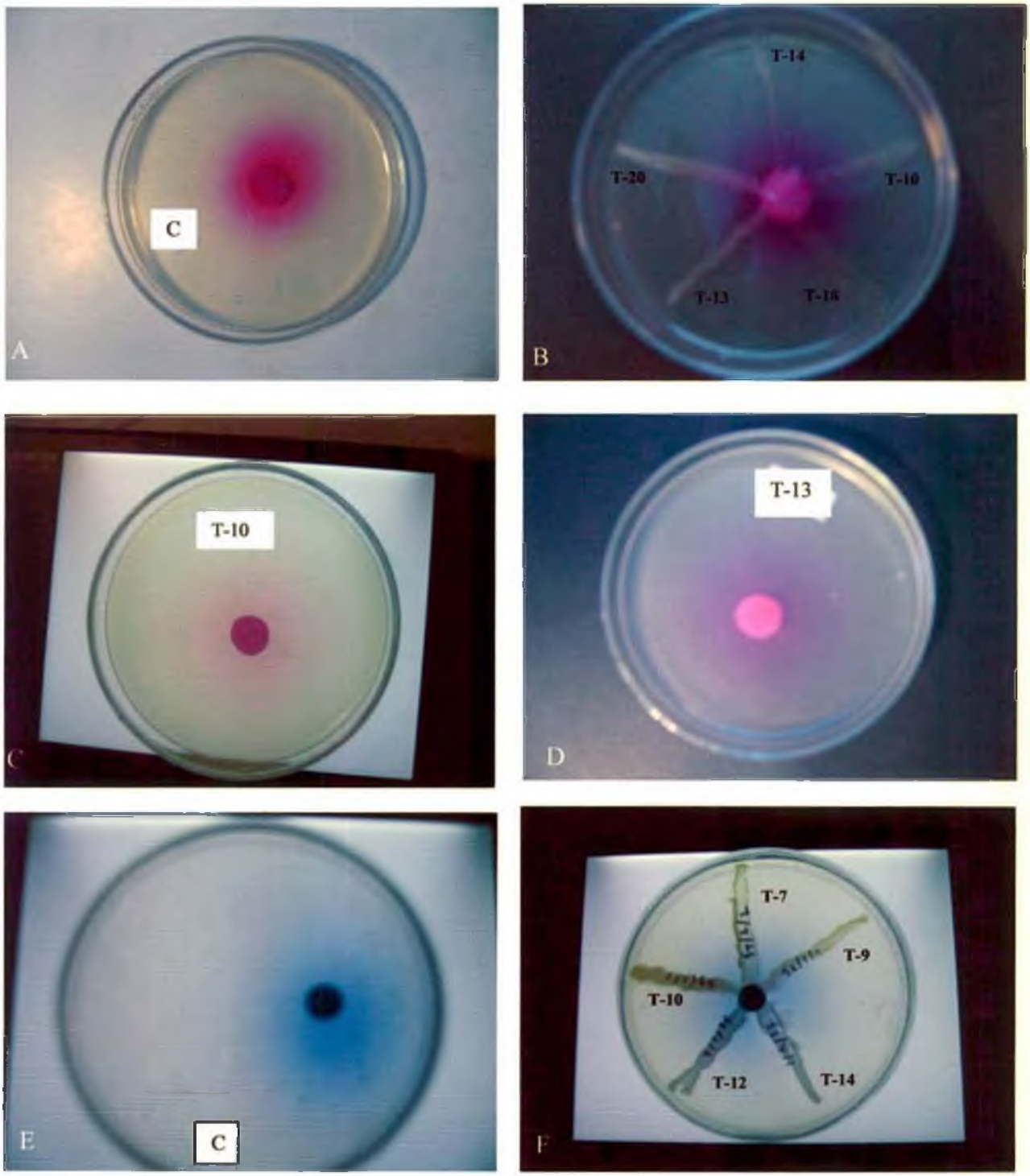

Fig.3.12 (A-F) Photographs showing decolourization of selected dyes viz. Red E6BL, Turquoise Blue BGF as observed by disc diffusion experiment.

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Table 3.13: Rate of dye decolourization by mixed bacterial culture.

Mixed Bacterial culture	Decolourization rate (%)		
	Red ME6BL	Orange ME2RL	Turquoise Blue BGF
G-1	93	80	72
G-2	90	92	75
G-3	95	90	80
G-4	85	80	70
G-5	75	72	60
G-6	93	89	65

G-1: *Pseudomanas aeruginosa, B. firmus, P. pseudoalcaligenes, B. cereus.*
G-2: *B. firmus, B. mycoides, B.badius, B. megaterium.*
G-3: *B. punilus, P. alcaligenes, B. cereus, B. subtilis.*
G-4: *B. lentus, B. thuringiensis, B. firmus, B. aeruginosa.*
G-5: *B. pseudoalcaligenes, B. lentus, B. mycoides, B. globisporus.*
G-6: *B. firmus, B. fastidiosus, P. pseudoalcaligenes, B. mycoides*

