Effect of Physical Parameters on the Decolorization of Multiple Synthetic Textile Dyes by Bacteria Recovered from Dye Contaminated Soil and Water

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Submitted by

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DECLARATION

I, Mostafa Kamal, hereby declare that the thesis project entitled "Effect of Physical Parameters on the Decolorization of Multiple Synthetic Textile Dyes by Bacteria Recovered from Dye Contaminated Soil and Water" has been carried out under the joint supervision and guidance of Dr. Mohammad Nazrul Islam Bhuiyan, Senior Scientific Officer, Industrial Microbiology Division, Institute of Food Science and Technology (IFST), Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka and Professor Dr. Naiyyum Choudhury, Former Coordinator, Biotechnology Program, BRAC University, Dhaka. I declare further that this work is entirely based on the original results I have found. The contents of this thesis have not been previously submitted elsewhere for publication or award of any degree.

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To

My Precious Parents
&
Taizaan

ACKNOWLEDGMENT

Finally, and most of all, I would like to thank my parents for their endless support, encouragement, and love; I would be lost without you.

Mostafa Kamal

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ABSTRACT

Textile wastewater is a complex mixture of many contaminating materials. These include a high concentration of salts, acids, heavy metals, organochlorine-based pesticides, pigments, dyes etc. Dye is the first pollutant to be recognized in wastewater and many synthetic dyes are toxic, mutagenic and carcinogenic in nature. This makes dyes imperative to be removed from textile wastewater. Current physicochemical textile dye removal methods are less efficient and expensive. Bioremediation through utilization of synthetic dye degrading bacteria can be considered as an effective aid to the clean-up process. The present study attempts to decolorize eight synthetic textile dyes: Novacron Brilliant Blue C-B, Novacron Dark Blue S-GL, Novacron Navy S-GI, Novacron Turquoise GN, Novacron Super Black-G, Novacron Ruby S-3B, Avitera Brilliant Yellow SE and Novacron Yellow S-3R by newly isolated bacterial strains from dye contaminated soil and water. Dye decolorizing ability of bacteria was screened on Nutrient broth containing mixed dyes. Effects of different physical parameters like dye concentration, temperature, pH, agitation and seed culture concentration on the dye degrading ability of selected isolates were tested. The potential isolates were selected for BiOLOG identification. *Pseudomonas aeruginosa* (S9A6) exhibited to be the most promising amongst the isolated bacteria for decolorizing synthetic dyes, followed by *Bacillus cereus/thuringiensis* (S1A6). *Pseudomonas aeruginosa* was tested further for effects of mixed dye concentration and the repeated addition of dye aliquots up to four cycles. The decolorization obtained in this study was expressed in percentages with *Pseudomonas aeruginosa*, S9A6 having up to 98.5% decolorization of Novacron Super Black-G, 98.4% of Novacron Yellow S-3R, 98.3% of Novacron Dark Blue S-GL, 97.4% of Novacron Navy S-GI, 96.7% of Novacron Ruby S-3B, 94.2% of Novacron Brilliant Blue C-B and 88.6% decolorization of six synthetic dye mixture for 100 ppm of dye concentration when incubated under stationary condition at 37°C for 72 hours. However, there was no observable color difference for Novacron Turquoise GN and Avitera Brilliant Yellow SE by any of the isolated bacteria. The prime candidate *Pseudomonas aeruginosa* also exhibited very good ability to decolorize repeated additions for six individual dyes up to the fourth cycle without supplementation of additional nutrients, which is remarkable criteria for commercial applicability of it in textile effluent treatment.

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Abbreviations	Descriptions
BUG	BiOLOG Universal Agar
cm	Centimeter
et al.,	And others
Etc	And the rest
FAS	Foreign Agricultural Service
FTIR	Fourier-Transform Infrared Spectroscopy
GAIN	Global Agricultural Information Network
GDP	*Gross domestic product*
GC-MS	Gas Chromatography-Mass Spectrometry
g/L	grams per liter
HPLC	High-Performance Liquid Chromatography
IF	Inoculating Fluid
mL	Milliliter
NA	Nutrient Agar
OD	Optical Density
pH	The negative logarithm of hydrogen ion concentration
Ppm	Parts per million
psi	Pound per square inch
PCR	Polymerase Chain Reaction
REP-PCR	Repetitive Element Palindromic-PCR
rpm	rotations per minute
USDA	United States Department of Agriculture
UV-Vis	Ultraviolet-Visible
viz.	videlicet, namely
v/v	Volume by volume
℃	Degree Celsius
%	Percentage

Introduction

The textile and apparel industry is one of the major industries in the world that play a vital role in the economy of many countries. In terms of production and employment, the textile industry is one of the largest industries in the world. It is the largest manufacturing sector in Bangladesh, contributing 12.36 percent of the country's GDP. It also accounted for 80.7 percent of the total export earnings in the Fiscal Year 2016-2017, making it Bangladesh's leading foreign exchange earner (Hossain, 2017). According to the USDA FAS-GAIN report approved by Wallace (2017), Bangladesh currently has 241 dyeing and finishing mills, 424 spinning mills, and 794 textile weaving mills. The report also states that a total of over 6,500 registered and over 500 un-registered garment and textile industries are present in Bangladesh and sixty-five percent of these factories are located in Dhaka district.

Pollution from Textile Industry

Industrial wastes from textile mills are a major source of pollution. In an article published in the Economist in 2017 entitled "Looking good can be bad for the planet", mentioned that "massive amounts of energy, water, and other resources are needed to make clothes". Numerous reports suggest that an average sized textile mill consumes about 1.6 million liters of water per day for the production of about 8000 kg of cloth (Khandare and Govindwar, 2015). The textile industry also uses a variety of chemicals and dyes in the manufacturing of textiles. As stated by Bharathi and Ramesh (2013), a total of about 10,000 different dyes and pigments are used in textile industry and more than 7×10^8 kilograms of synthetic dyes are manufactured worldwide annually (Seow and Lim, 2016). The textile dye-containing wastewaters have a complex chemical composition which also includes sizing agents, surfactants, volatile organic compounds, disodium terephthalate, ethylene glycol, etc. (Chen and Burns, 2006) and require on-site treatment before discharge into the sewage system. In Bangladesh, textile industrial units are mostly located at waterside and effluents produced from these mills are being discharged at random without treatments directly into the waterbodies through illegal pipes. Sultana *et al.*, (2009) reports that the textile dyeing factories of Narayanganj and Gazipur produce a large amount of effluents and sewage sludge on a regular basis. These pollutants are being directly discharged into the surrounding

channel, agricultural fields, irrigation channels, surface water and finally enter into Shitalakshya and Turag River. It is very common practice in Bangladesh because of poor implementation of environmental laws.

Figure 1.1 A pipe, dumps untreated toxic industrial effluents directly to the Buriganga River in Shyampur, Dhaka. Image downloaded from http://www.environmentmove.com/2015/01/16/natural-dyeing-in-fabric-for-reducing-pollution/ in August 2017.

Figure 1.2 Textile dye wastewater brought up to a road by rainwater and residents walk on the dye contaminated water flooded road in Jurain, Dhaka. Image downloaded from http://www.banglanews24.com/national/news/bd/592588.details/ in August 2017.

Dyes: A Short Overview

Worldwide over 10,000 different dyes and pigment are currently used in various industries like textile, rubber product, paper, printing, color photography, pharmaceuticals, cosmetics (Rafii, Hall and Cerniglia, 1997; Revankar and Lele, 2007). Earlier dyes used to be made usually from plants (e.g. indigo from wood and the red dye alizarin from madder) also from insects (e.g. the scarlet dye kermes from the shield-louse *Kermes vermilio*), fungi and lichens. Synthetic dyes are the man-made dyes e.g. azo dye, triphenylmethane dyes, nitroso dyes, disperse dyes, solvent dyes etc. Synthetic dye manufacturing started in 1856 by the English chemist William Henry Perkin. In an attempt to synthesize quinine, he accidentally discovered a bluish substance with excellent dyeing properties. He gave the name Aniline Purple to the bluish substance that later became known as Mauveine (Christie, 2001).

Textile Dyes

Dyes consist of two essential components: the chromophore and the auxochrome. A chromophore is a group of atoms, which controls the color of the dye, and it is usually an electron-withdrawing group. The most important chromophores are -C=C-, -C=N-, -C=O, -N=N-, $-NO_2$ and -NO groups. Azo (monoazo, disazo, triazo, polyazo), anthraquinone, phthalocyanine and triarylmethane dyes are quantitatively the most important chromophores. The compounds bearing chromophores are known as chromogens.

| Azo | Anthraquinone | Phthalocyanine | Triarylmethane |
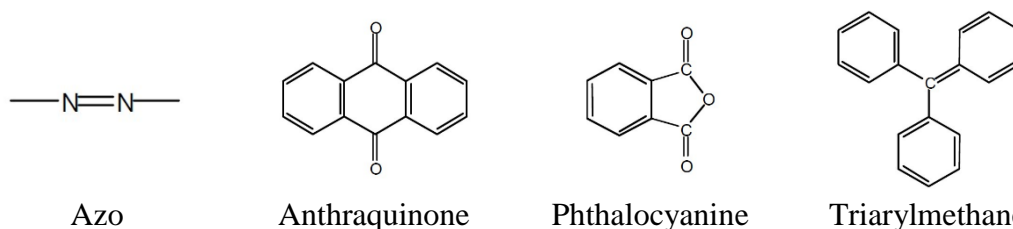
Figure 1.3 The most important chromophores: Azo, anthraquinone, phthalocyanine, and triarylmethane

The auxochrome is an electron-donating substituent that can intensify the color of the chromophore by altering the overall energy of the electron system and provides solubility and adherence of the dye to the fiber. Some examples are: -OH, -NHR, $-NH_2$,

-NR$_2$ (basic), -COOH, -SO$_3$H (acidic). A chromogen without auxochrome can never act as a dye.

Dyes can be classified according to their color, structure, by their usage or method of application. The most appropriate system for the classification of dyes is by chemical structure. It helps to identify dyes belonging to a group that has characteristic properties. According to Rivlin, J. (1992), dyes can be grouped under different classes by their chemical constituents: Azo dyes and pigments (mono azo, diazo, etc.), Anthraquinone Dyes, Benzodifuranone Dyes, Polycyclic Aromatic Carbonyl Dyes, Indigoid Dyes, Polymethine and Related Dyes, Styryl Dyes, Di- and Tri-Aryl Carbonium and Related Dyes, Phthalocyanines, Quinophthalones, Sulfur Dyes, Nitro and Nitroso Dyes and Miscellaneous Dyes (Stilbene and Formazan Dyes). On the basis of the method of application, dyes can be classified into Acid dyes (anionic), Basic dyes (cationic), Direct dyes, Azoic dyes, Vat dyes, Sulfur dyes, Reactive dyes and Disperse dyes.

Toxicity of Dyes: Health and Environmental Concern

Textile wastewater is a complex mixture of many contaminating materials ranging from organochlorine-based pesticides to heavy metals associated with dyes or the dyeing process (Correia, Stephenson and Judd, 1994). Dye is the first pollutant to be recognized in wastewater. The presence of very small amounts of dyes in water (less than 1 ppm for some dyes) is highly visible and affects the clearness of water. Dyes also affect the absorption and reflection of sunbeams, interfering with aquatic life in lakes and rivers. Moreover, many synthetic dyes are toxic, mutagenic and carcinogenic (Golka, Kopps and Myslak, 2004). The experimental work on animal model led by Raj *et al.* (2012) revealed that the azo dyes are directly linked to the urinary bladder cancer in humans. The azo dye group is also related to splenetic sarcomas and hepatocarcinomas. It is also the main cause of chromosomal aberrations in mammalian cells.

 Research has been carried out to find the effects of dye containing effluents on the activity of both aerobic and anaerobic bacteria in wastewater treatment systems. All fabric dyes used in the textile industry are manufactured to be chemically and photolytically stable, i.e., to resist the light, water, various chemicals and microbial attack. Therefore, the release of potentially hazardous dyes in the environment can be

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an ecotoxic risk and can affect man through the food chain (Van Der Zee and Villaverde, 2005). Taking that fact into account, removal of a certain percentage of dyes from industrial effluents is compulsory before being discharged into water systems. Biological methods have been the main focus of recent studies on dye removal.

Bioremediation of Dyes

Bioremediation is biologically mediated breakdown and therefore detoxify hazardous pollutants or waste (as in industrial effluent, oil spill, contaminated groundwater, or an industrial process) in the environment. Plants, microorganisms (bacteria, filamentous fungi, yeasts, actinomycetes, and algae) or enzymes are used to degrade xenobiotics from polluted environments. Bioremediation of synthetic dyes has been recognized as an efficient, cost-effective and eco-friendly solution. In recent years, there has been an increasing interest in the use of bacteria for the treatment of dye polluted wastewaters (Mendes *et al.*, 2011). For synthetic textile dye bioremediation, mixed cultures (consortia), isolated pure organisms or isolated enzymes can be used. Different modes of bioremediation of textile effluents are presented in Figure 1.4.
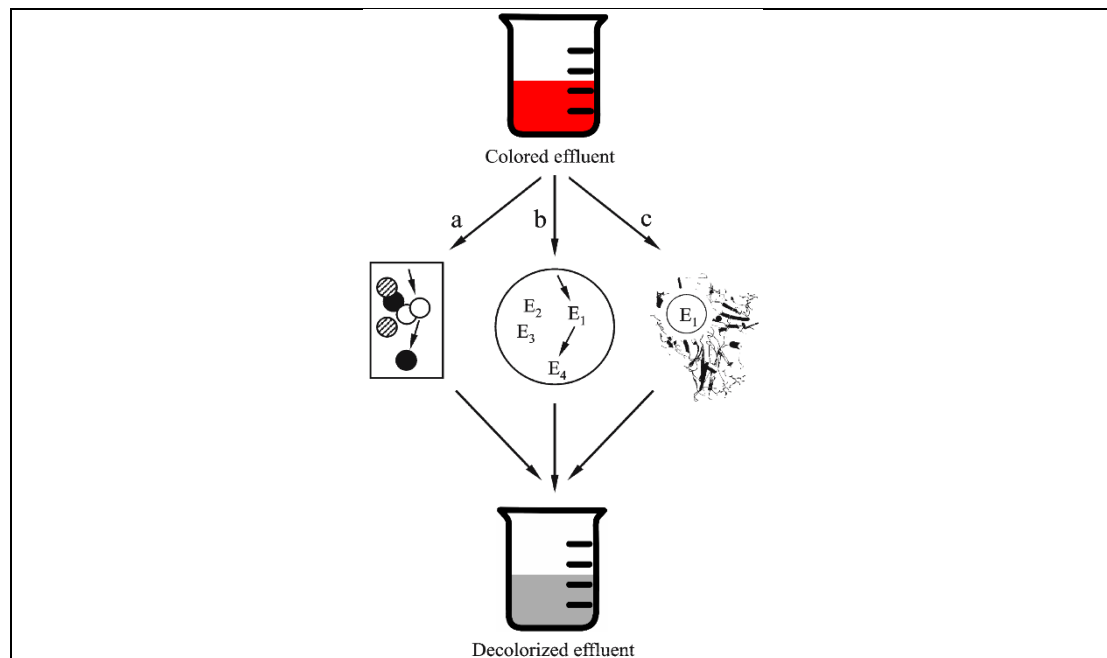
Figure 1.4 Dye bioremediation using -

a. mixed organisms (represented by the white circles, black circles, and lined circles),

b. isolated pure organisms (enzymes denoted as E_1, E_2, E_3 and E_4 expressed in an organism),

c. isolated pure enzymes from the organism. (Amended from Kandelbauer and Guebitz, 2005)

Utilization of mixed organisms often gives considerable advantages over the use of pure isolates in the remediation process of synthetic dyes. In many bioremediation processes, mixed cultures have demonstrated to be superior to pure cultures and exhibit higher stabilities towards environmental stress. As seen in Figure 1.4, one species may be involved in cleavage of the chromophoric group (white circles). Further biotransformation of the modified dye may be performed by another species (black circles). Other species (lined circles) present may not be involved in bioremediation at all but can stabilize the overall ecosystem. However, several researchers have isolated and identified single bacterial strains that have very high efficiency for decolorization of dyes. Many microbial strains that can degrade dyes now been commercialized. Decolorization with isolated pure organisms is useful in interpretation and correlation of the experimental data. It also facilitates detailed mechanisms of biodegradation study. Enzymes expressed in these microbes exhibit a high level of biocatalytic efficiency and a high degree of specificity. Isolated microbial enzymes can be used in dye remediation treatments to target specific pollutants. The main enzymes involved in enzymatic dye bioremediation are the bacterial azoreductases, and the lignin-modifying extracellular enzymes, for instance, laccase, lignin peroxidase, phenol oxidase, manganese-dependent peroxidase and manganese-independent peroxidase secreted by white rot fungi (Kandelbauer and Guebitz, 2005). However, enzymatic bioremediation is still expensive due to the high cost of isolation, purification, and production of enzymes.

Review of Literatures

Dye Reduction Techniques

At present, there are several techniques that can be used in the removal of dyes from textile effluents. However, not all techniques have the same efficacy due to the variety of dyes and to the complexity of the textile effluents. As a result, a combination of various techniques may be required. Several literatures reveal conflicting findings regarding the ability of textile effluent treatment processes. Existing techniques can be divided into three main groups: physical, chemical and biological. In general, each technique has limitations and the use of one individual method may often not be sufficient to achieve complete degradation of dyes. Dye removal approaches consist therefore usually of a combination of different techniques (Van Der Zee and Villaverde, 2005).

Physical and Chemical Dye Removal Approaches

A number of physical and chemical treatment strategies have been used to lower the color pollutants from wastewater. Earlier research efforts mostly depended on using physical or chemical-treatment procedures, occasionally in conjunction with biological treatment. Currently, the main approaches of textile wastewater treatment involve physical and/or chemical processes as adsorption, electroflotation, electrokinetics coagulation, electrochemical destruction, flocculation combined with flotation, flocculation with $Fe(II)/Ca(OH)_2$, ion-exchange, irradiation, membrane-filtration, ozonation, precipitation, and Katox treatment technique involving the use of air mixtures and activated carbon (Pandey, Singh and Iyengar, 2007). All the approaches show significant differences in capital costs, dye removal results, operating speeds, and volume capability. Some of these methods have been shown to be effective, although they have limitations like superfluous amount of chemical usage and sludge generation with obvious discarding difficulties; expensive plant requirements or operating costs; lack of effective dye reduction (Zhang *et al.*, 2004). Major merits and demerits of physical, chemical and photochemical approaches available for the treatment of dye wastewater (Robinson *et al.*, 2001; Kharub, 2012) are mentioned in Table 2.1.
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Table 2.1 Merits and demerits of various physicochemical methods used for the treatment of textile dyes

Physicochemical dye removal approaches	Merits	Demerits
	Physical methods	
Adsorption on activated Carbon	• The most effective adsorbent with good removal efficiency. • Suspended solids and organic materials well reduced.	• The regeneration procedure is expensive. • Too much solid waste production.
Coagulation/ Flocculation	• Simple • Good removal efficiencies. • Removes insoluble dyes.	• Cost of coagulants and chemicals for pH adjustment. • High sludge generation • Water removal and sludge handling problems.
Cucurbituril as adsorbent	• Good sorption capacity for various dyes.	• High cost.
Ion-exchange	• Regeneration with little loss of adsorbent resins.	• Specific application. • Not effective for all dye types.
Membrane filtration	• Removes of all dye types. • Recovery and reuse of chemicals and water is possible. • Wider application for complex wastes.	• Concentrated sludge generation. • Dissolved solids are not separated in this process. • Running cost is very high.
Nanoparticles (Ag, ZnO, TiO$_2$, coated ceramic filters) as adsorbent	• Offers an effective and economic way to environmental bioremediation protection.	• Leaching problem.
Nano- adsorbents	• Higher adsorption rates. • Small footprint.	• High production cost.
Peat as adsorbent	• Good adsorbent because of cellular structure.	• Specific surface area for adsorption are lower than activated carbon
Polymeric Nano adsorbents (dendrimers)	• Bi-functional (inner shell adsorbs organics and outer branches adsorb heavy metals). • Reusable.	• Complex multistage production process.
Silica gel as adsorbent	• Effective for basic colorant removal.	• Side reactions prevent commercial application

Physicochemical dye removal approaches	Merits	Demerits
Wood chips as adsorbent	• Good sorption capacity for acidic dyes.	• Longer retention times.
Zeolites as Adsorbent	• Controlled release of nanosilver. • Bactericidal.	• Reduced active surface through immobilization of nanosilver particles.
Chemical methods		
Advanced oxidation Processes	• Complete mineralization confirmed. • Increasing number of commercial applications. • Effective pretreatment methodology in integrated systems and enhances biodegradability.	• Economically unfeasible. • Generation of by-products. • Technical limitations.
Electrochemical Oxidation	• No supplementary chemicals required and end products are non-hazardous. • Capacity of adaptation to different volumes and pollution loads.	• Expensive process. • Iron hydroxide sludges.
Fenton's reagent	• Capable of decolorizing wide variety of wastes. • Effective for both soluble and insoluble colorants.	• Problem with sludge disposal that makes the process relatively expensive.
Sodium hypochlorite (NaOCl)	• Initiates and accelerates azo-bond cleavage. • Low temperature requirement.	• Process with high cost. • Release of aromatic amines.
Ozonation	• Effective for degradation of azo dyes. • No change in volume.	• Not suitable for dispersed colorants. • Releases aromatic amines. • Half-life is short (20 min.).
Photochemical methods		
Photocatalysis	• Process carried out at ambient conditions. • Inputs are atoxic and inexpensive. • Complete mineralization • Shorter retention times.	• Not effective for large amount of dyes. • Process is expensive.
Photochemical oxidation with H_2O_2	• No sludge production	• Generation of byproducts.

Biological Dye Removal Approaches

The use of biological techniques to remove dye from textile effluents is an inexpensive alternative. Biological methods present no major processing expenses. However, this method presents some drawbacks also, since a number of dyes are designed to resist microbial attack. Biological dye removal approaches involve the use of bacteria, fungi, and algae. The ability of bacteria to metabolize azo dyes has been investigated by a number of research groups. Bacterial biodegradation has been mostly applied in the removal of azo dyes. However, this biological method has been found useless in removing color from several dyes. The azo dyes usually resist aerobic degradation. Its degradation was observed in anaerobic conditions too, but aromatic amines are formed as a final product (Blümel, Knackmuss and Stolz, 2002) which can be colorless but toxic, mutagenic or carcinogenic (Işik and Sponza, 2007). Therefore, to achieve complete removal of azo dyes, another stage that involves aerobic biodegradation of the produced aromatic amines is needed (Shaw, Carliell and Wheatley, 2002; Libra *et al.*, 2004; Işik and Sponza, 2007). Fungi can remove textile dyes through its own action or by enzymes produced by them. Ligninolytic fungi are the most common in the dye degrading processes for textile dyes, even though some non-ligninolytic are also effective (Ambrósio and Campos-Takaki, 2004). Some algae are identified to be used for efficient dye degradation from textile wastewaters (Omar, 2008; El-Sheekh, Gharieb and Abou-El-Souod, 2009). The azo bond reduction is the mechanism of degradation followed by algae. Aromatic amines thus generated can be then completely mineralized (Wang *et al.*, 2007). Table 2.2 summarizes the merits and the demerits of the various biological dye removal treatment approaches for textile industrial effluents.

Table 2.2 Merits and demerits of various biological decolorization methods for industrial effluents.

Biological dye removal approaches	Merits	Demerits
Aerobic biodegradation	• Dye removal is facilitated along with COD removal.	• Require long retention time. • Comparatively less resistant to recalcitrant compounds.
Anaerobic biodegradation	• Resistant to a wide variety of complex dyes.	• Slower acclimatization phase.

Biological dye removal approaches	Merits	Demerits
	• Biogas produced is used for electricity and steam generation.	
Bacterial, fungal and algal biodegradation	• Good removal efficiency for low volumes and concentrations. • Very effective for specific dye degradation.	• Culture maintenance is expensive. • Cannot cope up with large volumes of colored textile effluents.

Biological Approach of Dye Removal by Using Pure Bacterial Cultures

Several bacteria capable of various dye decolorization have been described, but not many dye degradation bacteria have been identified. Efforts to isolate pure bacterial cultures, which degrade azo dyes, started in the 1970s with reports of a *Bacillus subtilis* (Horitsu *et al.*, 1977)*, Aeromonas hydrophila* (Idaka *et al.*, 1978) *and Bacillus cereus* (Wuhrmann, Mechsner and Kappeler, 1980). Most of the research work concerning anaerobic decolorization of dyes (mostly azo dyes) has been conducted using bacterial monocultures. Table 2.3 shows a list of various dyes and bacterial species having dye decolorizing potential.

Species of *Bacillus* and *Pseudomonas* were found to be active in anaerobic degradation of a number of textile dyes. Other microorganisms such as those belonging to the genus *Aeromonas* have also been found to effectively decolorize a range of dyes (Carliell *et al.*, 1995).

Hu (1994) isolated *Pseudomonas luteola* and used it for achieving 37-93% dye degradation of four reactive azo dyes namely Red G, RBB, RP_2B and V_2RP after 42 hours of incubation under static conditions. Dyes Amaranth, Orange II and Tartrazine were decolorized in 8 hours up to 80, 50, and 20%, respectively, by *Bacteroides fragilis* under anaerobic conditions.

Mali *et al.* (2000) isolated an efficient species of *Pseudomonas* from the soil which decolorized the dyes belonging to triphenylmethane and the azo group. Malachite green, fast green, brilliant green, congo red and methylene blue were decolorized in the

range of 30-70% under the aerobic condition (temperature range 30°C to 40°C and pH 6 to 8).

Valli Nachiyar and Suseela Rajkumar (2003) revealed that *Pseudomonas aeruginosa* degrades a commercial textile and tannery dye viz. Navitan Fast Blue S5R under aerobic conditions in the presence of glucose.

Aeromonas hydrophila was isolated and identified by Ren *et al.* (2006) from activated sludge sample and it showed the greatest color removal of triphenylmethane (more than 90% removal) within 10 hours under aerobic condition. It also exhibited good removal of azo and anthraquinone dyes (more than 85% decolorization) within 36 hours under anoxic condition. According to their research work, the most suitable pH and temperature for decolorization of Crystal Violet, Basic Fuchsin, Brilliant Green, and Malachite Green, Acid Amaranth, Great Red GR, Reactive Red KE-3B, and Reactive Brilliant Blue K-GR were pH 5.0–10.0, and 25–37°C, respectively.

Kalyani *et al.* (2008) screened a novel bacterial strain *Pseudomonas sp.* SUK1 from polluted sites of textile industry which can degrade reactive dye Red BLI (50 mg/L) to 99.28% within just 1 hour under static condition at pH range from 6.5 to 7.0 and 30°C.