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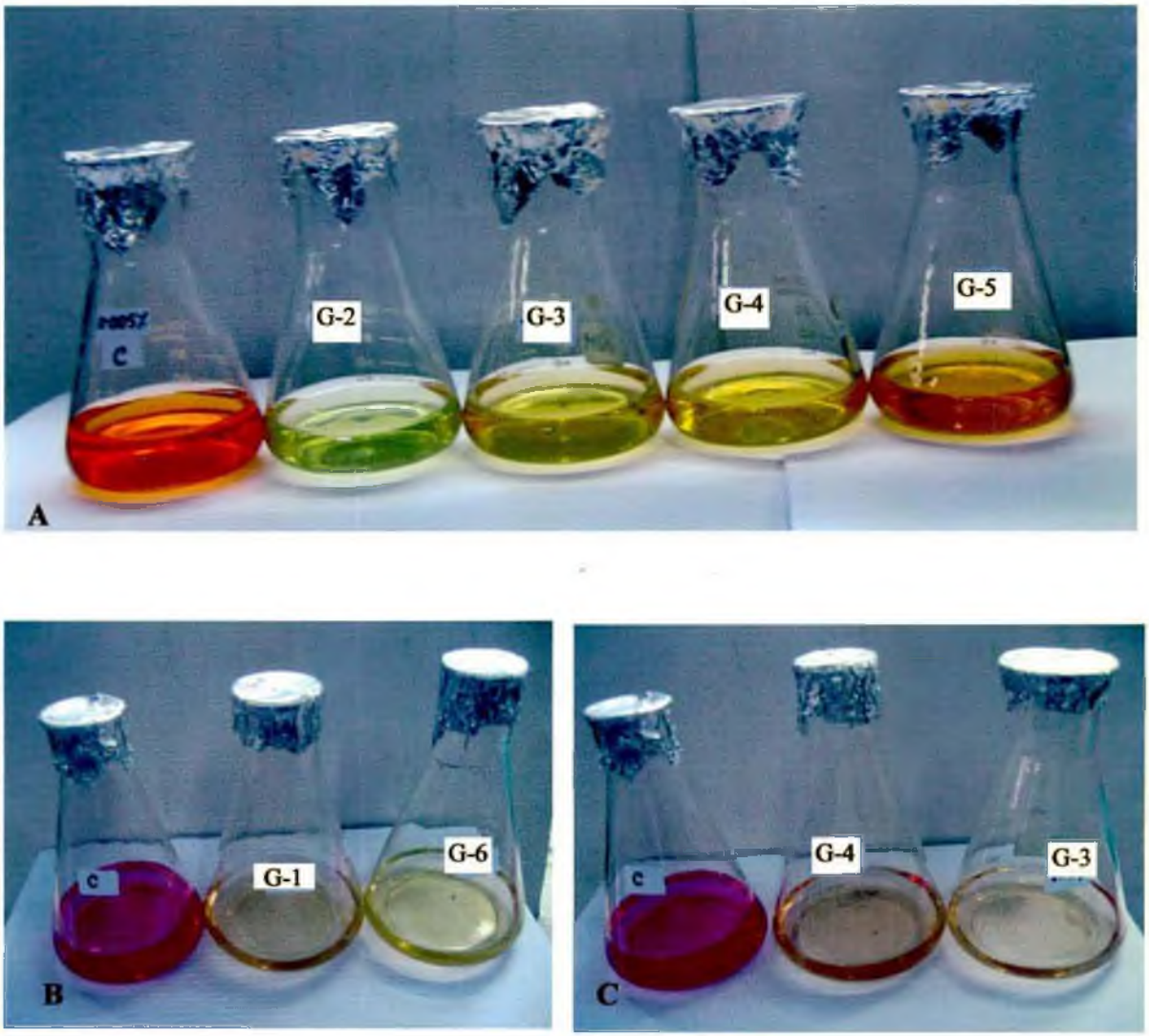

Fig. 3.13(A-C) Dye decolourization by single mixed culture. A. Orange ME2RL. B,C. Red ME6BL.
(C- Control)

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3.14. Effect of pH on Red ME6BL decolourization

The effect of pH on decolourization of Red ME6BL was examined. Result was shown in Fig 3.14, 3.16, 3.17 and 3.18. Effective decolourization was observed in between pH 6.5-9.5. This could be due to adaptation of their original habitat. (pH 6.85 – 10.52.)

3.15. Effect of temperature on Red ME6BL decolourization

Effect of temperature on dye decolourization was studied using dye Red ME6BL, Orange ME2RL and Turquoise Blue BGF the result was shown in the Fig.3.15, 3.19, 3.20 and 3.21. Better decolourization was observed between 30-40°C temperature.

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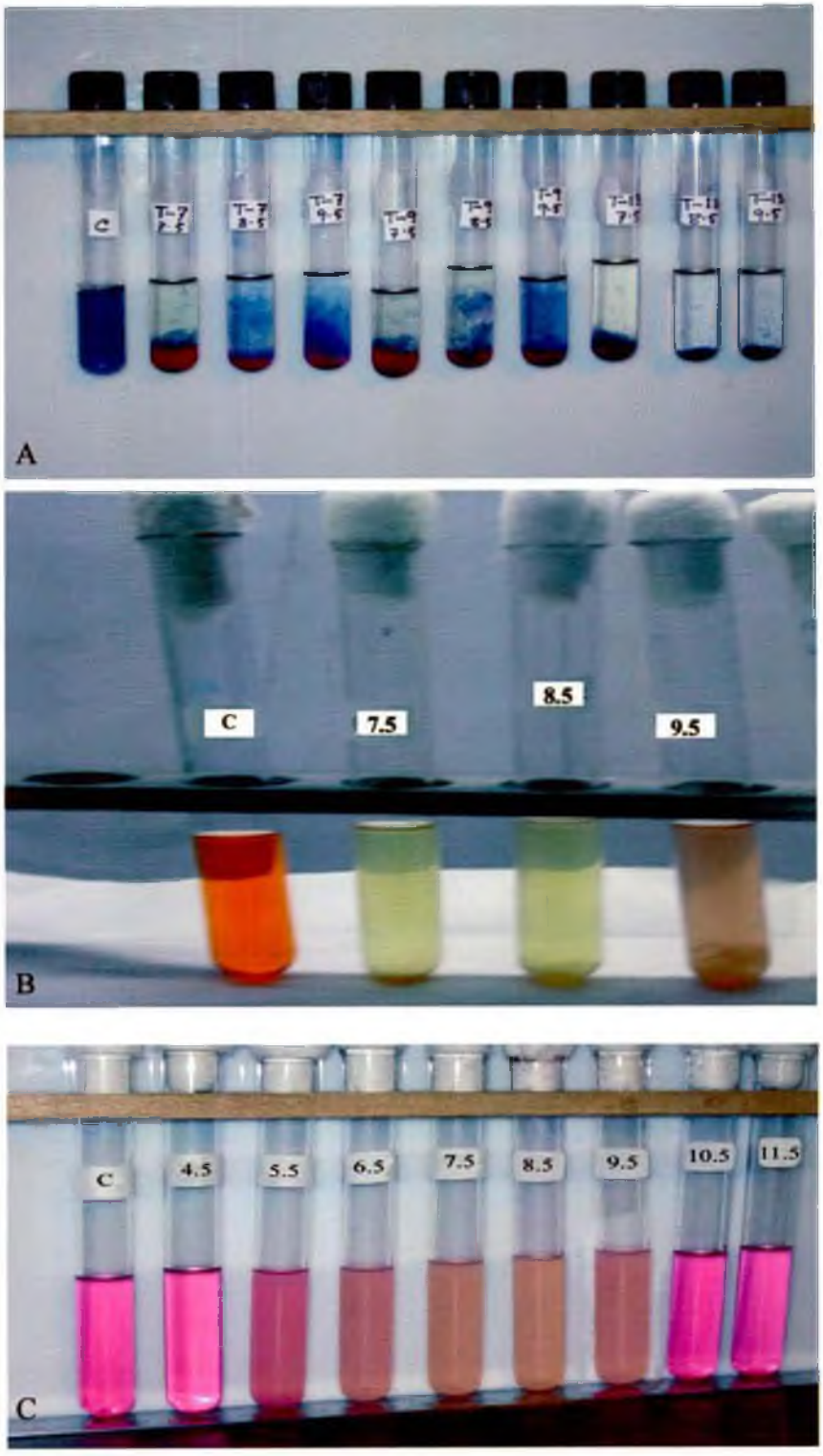

Fig. 3.14 (A-C) Effect of pH on A. Turquoise Blue BGF, B. Orange ME2RL and C. Red ME6BL.