Khalid, Arshad and Crowley (2008) isolated 288 strains of azo-dye degrading bacteria using a mixture of four structurally different dyes (Acid Red 88, Reactive Black 5, Direct Red 81, and Disperse Orange 3) as sole source of Carbon and Nitrogen and found that among all isolates *Shewanella putrefaciens* was the most efficient strain. The bacterial strain required 4 hours for complete degradation of Acid Red 88 and Direct Red 81 dyes (100 mg/L) under static conditions while complete decolorization of Reactive Black 5 and Dispersed Orange 3 was observed within 6 and 8 hours, respectively.

Handayani, Meitiniarti and Timotius (2007) examined the dye degradation efficiency of *Enterococcus faecalis* on azo dyes: Acid Red 27 and Reactive Red 2 as well as growth characteristics of *E. faecalis* on these dyes. High dye degrading efficiency (95–100%) was attained within 3 hours of incubation for Acid Red 27, and 12 hours for Reactive Red 2, at room temperature, neutral pH, static condition.

Kothari, Kothari and Pathak (2005) described the effect of static and shaking conditions on dye degradation of various textile dyes. Their results suggested that more decolorization is attained under static culture condition compared to shake flask condition.

According to the research conducted by a group of scientists He, Hu and Li (2004), in the case of bacterial monocultures, one loses the benefit of microbial metabolic diversity and synergistic interactions in dye degradation process. This is the main disadvantage of using pure bacterial culture as biological decolorant.

The main advantage to work with bacteria is that they can grow more rapidly as compared to other microorganisms and they are easy to culture. The dye decolorizing ability of bacteria can easily be enhanced by the molecular genetic manipulation.

Table 2.3 List of various textile dyes and potential dye degrading bacterial sp.

Decolorizing Bacteria	Textile Dyes	Reference
Aeromonas hydrophila	Reactive Red 198, Reactive Black 5, Reactive Red 141, Reactive Blue 171, Reactive Yellow 84	Hsueh, Chen and Yen, 2009
Aeromonas hydrophila	Reactive Red 141	Chen *et al.*, 2009
Bacillus endophyticus VITABR13	Acid Red 128	Prasad and Rao, 2011
Bacillus fusiformis KMK5	Disperse Blue 79, Acid Orange 10	Kolekar *et al.*, 2008
Bacillus subtilis	Crystal Violet	Kochher and Kumar, 2011
Bacillus sp.	Provisional Pink	Celia and Suruthi, 2016
Bacillus sp.	Methyl red, Navy blue	Ezhilarasu, 2016
Bacillus sp.	Drimarene Red	Oak *et al.*, 2016
Bacillus pumillus	Congo Red	Modi, Pathak and Fulekar, 2015

Decolorizing Bacteria	Textile Dyes	Reference
Brevibacillus laterosporus MTCC 2298	Golden Yellow HER	Gomare and Govindwar, 2009
Burkholderia sp.	Alizarin	Sharma and Sharma, 2015
Clostridium perfringens	Crystal Violet	Ali and Akthar, 2014
Enterobacter sp. Strain VP-64	Crystal Violet	Hemapriya *et al.*, 2014
Enterococcus sp.	Reactive Blue 19	Gulati and Jha, 2014
Klebsiella ozaenae	Congo Red, Direct Blue 80	Shinkafi, Mohammed and Audu, 2015
Klebsiella sp.	Reactive Blue 19	Gulati and Jha, 2014
Klebsiella sp.	Light Red Dye	Sethi *et al.*, 2012
Ochrobactrum sp.	Remazol Blue	Kiliç and Dönmez, 2012
Pseudomonas aeruginosa	Crystal Violet	Ali and Akthar, 2014
Pseudomonas aeruginosa	C.I. Acid Blue 113, C.I. Basic Red 46, C.I. Direct Blue 151, C.I. Direct Brown 2, Mixture of four dyes	Falavarjani, Khorasani and Ghoreishi, 2012
Pseudomonas aeruginosa	Remazol Blue	Kiliç and Dönmez, 2012
Pseudomonas putida	Direct Red 28, Direct Blue 80	Shinkafi, Mohammed and Audu, 2015
Pseudomonas putida	C.I. Acid Blue 113, C.I. Basic Red 46, C.I. Direct Blue 151, C.I. Direct Brown 2, Mixture of four dyes	Falavarjani, Khorasani and Ghoreishi, 2012
Shewanella sp. strain IFN4	Acid Red 88, Direct Red 81, Reactive Black 5	Imran *et al.*, 2014
Proteus sp.	Light Red Dye	Sethi *et al.*, 2012
Proteus vulgaris	Crystal Violet	Ali and Akthar, 2014

Objectives of the Present Study

General objectives

To explore the possibility of using a microbial system, developed though using bacteria isolated from different soils and wastewater of the textile dye industry.

Specific objectives

    i. Isolation of dye degrading bacteria from textile effluent and dye contaminated soil of handloom fabrics dyeing sites and dye wastewater disposal sites of different locations of Tangail, Narayanganj, and Savar.

   ii. Identification of bacterial isolates using modern identification techniques.

  iii. Degradation of commonly used dyes used in textile industries under different concentrations and conditions.

  iv. Optimization of growth conditions of the isolated bacterial strains under different media and growth conditions to maximize the dye degrading capability.

Materials and Methods

Place of Research

The present study was carried out in the Industrial Microbiology Laboratory, Institute of Food Science and Technology (IFST), Bangladesh Council of Scientific and Industrial Research (BCSIR). The materials and procedures employed in the present study are described below.

Media, Solutions, and Reagents

Most of the required solutions, reagents and media available in the laboratory were of reagent grade and were used without further purification. List of the chemicals is given in the Appendix-A.

Handling of Laboratory Apparatus, Glassware and Analysis Equipment

All glassware were washed with detergents, rinsed 4-5 times with tap water and dried in an oven before use. Glassware like Petri plates were heat sterilized at 180°C for 1 hour in hot air oven (Binder ED23, Germany) before use. McCartney's bottles, Durham bottles, conical flasks, micropipette tips, glass spreader, glass test tubes, falcon tubes and microfuge tubes were sterilized by autoclaving at 121°C for 15 minutes at 15 psi (Sturdy, SA-300VF, Taiwan). All the microbiological works were done inside Biological Safety Cabinet (LabTech, LCB-1803B-A2, Korea). For the measurement of absorption in UV-Visible region, a high-performance compact split beam spectrophotometer (PG Instruments, T60, UK) was used. List of the instruments used is given in the Appendix-B.

Sample Collection

Collection of Dye Waste Contaminated Soil Sample
The source of microorganism isolated in this study was soil samples of topsoil (0-10 cm depth) collected from dye contaminated places which is a dying site for handloom fabrics and dye wastewater disposal areas. These soils were stored in sterile reclosable plastic bags at 4°C for later use.

Collection of Textile Effluent Sample

The textile effluent was collected from YKK Bangladesh Pte. Ltd. (DEPZ, Savar, Dhaka) in a clean plastic bottle and designated as S9. A fresh plastic bottle was soaked earlier in 10% HNO_3 overnight and washed with deionized water (Fetyan *et al.*, 2017). The sample was transported to the laboratory and was processed within 3 hours of collection. It was then transferred into a Durham bottle and stored at 4°C for future analysis.

Table 3.1 List of soil and textile effluent samples that were collected for this study.

Soil Sample	Location
S1	Handloom fabrics dyeing site, Kumulli Namdar, Tangail.
S2	Handloom fabrics dyeing site, Kumulli Namdar, Tangail.
S3	Dye wastewater disposal site, Near Talukdar Market, Tangail.
S4	Handloom fabrics dyeing site, Alokdia, Tangail.
S5	Dye wastewater disposal site, Alokdia, Tangail.
S6	Dye wastewater disposal site, Rupganj, Narayanganj.
S7	Handloom fabrics dyeing site, Rupganj, Narayanganj.
S8	Dye wastewater disposal site, Rupganj, Narayanganj.
S9	Main unit of ETP, YKK Bangladesh Pte. Ltd. DEPZ, Savar.

Textile Dyes

Eight dye samples obtained from Swiss Colors Ltd. Bangladesh, namely Novacron Brilliant Blue C-B, Novacron Dark Blue S-GL, Novacron Navy S-GI, Novacron Turquoise GN, Novacron Super Black-G, Novacron Ruby S-3B, Avitera Brilliant Yellow SE and Novacron Yellow S-3R. Dyes were filter sterilized (RanDisc^{TM} Nylon Syringe Filter, India) inside a biosafety cabinet (LabTech, LCB-1803B-A2, Korea) and stored separately in autoclaved McCartney's bottles and sealed with plastic paraffin film. For sterility confirmation, 100 µL of working dye solution was spread on Nutrient agar (NA) plates and incubated for 48 hours at 37ºC before the start of every experiment. The absence of growth on agar plates ensured the dye to be uncontaminated. No seals of McCartney's bottles were broken outside of the biosafety cabinet.

Isolation of Bacteria from Locally Collected Samples

Isolation and Screening of Bacteria from Soil Samples

One gram from each of the soil samples was individually suspended and serially diluted in 9 ml Ringer's solution. 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} dilutions were plated on Nutrient agar plates through spread plate technique. These are then incubated at 37°C for 24 hours. The morphological characteristics of bacterial colonies grown on each plate were noted after 24 hours. Most of the colonies from the spread plate technique displayed recurring morphological characteristics. Thus the bacterial colonies were selected such a way that no two colonies showed the same characteristics. The selected colonies were numbered and streaked again on Nutrient agar plates to obtain pure isolates. Pure isolates were designated as SnAn (where, S = Sample ID, A = pure isolates, n = 1, 2, 3…).

Isolation and Screening of Bacteria from Textile Effluent Sample

The textile effluent sample was used for isolation and screening of dye decolorizing bacteria by enrichment culture techniques using Nutrient broth containing a mixture of eight experimental dyes with the final dye concentration of 100 ppm. Ten milliliters of textile effluent were inoculated into 250 ml Erlenmeyer flask containing 90 ml of Nutrient broth supplemented with a mixture of dyes and incubated at 37°C. After 72 hours of incubation, 1 ml of the incubated culture broth was serially diluted in 9 ml Nutrient broth and plated on Nutrient agar plates. Morphologically distinct bacterial isolates were selected for the screening of dye decolorization. Pure isolates were labeled as SnAn (where, S = Sample ID, A = pure isolates, n = 1, 2, 3…).

Preparation of Inoculum

The suspension of one-day-old cultures of bacteria was used to investigate their abilities to decolorize dyes. A loopful of bacterial culture was taken from the agar plate and transferred to the Nutrient broth. The broth was then incubated at 37 °C for 24 hours.

Decolorization Studies of Dyes

Screening of Bacteria with Dye Decolorizing Ability in Solid Media

In this study, we attempted to screen potential dye degrading bacterial strains on solid agar media. Each bacterium was streaked on Nutrient agar supplemented with mixed dye at the concentration of 100 and 150 ppm and incubated at 37°C for up to 5 days.

Abiotic Petri plates amended with the same amount of dye were also incubated as a control. On 5th day plates were observed for any change or disappearance of color. The isolates did not show any noticeable decolorizing ability on solid medium.

Screening of Bacteria with Dye Decolorizing Ability in Liquid Media

Ten milliliters of Nutrient broth along with a mixture of dyes (100 ppm) was inoculated with 20% of individual bacterial isolates. The McCartney's bottles were incubated at a temperature (37°C) under the static condition. After 24 hours of incubation, decolorization of mixed dye broth was observed visually. Bacterial isolate showing the clear broth were screened for their ability to decolorize individual dye in the culture broth. Of the thirty-four morphologically distinct bacterial strains isolated from the dye contaminated soil and wastewater, only seven isolates were found to possess more than 50% decolorization of mixed dye within 24 hours of incubation. Secondary screening of these bacteria for multiple synthetic dye decolorization was performed using eight individual dyes at a concentration of 100 ppm. Table 3.2 shows dye degrading capabilities of the purely isolated bacterial cultures.

Table 3.2 Seven isolates were selected based on their decolorization ability

Sample	Designation of isolates	Novacron Brilliant Blue C-B	Novacron Dark Blue S-GL	Novacron Navy S-GI	Novacron Turquoise GN	Novacron Super Black-G	Novacron Ruby S-3B	Avitera Brilliant Yellow SE	Novacron Yellow S-3R
S1	S1A2	N	Y	Y	N	Y	Y	N	Y
S1	S1A6	Y	Y	Y	N	Y	Y	N	N
S2	S2A7	N	Y	Y	N	N	N	N	Y
S4	S4A3	N	Y	Y	N	N	Y	N	Y
S6	S6A5	N	Y	Y	N	N	Y	N	N
S7	S7A4	Y	N	N	N	Y	Y	N	N
S9	S9A6	Y	Y	Y	N	Y	Y	N	Y

Y – Positive decolorization, N – No significant decolorization.

The isolates with dye degradation abilities were selected for studying effects of different physical parameters such as pH, temperature, concentration of dye, concentration of inoculum and presence of agitation. The strain S1A6 and S9A6 were chosen for further study because of their high efficiency in dye decolorization. The dye concentration was chosen according to the typical dye concentration found in textile industry wastewater. As these isolates have the ability to utilize a higher concentration of textile dyes, thus could better fit for the treatment of textile effluent. The actual concentration of dye in wastewater is difficult to predict, which generally depends upon the application and nature of the dye. According to Chokalingam (2010), Textile wastewater may contain 10-200 ppm concentration of dyes.

Culture Maintenance and Preservation of Selected Bacteria

Culture maintenance was carried out by using sub-culturing. This was performed by selecting an isolated colony using a loop and streaking onto a Nutrient agar plate by quadrant streak method to get more single colonies. The plates were incubated overnight at 37° C. On the next day, the plates were sealed with plastic paraffin film and stored at 4°C for subsequent use (Thiel, 1999). This short-term preservation method was followed for daily or weekly use of the bacteria. We stored the bacterial strains for long-term preservation as well.