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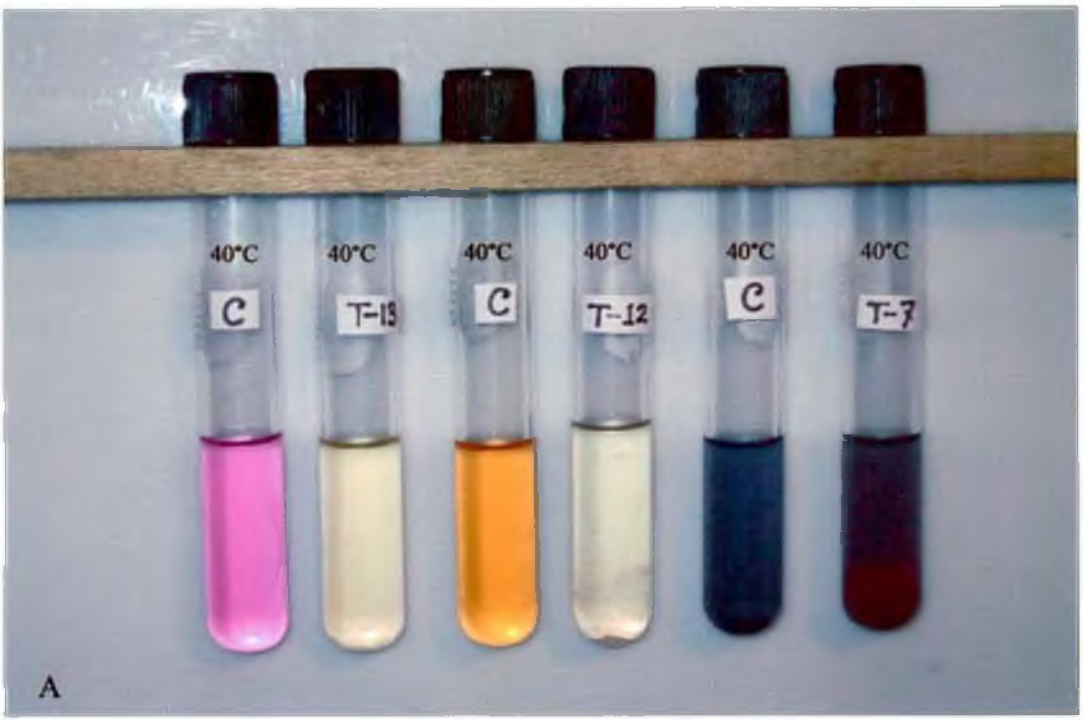

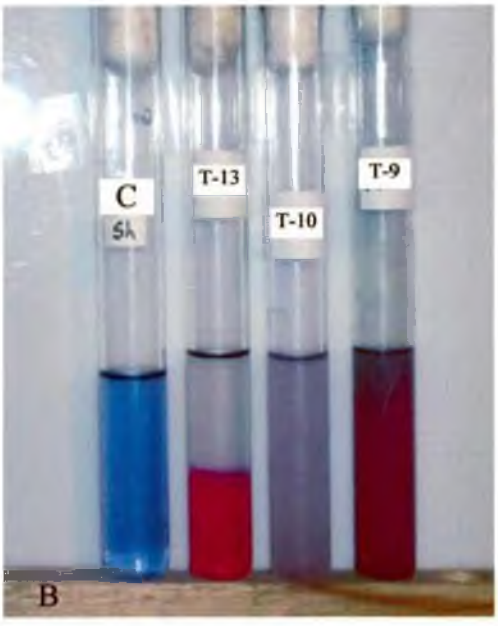

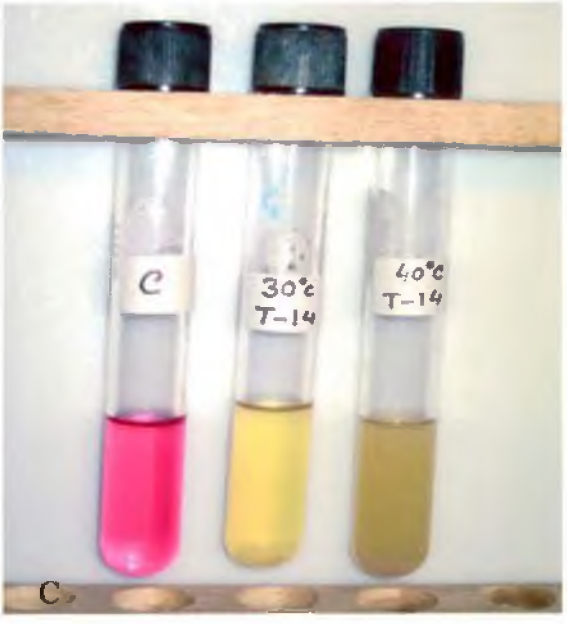

Fig. 3.15 (A-C) Effect of temperature on decolourization of Red ME6BL, Orange ME2RL and Turquoise Blue BGF.