Decolorization Assay via UV-Visible Spectrophotometry

For the measurement of absorption in UV-Visible region, a high-performance compact split beam spectrophotometer (PG Instruments, T60, UK) was used. A diagram of the components of a typical spectrometer is shown in Figure 3.1. The Beer-Lambert law is applied to determine quantitative concentration of an unknown analyte in given solution under examination.

$$A = \log (i_0/i) = \varepsilon c l$$

Where, A = Absorbance

i_0 = Intensity of light incident upon solution

i = Intensity of light leaving solution

ε = Molar extinction coefficient

c = Concentration of the analyte in solution

l = Length of light path through solution
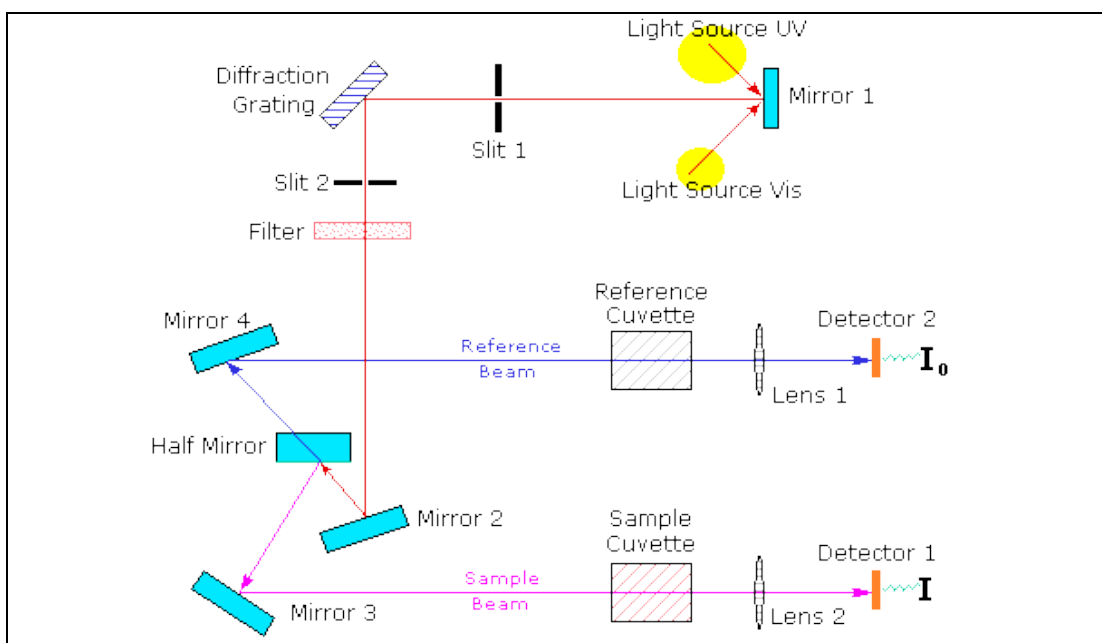
Figure 3.1 Schematic representation of working of a spectrophotometer

A portion of decolorized dye-broth was taken and centrifuged at 10,000 rpm for 15 minutes to separate the dye decolorizing bacterial cell mass. The supernatant was then run through a spectrophotometer to check absorbance at λ_{max} of selected dyes (400-800 nm). The uninoculated dye-free medium was used as a blank. Dye decolorization was recorded quantitatively by measuring at λ_{max} of that specific dye. Negative controls were also run as uninoculated cultures in two sets; one with dye and the other without dye. The following equation is used to express decolorization percentage (Chen *et al.*, 2003):

$$\%\text{Decolorization} = \frac{\text{Initial absorbance - Observed absorbance}}{\text{Initial absorbance}} \times 100$$

Incorporating Variations in Growth Conditions

Effect of Varying Concentration of Single Dyes

In order to examine the effect of dye concentration on decolorization, varying concentration (100 ppm, 200 ppm, 300 ppm, 400 ppm and 500 ppm) of eight textile dyes were added separately to the sterile Nutrient broth inoculated with 20% one-day old bacterial seed culture and incubated at 37°C for 72 hours under static condition.

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Abiotic dye controls (without microorganism) were always included. Uninoculated media served as blanks. A portion of decolorized dye-broth was taken after 72 hours and centrifuged at 10,000 rpm for 15 minutes to separate the dye decolorizing bacterial cell mass. The clear supernatant was used to measure the percent decolorization using UV-Visible spectrophotometer (PG Instruments, T60, UK).

Effect of Temperature On Degradation of Individual Dyes

To study the effect of temperature on decolorization of the eight textile dyes, 100 mg/L of each dye on Nutrient broth and 20% of one-day-old bacterial seed culture were incubated in screw-capped tubes under static condition for 72 hours. The dye-Nutrient broth solution along with bacterial seed culture was incubated at 25, 37 and 42ºC. Abiotic controls (without culture) were also set. The %decolorization was measured as mentioned earlier.

Effect of pH On Degradation of Individual Dyes

Twenty percent of one-day-old seed bacteria were cultured in separate 20 ml dye-Nutrient broth solution whose pH was adjusted to pH 6, pH 7 and pH 8 with HCl and K_2HPO_4. The screw-capped tubes were incubated at the static condition at 37°C for 72 hours. Abiotic controls (without microorganism) were always included. The %decolorization was measured as mentioned above.

Effect of Static Versus Agitating Condition On Dye Decolorization

In this study, two sets of 100 ppm dye concentrations of Novacron Brilliant Blue C-B, Novacron Dark Blue S-GL, Novacron Navy S-GI, Novacron Turquoise GN, Novacron Super Black-G, Novacron Ruby S-3B, Avitera Brilliant Yellow SE and Novacron Yellow S-3R were prepared separately with Nutrient broth and inoculated with 20% of one-day-old bacterial seed culture. Two sets of abiotic controls were also set. One biotic and one abiotic set were incubated at 37°C on shaking incubator (GFL, 3031, Germany) at 100 rpm. Other one biotic and one abiotic set were kept under a static condition at 37°C (DAIHAN Scientific Co. Ltd, WIR-150, Korea). The %decolorization was measured after 72 hours of incubation as mentioned earlier.

Effect of Varying Concentration of Seed Culture On Dye Decolorization

To determine the effect of seed culture concentration, 100 ppm Novacron Brilliant Blue C-B, Novacron Dark Blue S-GL, Novacron Navy S-GI, Novacron Turquoise GN, Novacron Super Black-G, Novacron Ruby S-3B, Avitera Brilliant Yellow SE and Novacron Yellow S-3R were prepared separately with Nutrient broth and inoculated with 10%, 15% and 20% of one-day-old bacterial seed culture. These are then incubated at the static condition at 37°C for 72 hours. The %decolorization of individual dyes was measured after 72 hours of incubation as mentioned earlier.

Effect of Repeated Addition of the Dye Aliquots

Repeated addition of 100 ppm of individual dyes was studied for the utilization of same bacterial isolates for more times maintaining other conditions constant. Highly efficient bacterial strain S9A6 was subjected to this purpose. For this purpose, four consecutive cycles of decolorization were evaluated. The first cycle was run in Nutrient broth containing 100 ppm of dyes (pH 7.0). At the end of each cycle (intervals of 72 hours) an additional aliquot of 100 ppm dye solution was added. The dye-broth were incubated at 37ºC under static condition. Dye free abiotic control broth and dye-containing control broth without bacteria were carried out in parallel. The absorbance of the dye solution was monitored at intervals of 72 hours.

Effect of Varying Concentration of Mixed Dyes On Decolorization

The textile industry uses a variety of dyes at a time for dyeing. Between 20 to 50 percent of the dye used by the textile industry is lost in the exhaust and wash water. The degradation ability of experimental isolate S9A6 towards eight experimental dyes was evaluated and dyes showing better decolorization (Novacron Brilliant Blue C-B, Novacron Dark Blue S-GL, Novacron Navy S-GI, Novacron Super Black-G, Novacron Ruby S-3B, and Novacron Yellow S-3R) were selected for the preparation of dye mixture. Twenty percent of bacterial seed cultures were inoculated in separate Nutrient broth containing a mixture of dyes at varying concentrations of 100, 200, 300, 400 and 500 ppm. One abiotic control containing dye was also set. These are then incubated at the static condition at 37°C for 72 hours. The %decolorization was measured as mentioned earlier.

Identification of Selected Strains with Dye Degradation Abilities

The bacteria screened for multiple dye degrading abilities were identified at species level using BiOLOG Semi-Automated Microbial Identification System. A Gram stain is not essential to select the proper protocol for testing bacteria when using the BiOLOG GEN III system but staining was done for microscopic visualization of bacteria. Bacterial culture tests for morphological characteristics of the colony were also performed.

Staining and Microscopic Observation of Bacteria

Gram staining is a differential staining method which is used to differentiate between Gram positive and Gram negative bacteria by their cell wall composition. Gram staining on the selected bacterial isolates was performed according to the methods mentioned in Microbiology Laboratory Manual (Cappuccino & Sherman, 2005). A small amount of a single bacterial colony was transferred onto a small drop of saline over a glass slide and made a smear using an inoculating loop. The bacterial smear was then dried up and heat fixed. Crystal violet (primary stain) was applied on the bacterial smear for 30 seconds and then washed off with distilled water for 10 seconds. Gram's iodine (mordant) was applied to flood the slide for 30 seconds. The smear was then decolorized using 95% alcohol and then Safranin (counter stain) was applied on the smear for 30 seconds and then washed off with distilled water. The stained slide was then air-dried. The size, shape, arrangement and Gram reaction properties of isolates were carefully observed under a compound microscope (OLYMPUS, EX41, Japan).

Identification Process of Bacteria Using BiOLOG Identification System

The BiOLOG MicroPlates were developed for the rapid identification of microbial isolates by sole-carbon-source utilization on a 96 well plate. Additionally, each well contains a colorless tetrazolium dye. Metabolism of the substrate in particular wells results in formazan production, producing purple color in tetrazolium dye.

The microbial identification process for GEN III MicroPlates involves four basic steps. The steps include: (a) isolation of a pure culture on BiOLOG media, (b) preparation of inoculum at specified cell density and determination of testing protocol, (c) inoculation

and incubation of GEN III MicroPlate and (d) MicroPlate reading and determination of ID.

a. Preparation of BiOLOG Universal Growth Medium and Isolation of a Pure Culture

BiOLOG Universal Growth Agar (BUG) is a general-purpose culture medium. This medium was prepared by simply dissolving BUG agar powder in distilled water (57 g/L). The mixture was boiled gently to dissolve the agar completely. The final pH of the BUG agar medium was adjusted to 7.3 with NaOH. The medium was then sterilized by autoclaving at 15 pounds of pressure, at 121° C, for 15 minutes. Sterile BUG agar allowed to cool to 45-50°C and dispensed into sterile Petri plates in a laminar flow hood (LabTech, LCB-1803B-A2, Korea). The bacterial isolates with highest degradation capabilities (S1A6 and S9A6) were streaked by quadrant streaking method on BUG agar plates. The plates were incubated aerobically for 24 hours at 33°C.

b. Determination of Testing Protocol and Preparation of Liquid Inoculum at Specified Cell Density

After culturing and isolating the desired bacterium, a liquid inoculum was prepared using a "gelling" inoculating fluid (IF) as per manufacturers instruction. Selection of the correct inoculating fluid to prepare liquid inoculum for the GEN III protocol is important to identify the microorganism. There are four GEN III protocols: Protocol-A is the default protocol. Protocol-B is followed to identify strongly reducing and capsule producing Gram-negative (e.g. some *Aeromonas*, *Vibrio*) and Gram-positive (e.g., some *Bacillus*, *Aneurinibacillus*, *Brevibacillus* etc) microorganisms. Protocol-C1 is for microaerophilic, capnophilic Gram-positive (e.g., *Dolosicoccus*, *Dolosigranulum* etc) microbial identification. Protocol-A, B, and C require a cell density of 90-98% (ideally 97%). IF-A and IF-B are used to prepare liquid inoculum for Protocol-A and Protocol-B, respectively. Fastidious, capnophilic, oxygen sensitive Gram-negative (e.g., *Actinobacillus*, *Aggregatibacter* etc) and Gram-positive (e.g., *Aerococcus*, *Corynebacterium* etc) microorganisms require Protocol-C2 for their identification. Protocol-C2 requires a higher cell density of 62-68%. Inoculating Fluid-C is used to make liquid inoculum for Protocol-C1 and Protocol-C2. In this study, dye degrading bacterial isolate S1A6 and S9A6 required Protocol-B and Protocol-A, respectively, for their identification.

The tube containing inoculating fluid was incubated at 33°C for 20 minutes before preparing the liquid inoculum. The properly calibrated Turbidimeter (BiOLOG, 21907, USA) was blanked with a clean tube (wiped clean of dirt and fingerprints) containing uninoculated IF. The transmittance adjustment knob was set to 100% so that the meter reads 100. The tubes containing inoculating fluid are not optically precise and can vary tube-to-tube and with rotation. For this reason, they were blanked individually with appropriate caution. Approximately 3 mm diameter area of selected bacterial cell growth picked up from the surface of the BUG agar plate using a sterile disposable cotton-tipped BiOLOG Inoculatorz swab stick. The bacterial colony was then released into the inoculating fluid by rubbing the swab tip against the bottom of the tube and a uniform bacterial cell suspension was made. The %Transmittance was measured using Turbidimeter and made sure that the isolated dye degrading bacterial cell density is 97%. This liquid inoculum was prepared in a laminar air flow.

c. Inoculation and Incubation of GEN III MicroPlate

The ninety-six wells of the GEN III MicroPlates contain all necessary nutrients and biochemicals required for the bacteria. The GEN III MicroPlates were incubated at 33°C for 20 minutes before inoculating the liquid bacterial cell suspension. The MicroPlate was placed in the laminar air flow cabinet and labeled with the identification number on the BiOLOG imprinted side. Maintaining all aseptic precaution, the cell suspension was then poured into a multichannel pipette reservoir. Eight sterile pipette tips were attached securely to the 8-Channel repeating pipette (VistaLab Technologies, Ovation, USA) and the tips were filled by drawing up the bacterial cell suspension from the reservoir. Hundred microliters of a bacterial cell suspension were dispensed into each well of the GEN III MicroPlate. The MicroPlate was then covered with its lid and incubated aerobically at 33°C.

d. MicroPlate Reading and Determination of ID

After incubation, the color densities in wells of the GEN III MicroPlates containing bacterial isolates were read with the BiOLOG Microbial Identification System, MicroStation Reader (BiOLOG Inc., ELx808BLG, USA) / MicroLog 3 (Version 5.2.2) and compared to its database. The first step to reading a MicroPlate is initializing the MicroStation machine. The MicroStation reader machine was switched on. The MicroStation/MicroLog software was opened by double clicking the shortcut icon on

the desktop. After entering valid Username and Password the main tab appeared. The Reader tab was selected from the Setup tab. The Com Port was determined by opening the Control Panel. Double-clicked on the System icon and the System Properties dialog box appeared. The Hardware tab was selected and chose the Device Manager button. A new Device Manager dialog box displayed. Double-clicked on Ports to reveal the USB Serial Port and Com Port number (for USB reader connection). For serial port reader connection, it will state Communications Port and Com Port number. In this example, the Com Port number is 5. The Com Port number was selected from the Com Port drop-down menu after returning to the Reader tab. The correct MicroStation was selected by clicking on the appropriate picture. The Initialize Reader button was clicked so that the messages turned green and displayed Com Port: Open and Ready. The Show Detailed Reader Status button was clicked to display the Reader status information on the right-side of the screen.
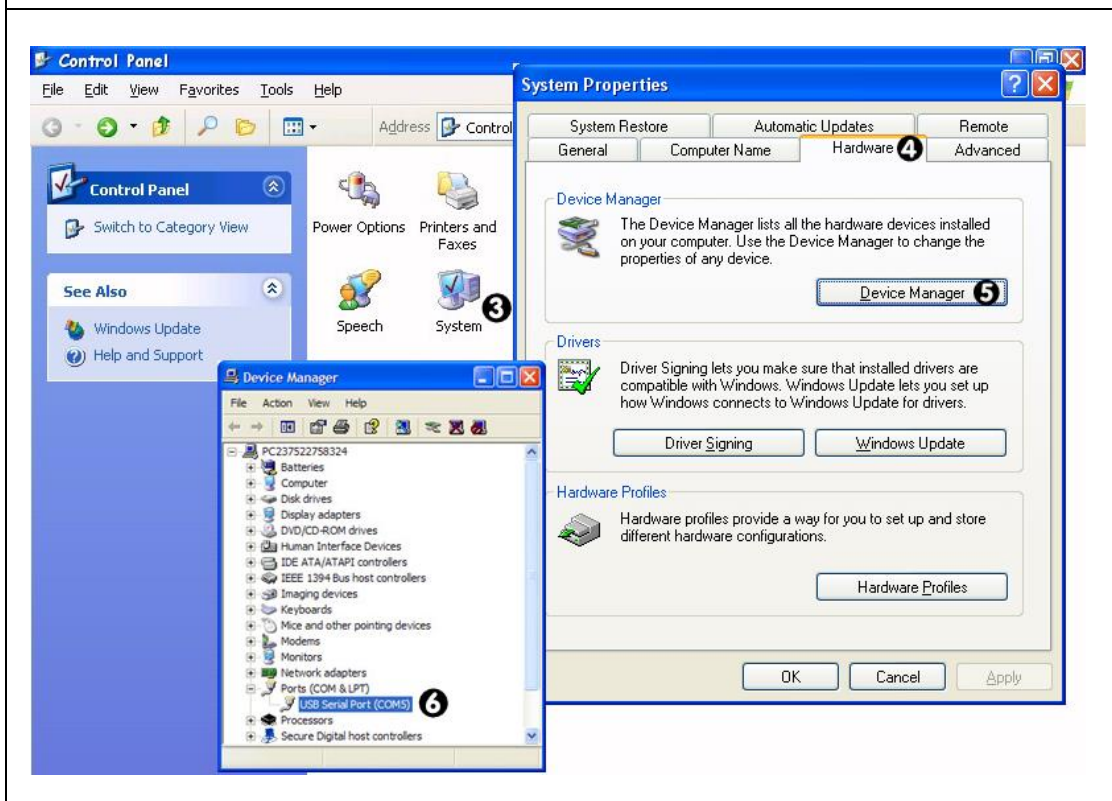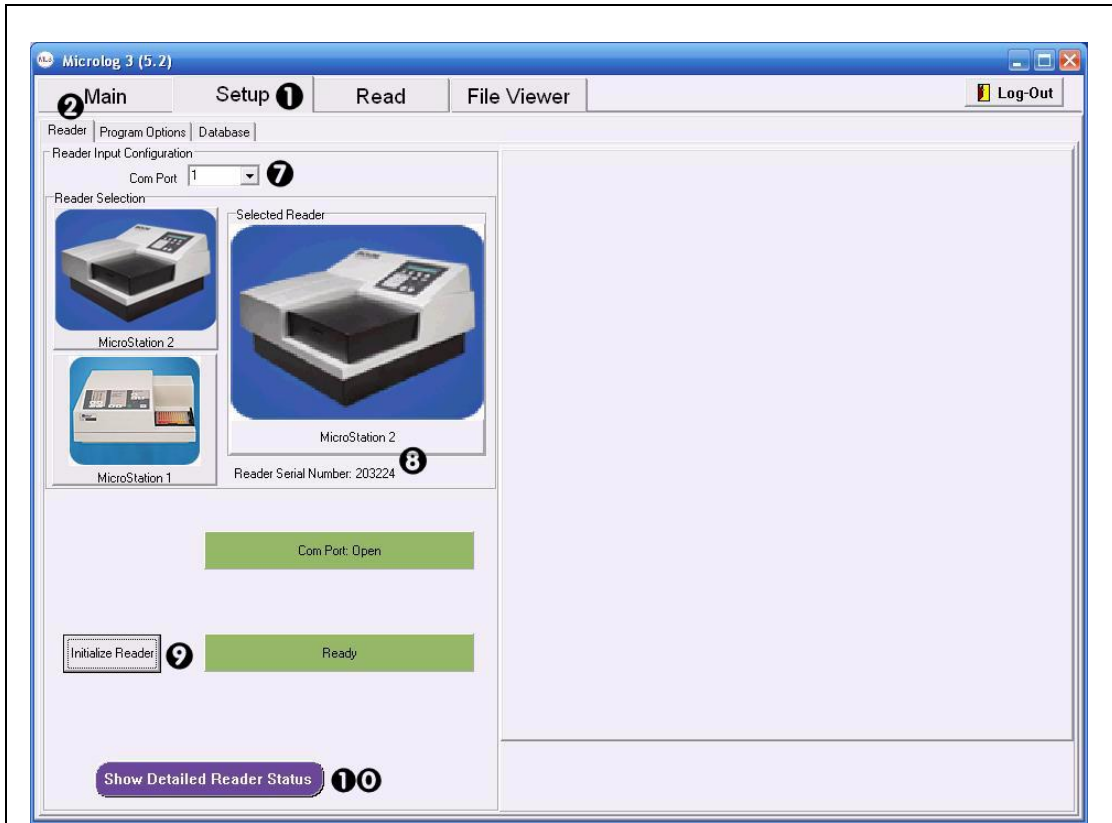
Figure 3.2 Steps of initializing the MicroStation Reader: Steps 1-10

(The lower figure shows the steps (3-6) of Com Port Determination)

The identification process can be run in two modes; either manually or using the MicroStation Reader. Reader mode is used in conjunction with the MicroStation Reader, where MicroPlate reactions are read and entered automatically. Similar to Manual mode, we can read a MicroPlate with or without a batch worksheet. Batch worksheets offer an efficient way to read multiple MicroPlates. Manual mode is used to enter MicroPlate reactions by hand. Manual mode is normally used when no MicroStation Reader is available or if the Reader has a mechanical problem.

Reading plates using the MicroStation without a batch worksheet

The Read Setup tab was selected from the Read tab. After that, the Reader option was selected from the Input Mode drop-down box. We wanted to read the plate without a batch worksheet so the option 'No' was selected from the Reader option. 'Yes' option was chosen from the Save To Data File drop-down menu and the Data File Name field appeared. The Folder icon was clicked to populate the Data File Name field. The desired file name (e.g., UsernameDate) in the File Name text box was entered (D5E extension was automatically appended to the file name; i.e., UsernameDate.D5E) and clicked on Save button. It was observed that the file name displayed in the Data File Name label and the Data File Name label turned black. After clicking the Read New Plate button and the Plate Information screen appeared. Sample identifiers were entered for the following fields i.e., Project, Plate Number, Plate Type, Protocol, Incubation Hours, Strain Type, User Value accordingly. The MicroPlate was then loaded on the reader tray of the BiOLOG MicroStation and the Read button was clicked. The reader calibrates itself, takes a reading, and displays the read results on the Read Information tab.

Results

The focus of this study was to isolate bacteria from locally collected samples which can degrade dyes efficiently and to determine the effects of its various physical parameters on the decolorization of multiple synthetic textile dyes. Seven isolates with degradation capabilities were isolated from thirty-four morphologically distinct bacteria for studying effects of different physical parameters such as pH, temperature, the concentration of dye, the concentration of inoculum and presence of agitation. In general, the experimental results correspond well with the findings reported in the literature.

Decolorization Studies of Dyes

In this study, thirty-four morphologically distinct bacterial isolates from eight soil and one water samples were separately cultured for 24 hours in Nutrient broth containing mixed textile dyes. The ability to decolorize mixed textile dyes was observed visually. Of them, only seven bacterial isolates have the ability to decolorize dyes. The effect of varying concentration of dyes (100-500 ppm), temperatures (25, 37 and 42ºC), pH (6, 7 and 8), agitation, seed culture concentration (10, 15 and 20% v/v) on decolorization process by selected seven bacterial isolates were studied.

Effect of Varying Concentration of Dyes

The effect of dye concentration on growth of a bacteria is an important consideration for its field application because the higher concentration of dye inhibits nucleic acid biosynthesis and cell growth (Chen *et al.*, 2003). In order to study the effect of varying concentration of dyes, the experiments were carried out at a fixed bacterial seed concentration (20%) at different dye concentrations (100, 200, 300, 400 and 500 ppm) at 37°C under static culture condition for 72 hours. Chart 4.1 exhibit the effects of varying concentration of dyes on decolorization. All the selected isolates showed maximum decolorization at a concentration of 100 ppm. The decolorization percentage was decreased with an increase in dye concentration. Decolorization of Novacron Super Black-G by S9A6 was observed 98.5% and 60.7% at 100 ppm and 500 ppm dye concentration, respectively. Khehra *et al.*, (2005) suggested that the decrease in decolorization efficiency might be due to the toxic effect of dyes. The %decolorization

by all the experimental bacteria was strongly inhibited at 500 ppm dye in the Nutrient broth except for the S9A6. No noticeable decolorization was observed for Novacron Turquoise GN and Avitera Brilliant Yellow SE by any of the microorganisms (data not shown).

	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
S1A6	73.6	65.2	51.9	42.3	0.0
S7A4	67.2	49.8	37.0	0.0	0.0
S9A6	94.2	88.5	72.9	65.3	49.7

Novacron Brilliant Blue C-B

	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
S1A2	80.8	66.3	50.8	35.6	0.0
S1A6	77.4	71.0	56.6	38.7	0.0
S2A7	88.1	70.2	61.8	42.2	0.0
S4A3	72.2	61.8	58.4	0.0	0.0
S6A5	86.2	63.8	51.4	0.0	0.0
S9A6	98.3	97.6	87.8	76.4	62.4

Novacron Dark Blue S-GL

	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
S1A2	71.3	58.4	46.3	0.0	0.0
S1A6	86.2	70.9	62.6	54.9	0.0
S2A7	76.0	62.4	53.7	0.0	0.0
S4A3	59.8	52.4	0.0	0.0	0.0
S6A5	51.1	0.0	0.0	0.0	0.0
S9A6	97.4	97.0	88.4	78.7	63.2

Novacron Navy S-GI

Novacron Super Black-G	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
S1A2	66.7	47.5	33.7	0.0	0.0
S1A6	70.2	61.3	48.7	35.8	0.0
S7A4	69.6	52.2	34.6	0.0	0.0
S9A6	98.5	97.4	87.2	73.7	60.7

Novacron Super Black-G

Novacron Ruby S-3B	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
S1A2	89.3	60.3	43.2	0.0	0.0
S1A6	72.6	57.1	38.1	29.4	0.0
S4A3	47.4	0.0	0.0	0.0	0.0
S6A5	65.2	52.0	0.0	0.0	0.0
S7A4	59.4	48.4	0.0	0.0	0.0
S9A6	96.7	90.4	84.3	78.4	52.7

Novacron Ruby S-3B

Novacron Yellow S-3R	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm
S1A2	77.8	51.2	30.4	0.0	0.0
S2A7	69.4	59.8	42.0	30.1	0.0
S4A3	78.2	52.0	0.0	0.0	0.0
S9A6	98.4	96.2	91.0	80.1	74.6

Novacron Yellow S-3R

Chart 4.1 Effect of varying concentration of dyes on decolorization

Effect of Temperature On Degradation of Individual Dyes

In order to study the effect of temperature, decolorization assays were performed over 25-42 ℃ temperature range at pH 7. Chart 4.2 shows that the decolorization percentage increases with increase in temperature from 25°C to 37°C. At 42°C, the decolorization ability was reduced for all the experimental microorganisms. This might have occurred due to the adverse effect of temperature on cell viability and the enzymatic activities (Çetin and Dönmez, 2006). Maximum dye decolorization (98.5%) of Novacron Super Black-G by S9A6 was observed at 37°C for 100 ppm dye removal at static condition. 50.8% dye decolorization was observed for the same bacteria at 42°C. Dye decolorization was 85.5% for Novacron Super Black-G by S9A6 at 25°C. This could be due to a lower temperature. At a lower temperature, microbial activity is reduced and affects decolorization.