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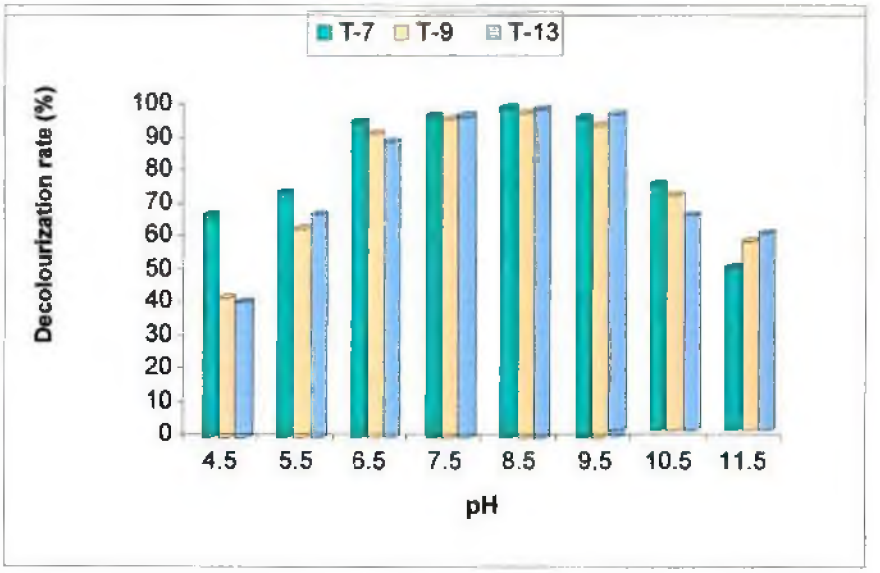

Fig. 3.16 Effect of pH on decolourization of Turquoise Blue BGF by *B. firmus, B. mycoides and B. subtilis.*

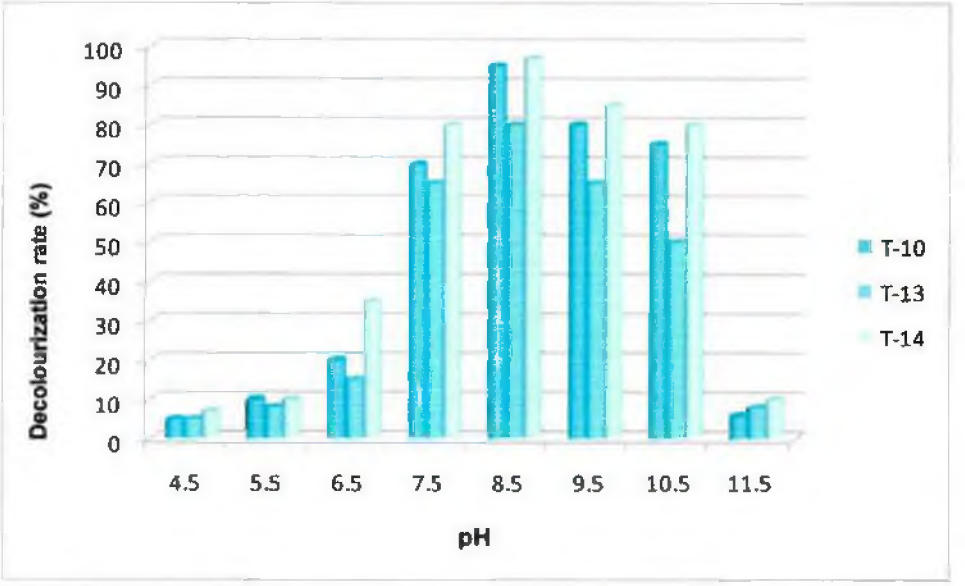

Fig. 3.17 Effect of pH on decolourization of Orange ME2RL by *B. cereus, B. subtilis and B. badius.*

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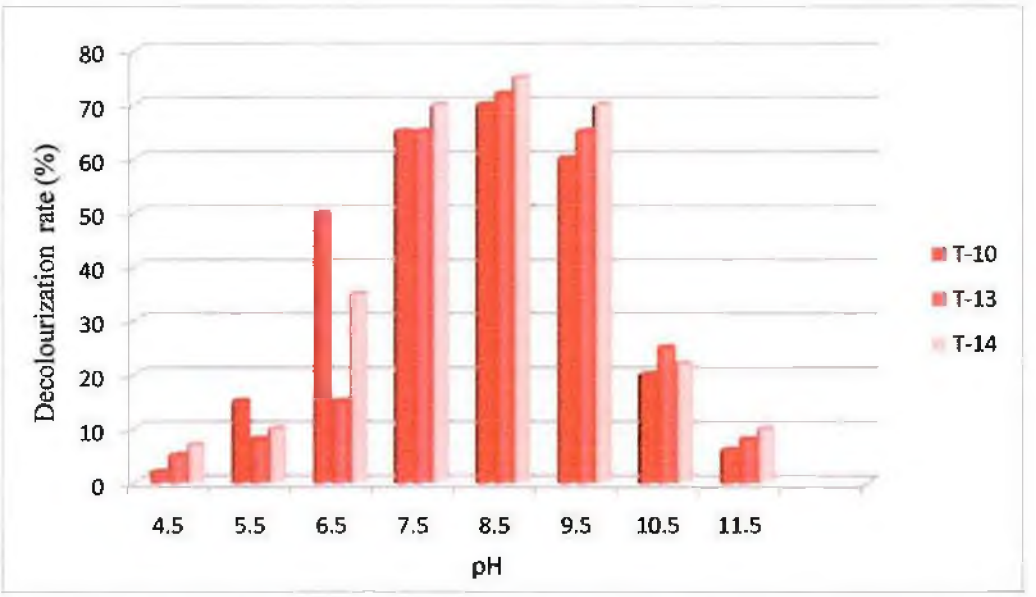

Fig. 3.18 Effect of pH on decolourization of Red ME6BL by *B. cereus*, *B. subtilis* and *B. badius*.