	25°C	37°C	42°C
S1A6	56.4	73.6	36.3
S7A4	51.8	67.2	55.2
S9A6	89.2	94.2	62.0

Novacron Brilliant Blue C-B

	25°C	37°C	42°C
S1A2	69.3	80.8	53.4
S1A6	49.7	77.4	37.1
S2A7	70.8	88.1	77.3
S4A3	42.8	72.2	48.3
S6A5	76.8	86.2	58.3
S9A6	91.6	98.3	65.3

Novacron Dark Blue S-GL

	25°C	37°C	42°C
■ S1A2	64.6	71.3	46.8
■ S1A6	62.7	86.2	42.9
■ S2A7	56.4	76.0	65.3
■ S4A3	34.4	59.8	33.3
■ S6A5	41.4	51.1	28.3
■ S9A6	89.7	97.4	59.3

Novacron Navy S-GI

	25°C	37°C	42°C
■ S1A2	60.3	66.7	44.5
■ S1A6	51.9	70.2	35.8
■ S7A4	58.4	69.6	57.5
■ S9A6	85.5	98.5	50.8

Novacron Super Black-G

	25°C	37°C	42°C
■ S1A2	67.1	89.3	60.2
■ S1A6	44.5	72.6	31.4
■ S4A3	33.1	47.4	0.0
■ S6A5	57.0	65.2	46.6
■ S7A4	43.2	59.4	42.4
■ S9A6	88.4	96.7	47.4

Novacron Ruby S-3B

	25°C	37°C	42°C
■ S1A2	51.3	77.8	49.7
■ S2A7	50.0	69.4	39.6
■ S4A3	51.0	78.2	50.6
■ S9A6	93.7	98.4	51.6

Novacron Yellow S-3R

Chart 4.2 Effect of temperature on degradation of individual dyes

Effect of pH On Degradation of Individual Dyes

Decolorization of eight experimental dyes at 100 ppm concentration was evaluated at the pH range from 6 to 8. The maximum dye decolorization 98.5% was observed for Novacron Super Black-G at pH 7 by S9A6. Similar result was previously reported by Kalyani *et al.*, (2008). At pH 8, 96.6% Novacron Super Black-G dye decolorization was observed by the same bacteria. The optimum pH for decolorization of other experimental dyes by isolated bacteria was observed at nearly neutral pH 7 and decolorization decreased at strongly acidic pH 6 and at high alkaline pH 8. The optimum pH for color removal is often the neutral pH value or a slightly alkaline pH value and the rate of color removal tends to decrease rapidly at strongly acid or strongly alkaline pH values (Evans and Furlong, 2003). Chart 4.3 depicted the effect of various pH on decolorization process.

	pH 6	pH 7	pH 8
■ S1A6	66.4	73.6	57.2
■ S7A4	46.5	67.2	48.3
■ S9A6	77.0	94.2	89.3

Novacron Brilliant Blue C-B

	pH 6	pH 7	pH 8
■ S1A2	68.7	80.8	71.3
■ S1A6	62.7	77.4	59.7
■ S2A7	67.6	88.1	68.3
■ S4A3	63.6	72.2	63.3
■ S6A5	72.6	86.2	68.3
■ S9A6	82.7	98.3	96.2

Novacron Dark Blue S-GL

	pH 6	pH 7	pH 8
■ S1A2	56.3	71.3	61.7
■ S1A6	70.9	86.2	63.4
■ S2A7	55.7	76.0	60.3
■ S4A3	45.7	59.8	62.3
■ S6A5	43.7	51.1	45.3
■ S9A6	83.2	97.4	91.6

Novacron Navy S-GI

	pH 6	pH 7	pH 8
■ S1A2	48.7	66.7	49.8
■ S1A6	54.5	70.2	47.1
■ S7A4	44.7	69.6	52.3
■ S9A6	79.7	98.5	96.6

Novacron Super Black-G

	pH 6	pH 7	pH 8
■ S1A2	72.6	89.3	66.4
■ S1A6	61.6	72.6	48.6
■ S4A3	36.8	47.4	50.2
■ S6A5	46.7	65.2	53.8
■ S7A4	32.2	59.4	43.8
■ S9A6	80.1	96.7	91.0

Novacron Ruby S-3B

	pH 6	pH 7	pH 8
■ S1A2	64.8	77.8	51.9
■ S2A7	53.7	69.4	56.8
■ S4A3	58.7	78.2	46.8
■ S9A6	76.2	98.4	95.0

Novacron Yellow S-3R

Chart 4.3 Effect of pH on degradation of individual dyes

Effect of Static Versus Agitating Condition On Dye Decolorization

In order to test the effect of agitation, selected bacterial isolates were kept in dye-broth under static and shaking conditions. Results obtained are shown in Chart 4.4. It is quite clear from the data that under static conditions better decolorization could be achieved with six experimental dyes and seven bacterial strains. No noticeable decolorization was observed for Novacron Turquois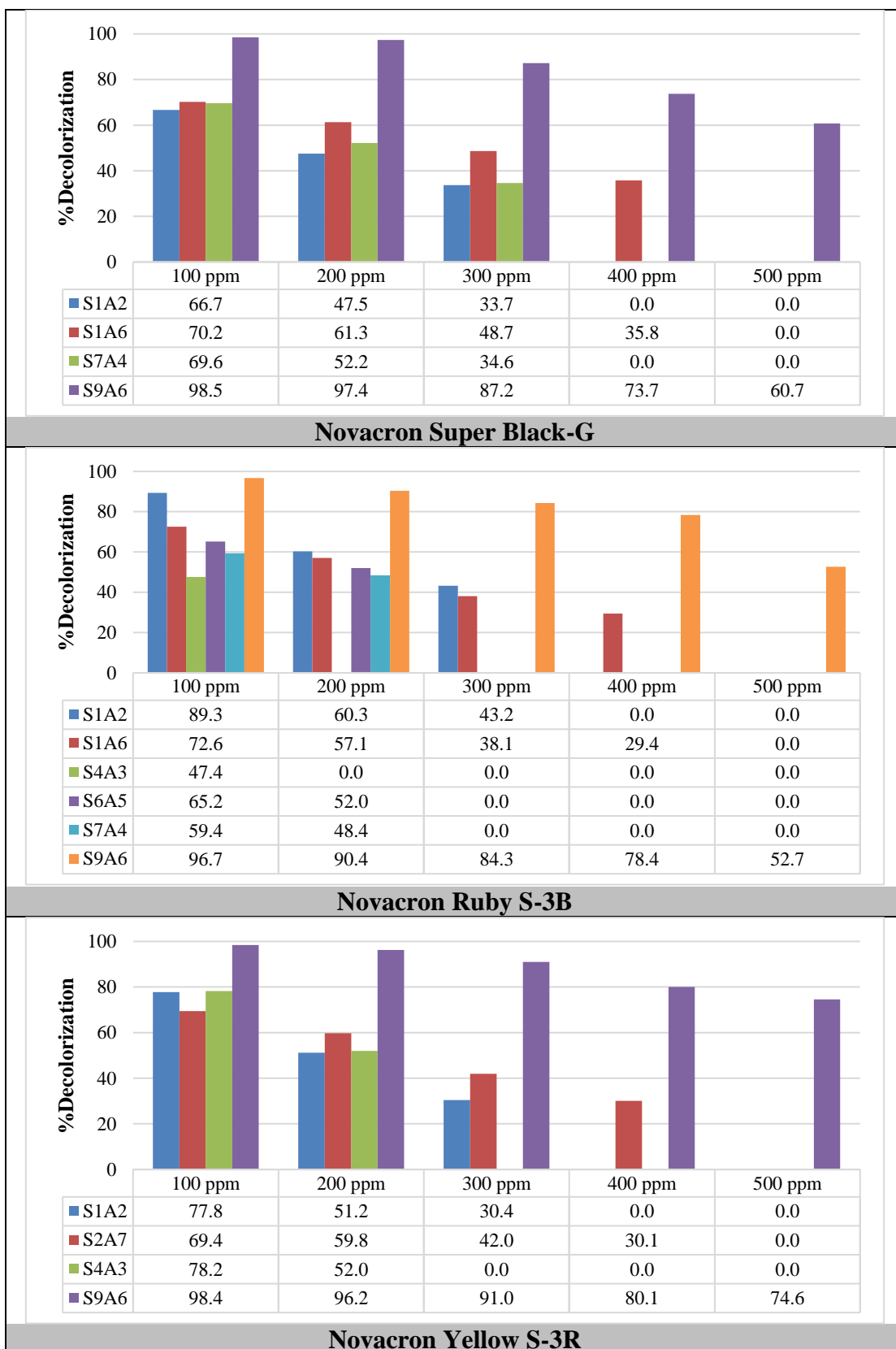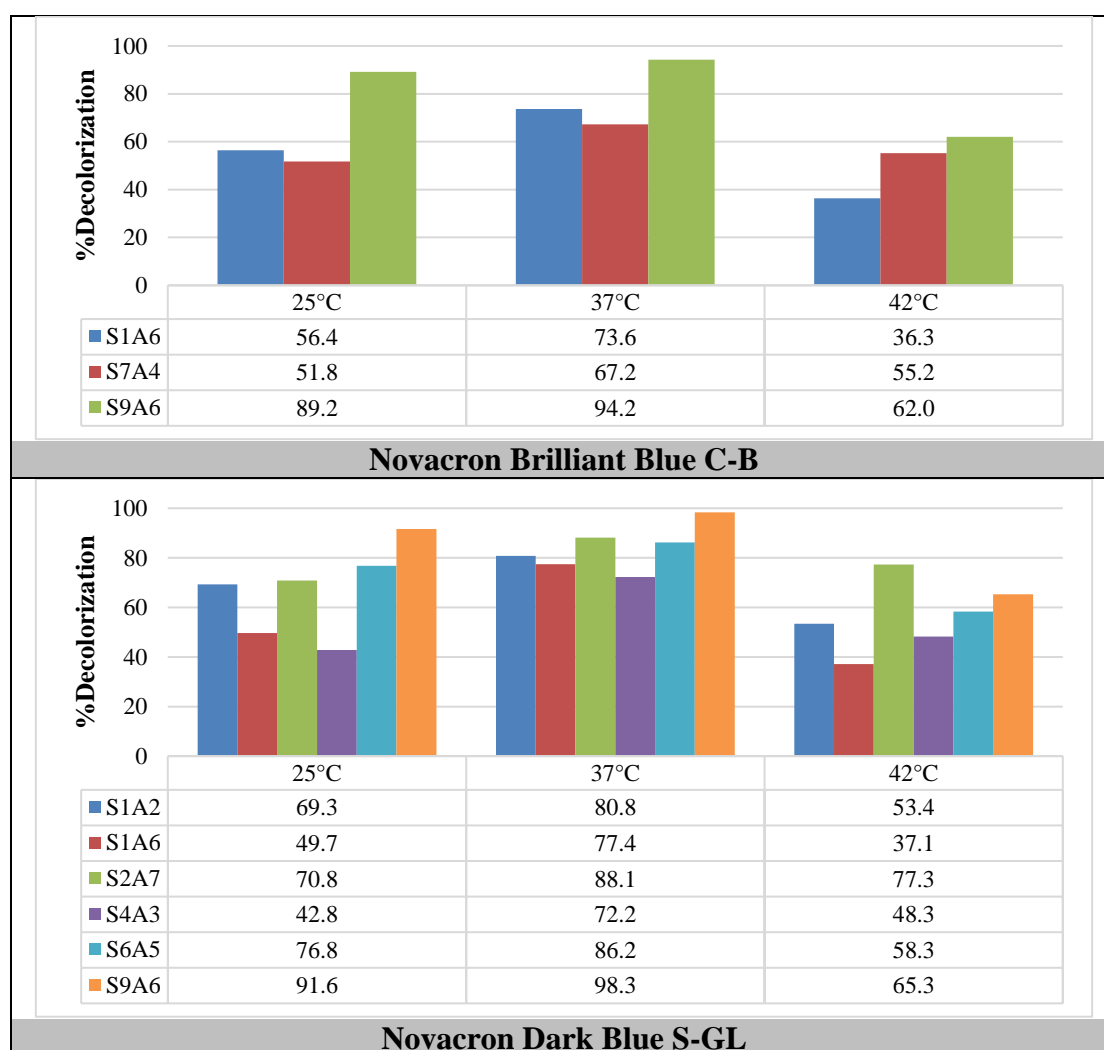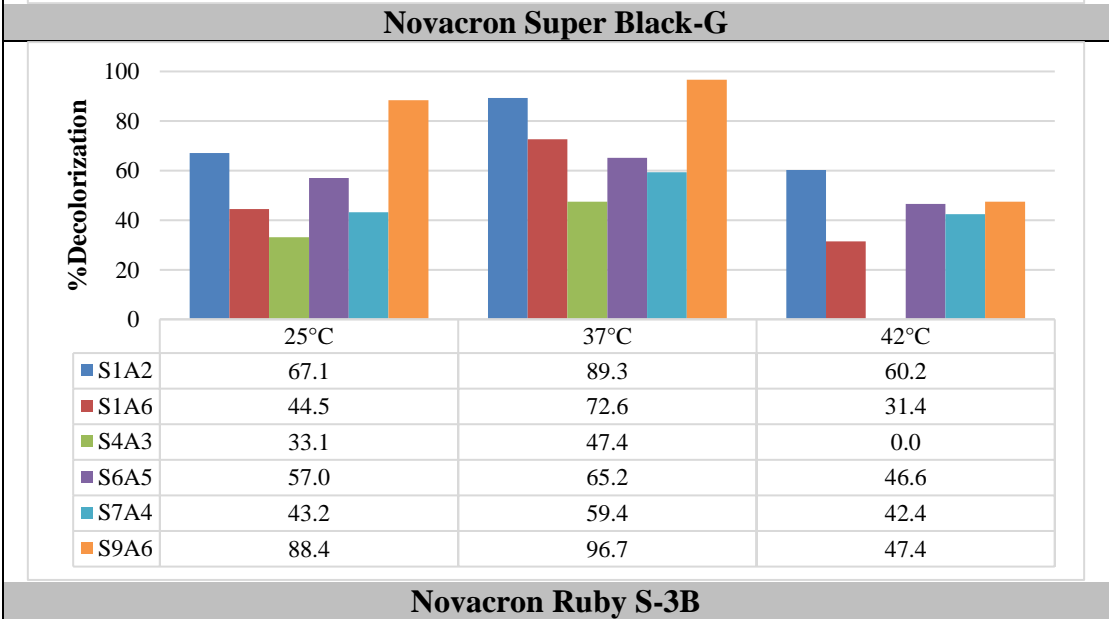e GN and Avitera Brilliant Yellow SE by any of the bacteria. Dye decolorization was steadily increased up to 48 hours in a static condition. With further increase in incubation time, the dye decolorization process remained constant. Compared to static conditions, agitated cultures grew well but showed decreased decolorization efficiency. Kothari, Kothari and Pathak (2005) reported the same. Only 40.5%, 33.3%, 31.0%, 58.4%, 46.0%, and 40.6% color removal was recorded for Novacron Brilliant Blue C-B, Novacron Dark Blue S-GL,