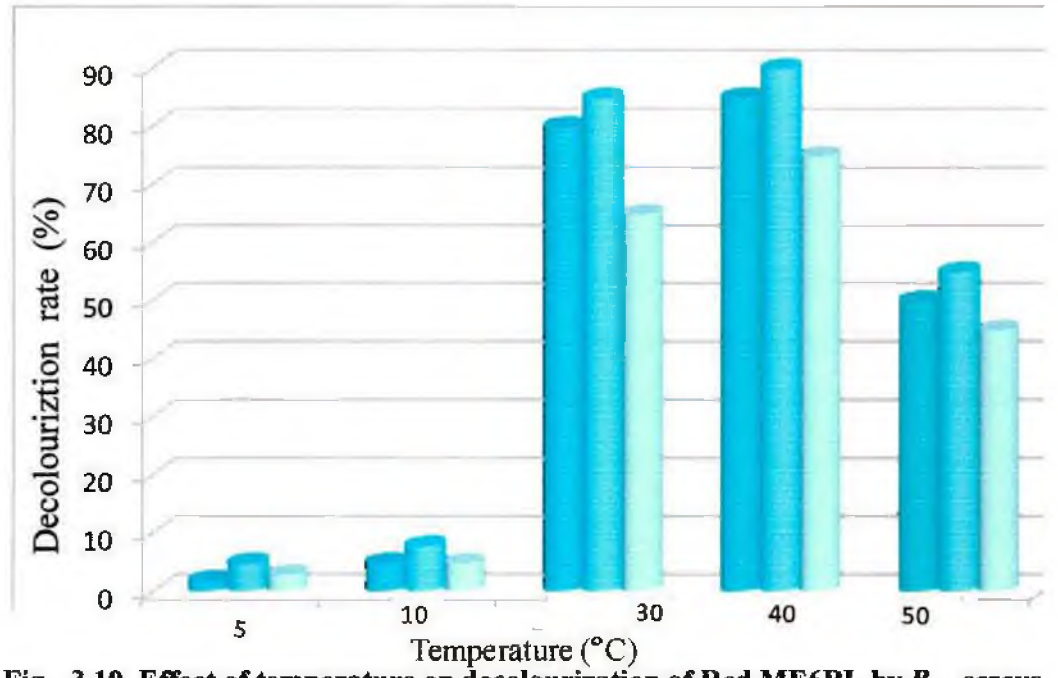

Fig. 3.19 Effect of temperature on decolourization of Red ME6BL by *B. cereus* *B. badius* and *B. globisporus*.

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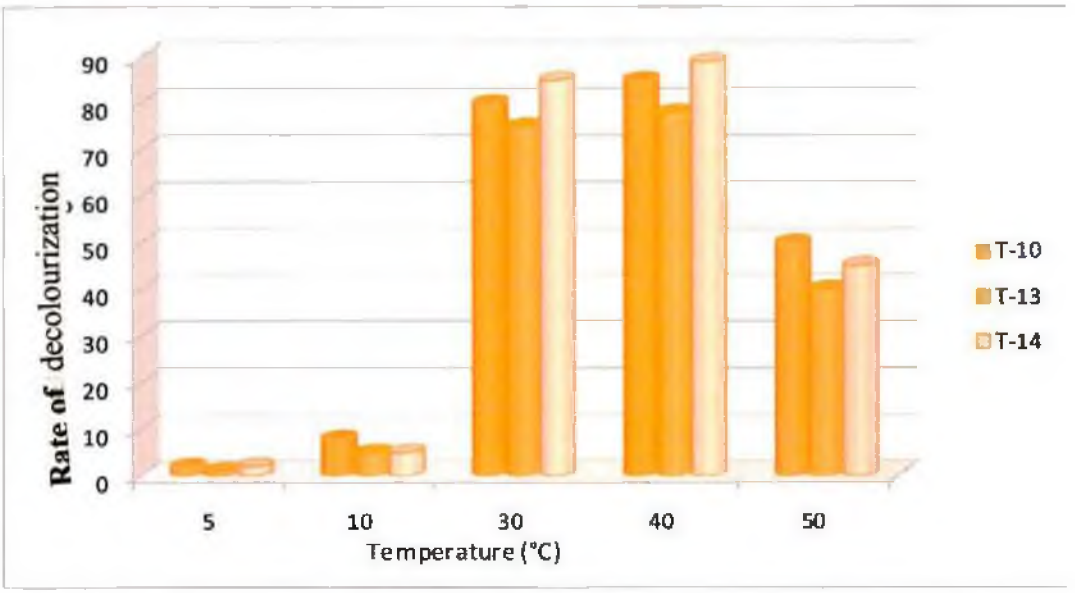

Fig. 3.20 Effect of temperature on decolourization of Orange ME2RL by *B. cereus, B. subtilis* and *B. badius.*

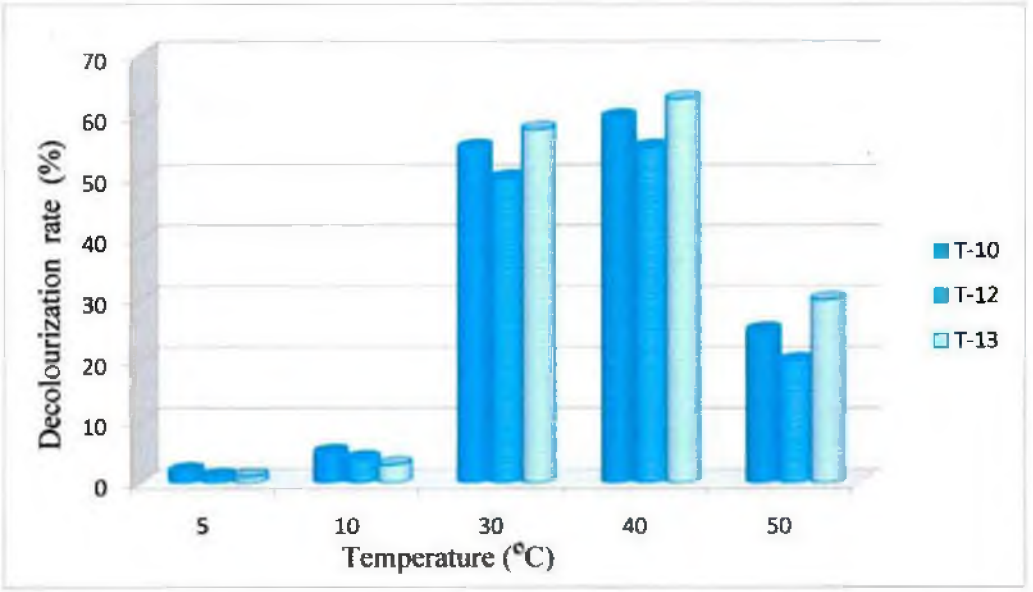

Fig. 3.21 Effect of temperature on decolourization of Turquoise Blue BGF by *B. cereus, B.lentus* and *B. subtilis.*

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Chapter - 4
Discussion

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Discussion:

Bangladesh is one of the underdeveloped countries. Its economy is primarily based on agriculture. At present some important small scale industries viz. textile dyeing, Leather industries etc. are coming up. Among these textile dyeing industry plays an important role to support our economy. We must have to concentrate our knowledge in this industry and it's surrounding environment. The discharge of the coloured waste effluents of these industries into the receiving water bodies is objectionable not only from aesthetic consideration, but also for the fact that it reduces penetration of sunlight and retards biological activity in the aquatic environment. The presence of dyes and chemicals in the waste effluents even at very low concentration is harmful to both aquatic and terrestrial life (Heldenbrand *et al.* 1999, Hatch and Maibach 2000). Textile production is one of the most polluting industries (Vandevivere *et al.* 1998). Production processes not only generate heavily polluted waste water, but also waste heat, solid waste and exhaust gas.The release of coloured compounds into the environment is undesirable not only because of their coloures, which may affect photosynthesis of aquatic plants, but also because many dyes and their breakdown products may be toxic and /or mutagenic to life (Weisburger 2002). About 10-15% of all dyes are directly lost to waste water in the dyeing process. Thus the waste water must be treated before releasing into the natural environment. For biological treatment of the waste water containing dyes, the microbial decolorization and degradation of dyes have been of considerable interest.

The removal of the polluting dyes is an important problem particularly for small scale textile industries where working condition and economic status do not allow them to treat their waste water before disposal and they have no choice other than dumping all effluents into the main stream of water sources. Microbial decolourization and

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degradation is environment friendly and cost competitive alternative to chemical composition processes. Moreover, decolourization and degradation can also detoxify the effluent. Recent fundamental work has revealed the existence of a wide variety of microorganisms capable of decolorizing a wide range of dyes (Banat *et al.* 1996).

Many factors including: the environment (for example oxygen, temperature, moisture, trace minerals and salts, nutrients and co-metabolites, P^H, redox potential, pressure and light); influence the rates of biodegradation. For materials exposed to a natural environment, biodegradation means fragmentation, loss of material's properties or chemical modification through the action of microorganisms. In the biomedical area, "biodegradation refers to hydrolysis and oxidations that are the primary polymer degradation processes (Cha and Pitt 1990).

In a natural environment, microorganisms on a substrate tend to form a biofilm, consisting of bacteria and fungi in a highly hydrated (85-98% water) matrix of extracellular polymers. Both hydrolysis and oxidation of the substrate can be mediated by the biofilm, by release of extracellular enzymes or free radicals. The extracellular nature of these substances enable microorganisms to tolerate higher concentrations of toxic chemicals than would be possible if the compounds had to be brought into the cell before degradation (Mas-Castella *et al.* 1995)

There were very little differences of pH and temperatures of all the samples collected from different sites of the textile dyeing industries. The collected samples were mostly alkaline. Similar result was also reported by other workers (Sarnaik and Kanekar 1995) and (Saha *et al.* 2006).

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Maximum temperature of the studied sample was 38°C and minimum temperature was 36°C. It was observed that maximum growth of the isolated strains were found at temperature 30-40°C.

During this study the bacterial count showed a range of 4.6×10^5 to 3.85×10^7 cfu/ml in textile dyeing water sample. In a study Saha *et al.* (2006) reported the average bacterial load in the textile effluent as in the range of 6.1×10^7 to 19.4×10^7 cfu/ml. The present study showed a very close bacterial count to the study carried out by Saha *et al.*(2006)

In the present study 25 aerobic heterotrophic bacterial isolates were finally selected for detail study. Following Bergey's manual (Vol. 1& 2) and exploiting the available facilities all the isolated organisms were provisionally identified. Among them 21 were aerobic heterotrophic, Gram-positive, spore former and rods of different size. It was very interesting to note that all these 21 bacteria belonged to a genus *Bacillus*. Remaining 4 were Gram- negative, rod shaped, non-spore former and belonging to the genus *Pseudomonas*.

Under the genus *Bacillus* 21 distinct species viz. *B. badius*(3), *B. firmus*(4), *B. thuringiensis*(1), *B. lentus*(3), *B. pumilus*(3), *B. mycoides*(2), *B. cereus*(1), *B. subtilis*(1), *B. globisporus*(1), *B. fastidiosus*(1), and *B. megaterium* (1) were found. The isolated organisms showed some minor differences in biochemical characters from those cited in the Bergey's manual of systematic bacteriology Vol.2. (Sneath *et al* 1986).

Among 21 strains 3 (T-1, T-4, T-14) were provisionally identified as *Bacillus badius*. The strains could not utilize citrate and could not produce acid from D-Glucose, L-Arabinose, D-Xylose and D-Mannitol. The strains T-2, T-5, T-7 and T-25 were very

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similar to *Bacillus firmus*. The strain T-2 differed from the description found in the Bergey's manual in its inability to produce acid from D-glucose, D-mannitol and starch hydrolysis. The strain T-25 could produce L-arabinose but reference organism could not produce.

The strain T-3 was provisionally identified as *Bacillus thuringiensis*. This organism could unable to hydrolyse of starch, casein and tyrosin, while the reference organism could hydrolyse. The strain also differed in its ability to produce acid from D-arabinose but the reference organism could not produce.

The strains T-8, T-11 and T-17 were found to resemble to the standard strain of *Bacillus pumilus*. The strains T-8 and T-17 could not produce acid from D-arabinose, D-xylose. The strain T-11 was unable to produce oxidase.

Considering maximum similar characters the isolates T-9 and T-19 were found to most resemble to the reference strain of *Bacillus mycoides*. This strain could able to hydrolyse tyrosin.

The isolated strain T-10 was most resemble to *Bacillus cereus*. This strain was also similar with *Bacillus subtilis*. This strain could not produce lecithinase and was unable to hydrolyse starch and utilize citrate.

The strain T-13 was very similar to *Bacillus subtilis*.

The isolated strains T-6, T-12 and T-16 were professionally identified as *Bacillus lentus*. The strain T-6 could not produce acid from D-glucose, D-arabinose, D-xylose and D-mannitol but standard organism could produce. The strains T-12 and T-16 could not produce acid from D-arabinose, D-xylose and D-manitol.

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The strains T-6, T-12 and T-16 were unable to hydrolyse starch while the reference organism could. The strains T-6 and T-16 could not hydrolyse starch and produce caseiase enzyme.

The isolated strain T-18 belonged to *Bacillus globisporius*, but it differed from the reference strain on the hydrolysis of starch.

The strain T-23 was found to closely resemble to the reference strain of *Bacillus fastidiosus*. This strain was unable to hydrolyze casein and gelatin.

The strain T-24 was provisionally identified as *Bacillus megaterium*. T-24 could not utilize casein and propionate.

During this period of study dye decolourization test was carried out by textile dyeing waste water sample as inocula. The waste water sample was added to the seven selected textile dyes solution viz. Red ME6BL, Orange ME2RL, Turquoise Blue BGF, Turquoise Blue RSPL, Violate S3RL, Yellow 4G and Green 3R were completely decolourized within 24h (Table 3.11 and Fig.3.7).

It was observed that rate of decolourization increased with increase of inocula. The maximum rate of decolourization was observed at 20% (v/v) inoculum concentration. Maximum colour removal was in the range from 90 to 95%. A similar results have been reported by Sani and Banerjee (1999).