Novacron Navy S-GI, Novacron Super Black-G, Novacron Ruby S-3B, and Novacron Yellow S-3R, respectively, with S9A6 under agitating conditions. Reports revealed that agitation might enhance the competition between abundant oxygen and the azo compounds for reduced electron carriers under aerobic conditions (Chang *et al.*, 2001) and consequently decreased the decolorization process (Khalid, Arshad and Crowley, 2008). Chen *et al.*, 1999 and Khehra *et al.*, 2005 reported that agitation and vigorous aeration should be avoided to achieve an effective color removal.

	Static	Agitation
■ S1A6	73.6	31.8
■ S7A4	67.2	41.6
■ S9A6	94.2	40.5

Novacron Brilliant Blue C-B

	Static	Agitation
■ S1A2	80.8	37.5
■ S1A6	77.4	65.3
■ S2A7	88.1	60.6
■ S4A3	72.2	54.6
■ S6A5	86.2	56.6
■ S9A6	98.3	33.3

Novacron Dark Blue S-GL

	Static	Agitation
■ S1A2	71.3	36.4
■ S1A6	86.2	68.4
■ S2A7	76.0	64.4
■ S4A3	59.8	46.4
■ S6A5	51.1	37.4
■ S9A6	97.4	31.0

Novacron Navy S-GI

	Static	Agitation
■ S1A2	66.7	43.0
■ S1A6	70.2	54.6
■ S7A4	69.6	38.6
■ S9A6	98.5	58.4

Novacron Super Black-G

	Static	Agitation
■ S1A2	89.3	56.8
■ S1A6	72.6	51.2
■ S4A3	47.4	28.9
■ S6A5	65.2	41.4
■ S7A4	59.4	43.2
■ S9A6	96.7	46.0

Novacron Ruby S-3B

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	Static	Agitation
■ S1A2	77.8	43.7
■ S2A7	69.4	52.4
■ S4A3	78.2	55.4
■ S9A6	98.4	40.6

Novacron Yellow S-3R
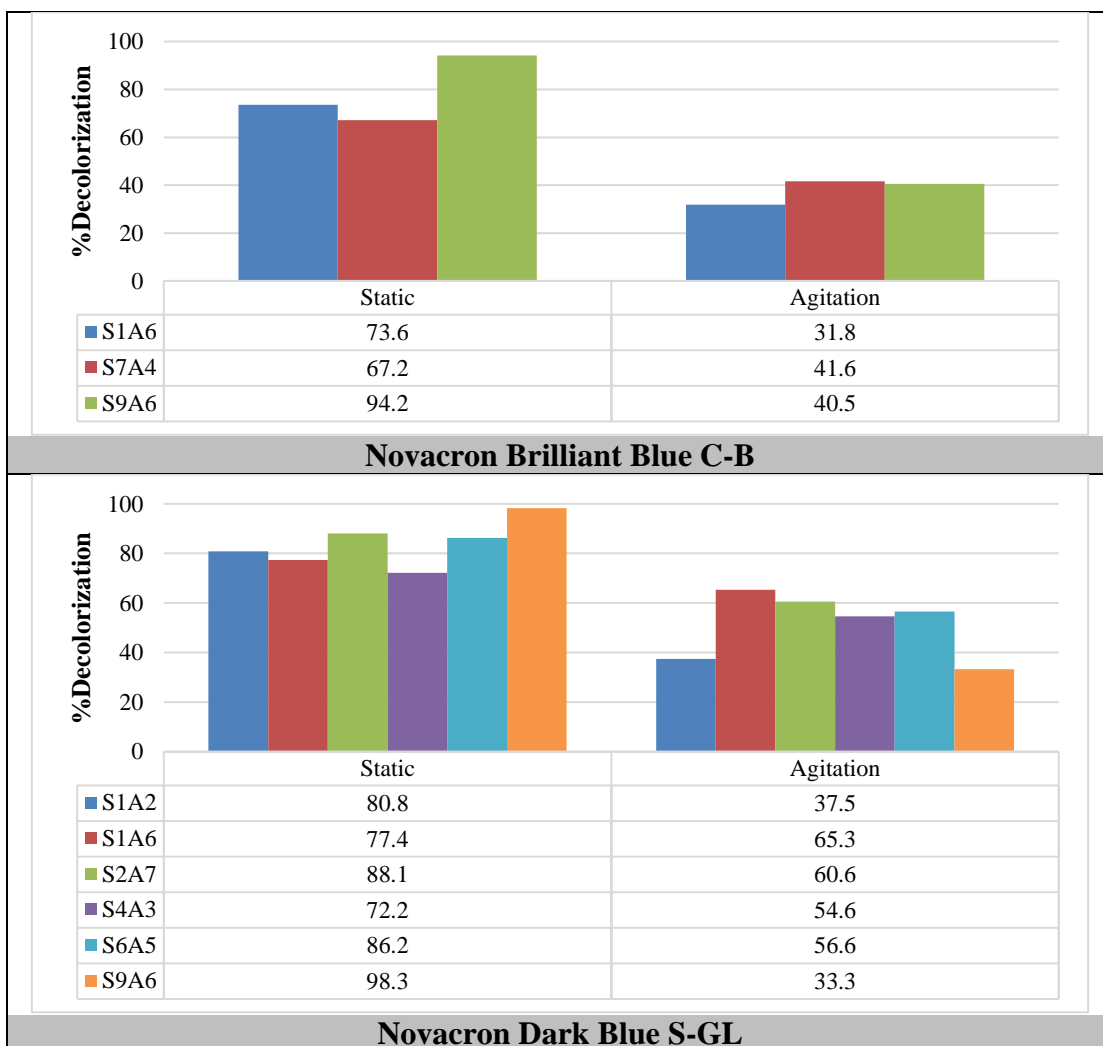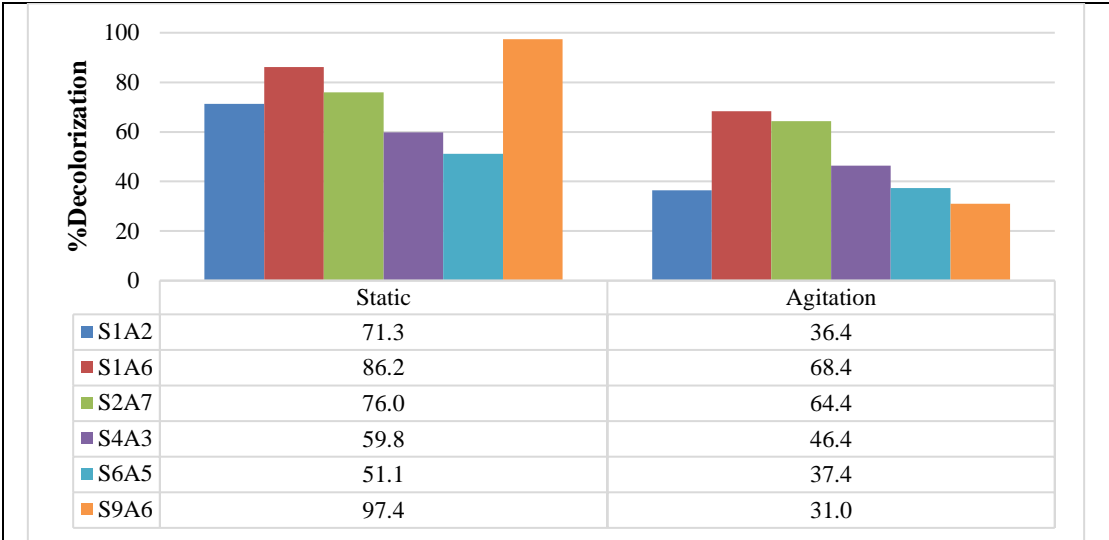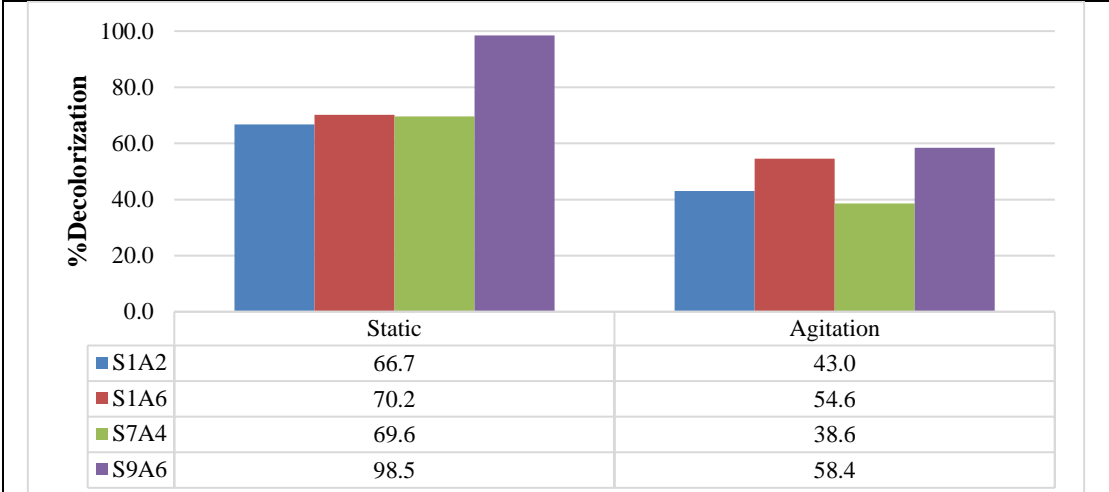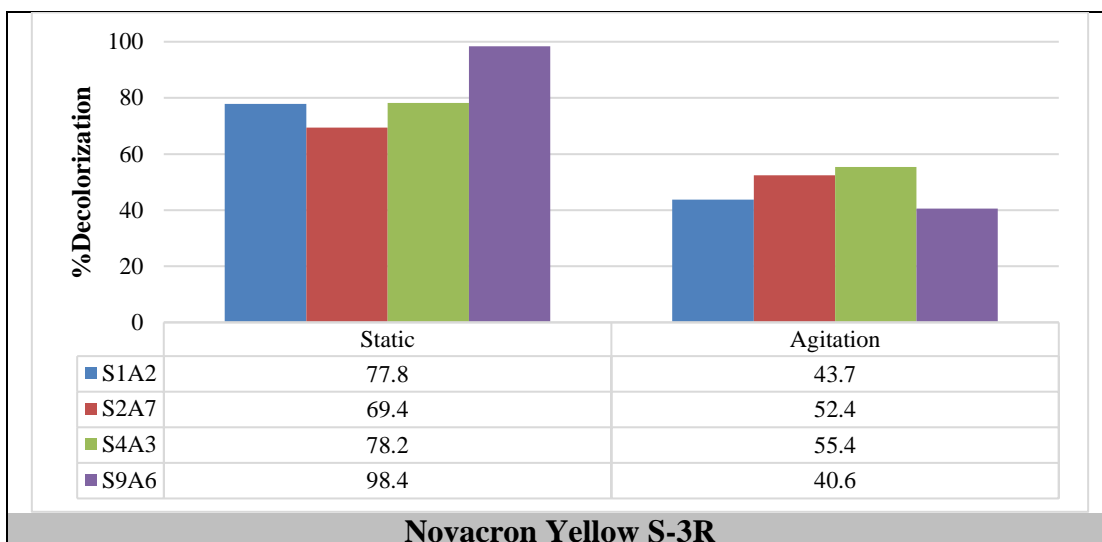
Chart 4.4 Effect of static versus agitating condition on dye decolorization

Effect of Varying Concentration of Seed Culture On Dye Decolorization

To examine the effect of varying concentration of seed culture on eight experimental dye decolorization, 100 ppm dye concentration and three different inoculum sizes (10%, 15%, and 20%) were applied. The results represented in Chart 4.5 indicated that, under the static cultural condition, the dye decolorization capacity remained mostly unaffected when 15% and 20% of the seed culture used. In case of 10% seed culture, reduced decolorization was observed. It was observed that as the seed culture concentration was increased, there was an increase in dye removal rate. A similar pattern was reported by Ayed *et al.*, (2009) where dye removal capacity increased significantly with the increase in initial biomass.