In a static condition decolourization test *B. cereus, B. subtilis and B. badius* could decolourize upto 90-95% Red ME6BL and Orange ME2RL within 48 hrs while Turquoise Blue BGF up to 50-60% within 72 hrs.

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In shake culture incubation same strains decolourized Red ME6BL & Orange ME2RL up to 70-75% within 48 hrs and Turquoise Blue BGF was decolourized up to 40-50% in 72 hrs.

In aeration condition Red ME6BL & Orange ME2RL were decolourized up to 70-75% within 48 hrs and Turquoise Blue BGF up to 40-45% within 72 hrs. (Table 3.12.1, 3.12.2 and 3.12.3, Fig 3.8).

During this study seven dyes viz. Red ME6BL, Orange ME2RL, Turquoise Blue BGF, Turquoise Blue RSPL, Violate S3RL, Yellow 4G and Green 3R were used for decolourization. Out of there seven test dye compounds Turquoise Blue RSPL, Yellow 4G and Green 3R were found to be recalcitrant for the test organisms. Three other tested dyes were found to be decolourize by most of the isolates (Table.3.12.4 and Fig. 3.9).

The incubation period of 5-7 days is quite long and unsuitable for any industry for actual application purpose (Sarnaik and Kanekar 1998). In this study it was found that complete decolourization of Red ME6BL and Orange ME2RL within 16 hrs by the *B. cereus* and *B. badius* (Table 3.12.5).

Considering the dye compounds, Red ME6BL (91%) was found to decolourize easily by *B. cereus, B. subtilis*, and *B. badius.*

Orange ME2RL was decolourized by *B. firmus, B. mycoides, B. cereus, B. lentus, B.subtilis, B. badius, B. globisporus* and *Pseudomonas alcaligenes.*

This was supported by Sugiura *et al.* (1999), as they had identified azo dye degrading bacteria *Bacillus sp.* OY1-2, *Pseudomonas sp.* and *Xanthomonas sp.* NR25-2 from soil and sewage samples.

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In the present study although *Xanthomonas* was not found but Bacillus and *Pseudomonas* were isolated with dye degradation potential observed.

Same test was carried out but concentration of dye compounds were variable viz. 0.005%, 0.001%, and 0.015% In this experiment average 1.0×10^7 cfu/ml inocula were used.

With initial concentration of 0.005%, *B. firmus, B. mycoides and B. cereus* showed decolourization rate up to 80-82% and *B.lentus, B.badius and B. globisporus* showed 88-91% of the dye Red ME6BL.

72-79% decolourization of Red ME6BL was noticed by *B. firmus, B. mycoides* and *B. cereus* and *B. lentus.*

93% and 94% decolourization were showed by *B. badius and B. globisporus* respectively with initial concentration 0.01%.

67-76% decolourization of Red ME6BL was shown by *B. firmus, B. mycoides and B. cereus* and *B .lentus. B. badius and B. globisporus* showed 81% and 90% decolourization respectively, where initial dye concentration was 0.015%.

B. firmus, B. cereus and *B. subtilis* decolourized (79%) OrangeME2RL (initial conc.0.005%) while 87% by *B. lentus* and *B. globisporus.*

Orange ME2RL(initial conc.0.01%) was decolourized up to 72-76% by the *B. firmus, B. mycoides, B. cereus, and B. subtilis* while 80-82% decolourization was achieved by *B. lentus, B. badius* and *B. globisporus.*

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With initial concentration of 0.015% of the dye Orange ME2RL, *B. firmus B. lentus, B. badius* and *B. subtilis* showed the decolourization rate of 76-79% and *B. badius, B. cereus and B. globisporus* showed 81%.

For decolourization test disc method was also employed, where discs were soaked with different dyes (viz. Red ME6BL, Orange ME2RL, Turquoise Blue BGF) and placed on sloppy NA plate where selected strains were inoculated by crowded plate and streak method.

.It was observed that when the decolourization test was carried out by the mixed culture in different combinations of the the result positively indicated a synergistic effect of the mixed cultures.

It was noticed that G-1 to G-6 required 2-3 days to decolourize Red ME6BL and Orange ME2RL. Turquoise Blue BGF was decolourized by G-1 to G-6 for 3-5 days.

It was interesting to note that dye solutions were decolourized at different rates. During the present study the active decolourization of different dyes by the tested organisms could also be considered as the degradation of dye stuff at least from the colour point of view.

It was observed that there was no decolourization when the initial pH of the medium was below 5.5. The rate of decolourization increased with increase in pH from 6.5 to 9.5 with a maximum rate of de colourization at pH 8.5. However a further increase in pH, decreased the decolourization rate. Mali *et al.* (1999), found that pH between 6.0 and 8.0 was optimum for decolourization of triphenylmethane and azo dyes by *Pseudomonas sp.*

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The temperature ranging between 30°-40°C was found to be suitable for the decolourization by *Bacillus spp*. And a further increase in the temperature drastically affected decolourization activity of bacterial culture. It was observed that *Klebsiella pneumoniae* RS-1 and *Alkaligenes liquefaciens* S1 showed no decolourization of Methyl Red at 45°C (Wong and Yuen 1998).

There are more than 200 textile dyeing industries in the sampling site for a period of 20-25 years. The soil and water body in the premises of these industries have been charged with toxic chemicals like azo, sulfo and nitro dyes. Hence the variety of microorganisms which are expected to be present in this environmental condition possibly have adapted to tolerate and utilize these chemicals as their substrates.

During this study a good number of potential bacteria were isolated which were capable of decolourization or detoxification of the dyes or chemicals present in the textile dyeing effluent. The potential bacteria could be used for future study for the bioremediation of the toxic chemicals present in the industrial waste samples.

In a third world country like Bangladesh biotechnology is the technology of choice on which we can depend. Since microbial biotechnology plays a vital role on our civilized lives. We can plan to employ our potential microorganisms for bioremediation of textile effluents and thus clear up our environment for future generation.

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Chapter - 5
References

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Appendix

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APPENDIX

Composition of the media and reagents used in this study are as follows. In each case, pH was adjusted before sterilization

1. Ammonium oxalate crystal violet solution (Claus 1995)

Solution A

Crystal violet (85% dye content)	2.0 g
Ethyl alcohol (95%)	20 ml

Solution B

Ammonium oxalate	0.8 g
Distilled water	80 ml

Solution **A** and **B** were mixed.

2. Alkaline Methylene blue solution (SAB 1957)

Solution A

Methylene blue	0.3 g (90% dye content)
Ethyl alcohol (95%)	40 ml

Solution B

KOH (0.01 % by wt)	100 ml

Solution **A** and **B** were mixed.

3. Basal medium for fermentation (SAB 1957)

Beef extract	3.0 g
Ammonium dihydrogen phosphate	1.0 g
Magnesium sulphate	0.2 g
Potassium chloride	0.3 g
Carbohydrate	10.0 g
Bromothymol blue	2.0 ml
Distilled water	1000 ml

4. Carbol-fuchsin solution (SAB 1957)

Solution-A

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Basic fuchsin (90% dye content)	0.3 mg
Ethyl alcohol (95%)	10 ml

Solution-B

Phenol	5.0 g
Distilled water	95.0ml

Solution **A** and **B** were mixed.

5. Congo red solution (SAB 1957)

Congo red (80% dye content)	2.0 g
Distilled water	100 ml

6. Deep glucose agar medium (Khan 1962)

Beef extract	3.0 g
Peptone	5.0 g
Glucose	10.0 g
Agar	15.0 g
Distilled water	1000 ml

7. Egg-yolk reaction medium (SAB 1957)

Peptone	40.0 g
Na_2HPO_4	5.0 g
KH_2PO_4	1.0 g
NaCl	2.1 g
$MgSo_4$	0.1 g
Glucose	2.0 g
Agar	25.0 g
Distilled water	1000 ml
pH	7.6

8. Gas Production From Glucose (SAB 1957)

Beef extract	3.0g
Peptone	5.0g
Carbohydrate (glucose)	5.0g
Bromothymol blue	1.25g

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Distilled Water	1000ml
pH	6.8

9. Iodine Solulion (Claus 1995)

Iodine	1.0 g
Potassium Iodide	2.0 g
Distilled water	300 ml

10. Indole Nitrate broth (Biolife Manual)

Peptone	20.0g
NaH2PO4	2.0g
Glucose	1.0g
KNO3	1.0g
Agar	1.0g
Dist. Water	1000ml
pH	7.2

11. Kovac's reagent (SAB 1957)

Paradimethyl amino-benzaldehyde	5.0 g
Butyl alcohol	5 ml
HCl (Conc.)	25 ml

12. Levan medium (Schaad 1988)

Beef extract	3.0 g
Peptone	5.0 g
Sucrose	50.0 g
Agar	15.0 g
Distilled water	1000 ml
pH	9.5

12. Malachite green solution (Claus 1995)

Malachite green	5.0 g
Distilled water	100 ml

13. Mercurochrome solution (SAB 1957)

Mercurochrome	0.5 g

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Distilled water	100 ml

14. Methyl Red/Voges-Proskauer broth medium (Bryan 1950)

Peptone	7.0 g
Glucose	5.0 g
Dipotassium phosphate	5.0 g
Distilled water	1000ml

15. Methyl red solution (Bryan 1950)

Methyl red	0.1 g
Ethyl alcohol (95%)	300 ml
Distilled water	200 ml

16. Modified Nutrient Agar medium

Beef extact	3.0 g
Peptone	5.0 g
NaCl	5.0 g
Agar	15 g
Textile effluent (sterile)	1000 ml
pH	8.5

17. Modified Nutrient Broth medium

Beef extact	3.0 g
Peptone	5.0 g
NaCl	5.0 g
Textile effluent (sterile)	1000 ml
pH	8.5

18. Motility medium (Eklund and Lankford 1967)

Nutrient broth	100 ml
Agar	0.5 g
Tetrazolium chloride	0.001g

19. Nutrient agar medium (Bryan 1950)

Beef extract	3.0 g
Peptone	5.0 g

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Nacl	5.0g
Agar	15.0g
Distilled water	1000 ml
pH	7.2

20. Nutrient broth medium (Bryan 1950)

Beef extract	3.0 g
Peptone	5.0 g
Nacl	5.0g
Distilled water	1000 ml
pH	7.2

21. Nutrient gelatin agar medium (Collins and Lyne 1984)

Beef extract	3.0 g
Peptone	5.0 g
Gelatin	8.0 g
Agar	15.0 g
Distilled water	1000ml

22. Nitrate broth medium (SAB 1957)

Peptone	5.0 g
Beef extract	3.0 g
Potassium nitrate	1.0 g
Distilled water	1000ml

23. α-Napthol solution (Bryan 1950)

α –Napthol	5.0 g
Ethyl alcohol (95%)	100ml

24. Oxidase test reagent (Collins and Lyne 1984)

Tetramethyl-p-phenylene-diamine dihydro-chloride	1.0 g
Distilled water	100 ml

25. Peptone broth

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Peptone	2.0 g
Water	100 ml

26. Phenylalanine agar medium (Sneath *et al.* 1986)

Yeast extract	3.0 g
Di-phenylalanine	2.0 g
Na2HPO4	1.0 g
NaCl	5.0 g
Agar	12.0 g
Distilled water	1000 ml
pH	7.3

27. Physiological saline

Sodium chloride	0.85 g
Distilled water	100 ml

28. Propionate agar medium (Sneath *et al.* 1986)

Sodium propionate	2.0 g
$MgS0_4.7H_20$	1.2 g
$NH_4 HP0_4$	0.5 g
KCl	1.0 g
Trace element solution	40.0 ml
Agar	15.0 g
Distilled water	920 ml
Phenol red (0.04% w/v)	20 ml
pH	6.8

29. Safranin solution (SAB 1957)

Safranin	0.5 g
Distilled water	100 ml

30. Simmon's citrate agar (Claus 1995)

$MgSO_4$	0.2 g
Mono-ammonium phosphate	1.0 g
Di-Potasssium phosphate	1.0 g

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Na-citrate	2.0 g
NaCl	5.0 g
Bromo-thymol-blue	0.08 g
Agar	15.0 g
Distilled water	1000 ml.

31. Starch agar medium (Claus 1995)

Beef extract	3.0 g
Peptone	5.0 g
Soluble starch	10.0 g
Agar	15.0 g
Distilled water	1000 ml

32. Skim Milk Agar (SMA) medium (Collins and Lyne 1984)

Skim Milk	6.6 ml
Nutrient agar	100 ml
pH	9.5

33. Tyrosine agar medium (Sneath *et al.* 1986)

L-tyrosine	0.5 g
Distilled water	10 ml
Nutrient agar	100 ml

Tyrosine was sterilized separately and was added to the medium before inoculation.

34. Tryptone broth medium (SAB 1957)

Tryptone	10 g
Distilled water	1000 ml
pH	7.2