	10%	15%	20%
■ S1A6	62.4	70.8	73.6
■ S7A4	58.6	60.5	67.2
■ S9A6	88.6	89.9	94.2

Novacron Brilliant Blue C-B

	10%	15%	20%
S1A2	73.4	78.3	80.8
S1A6	64.1	68.9	77.4
S2A7	78.6	85.1	88.1
S4A3	62.6	70.0	72.2
S6A5	75.6	79.3	86.2
S9A6	96.1	98.0	98.3

Novacron Dark Blue S-GL

	10%	15%	20%
S1A2	66.0	69.2	71.3
S1A6	76.8	78.8	86.2
S2A7	65.7	70.2	76.0
S4A3	45.7	52.8	59.8
S6A5	45.1	49.7	51.1
S9A6	94.5	95.2	97.4

Novacron Navy S-GI

	10%	15%	20%
S1A2	56.8	61.2	66.7
S1A6	57.1	66.8	70.2
S7A4	55.5	62.5	69.6
S9A6	86.6	96.3	98.5

Novacron Super Black-G

	10%	15%	20%
■ S1A2	76.4	82.5	89.3
■ S1A6	65.3	71.6	72.6
■ S4A3	37.6	40.3	47.4
■ S6A5	55.3	59.3	65.2
■ S7A4	46.4	55.1	59.4
■ S9A6	91.5	94.5	96.7

Novacron Ruby S-3B

	10%	15%	20%
■ S1A2	60.2	71.2	77.8
■ S2A7	60.7	66.0	69.4
■ S4A3	60.7	70.3	78.2
■ S9A6	93.0	96.4	98.4

Novacron Yellow S-3R
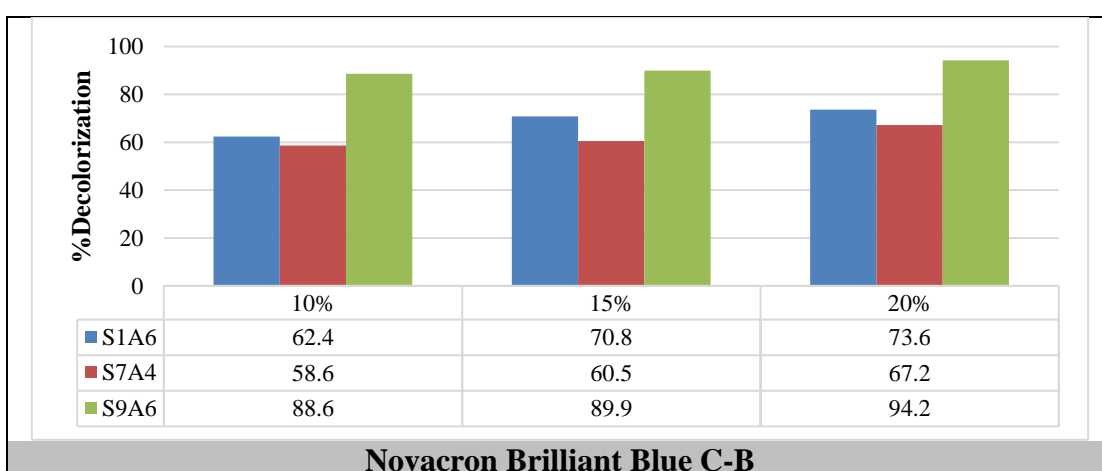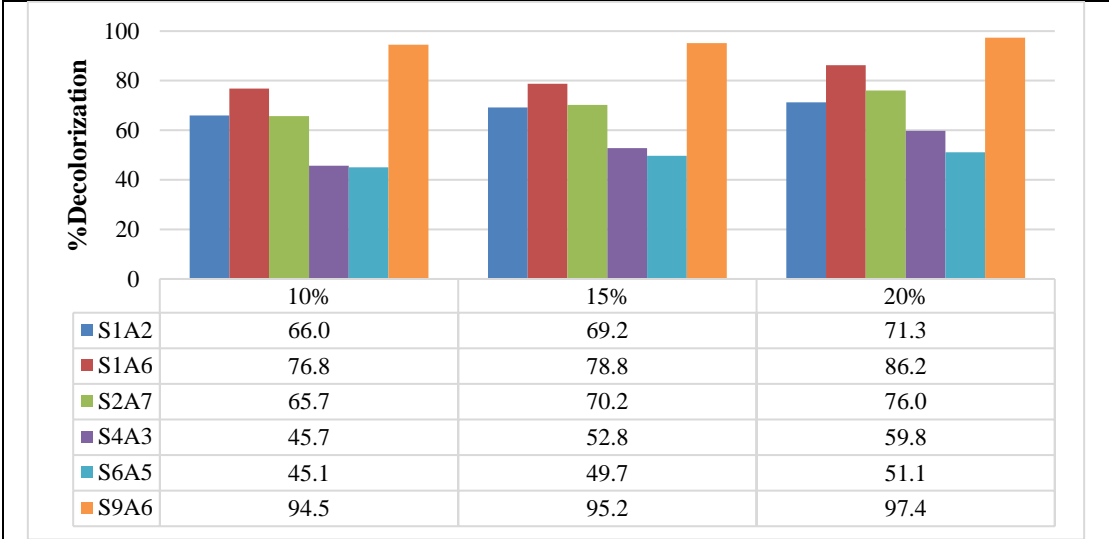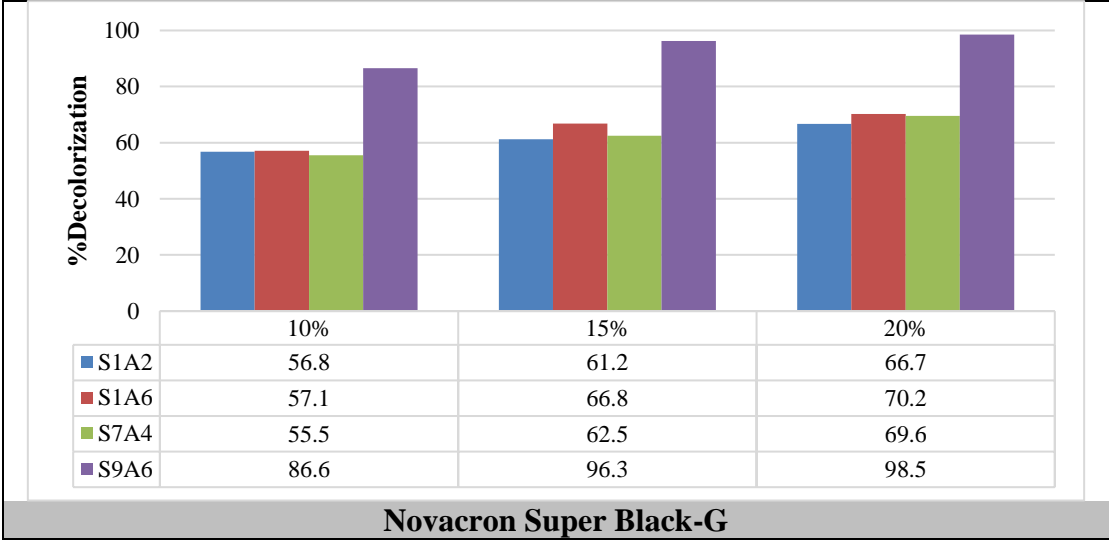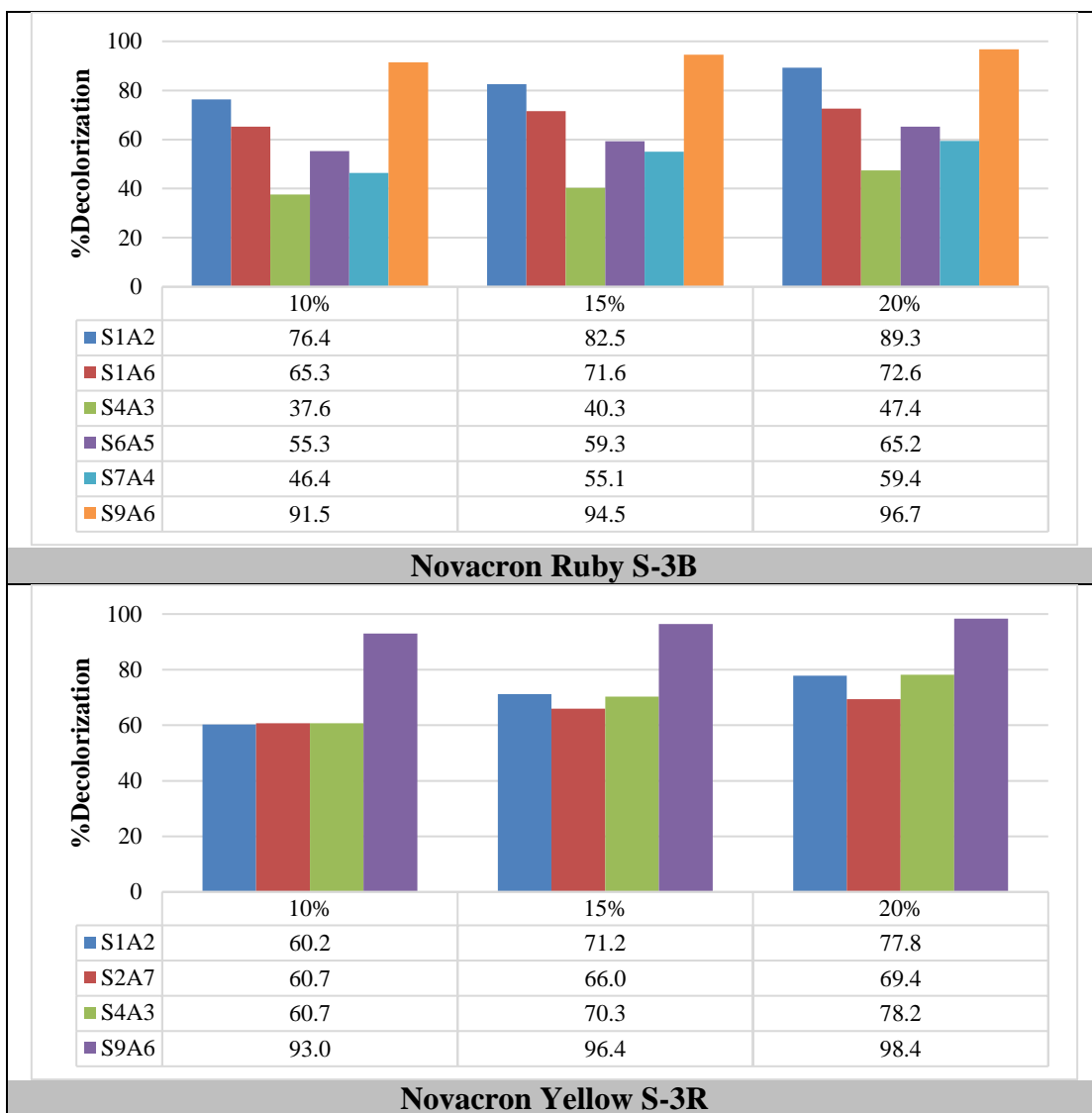
Chart 4.5 Effect of varying concentration of seed culture on dye decolorization

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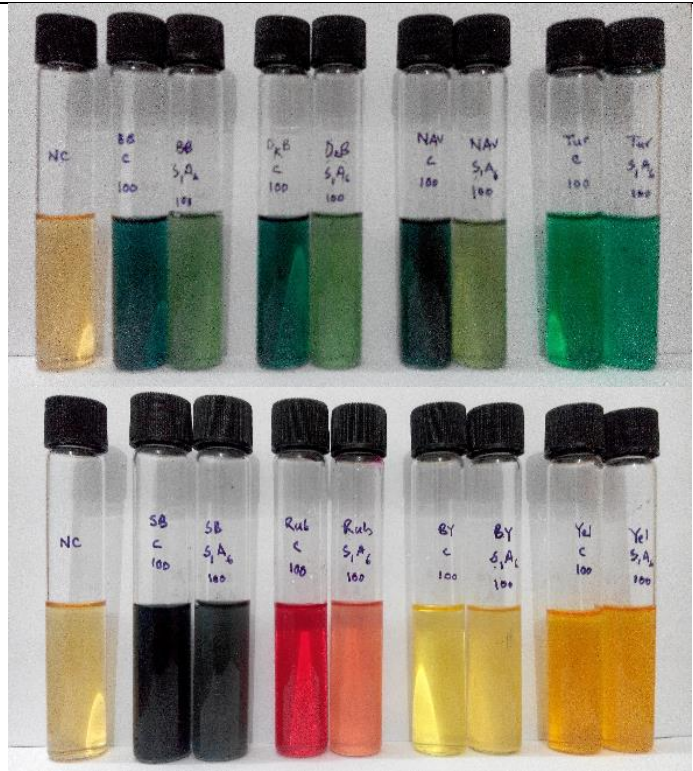
(I) Decolorization by bacterial isolate S1A6
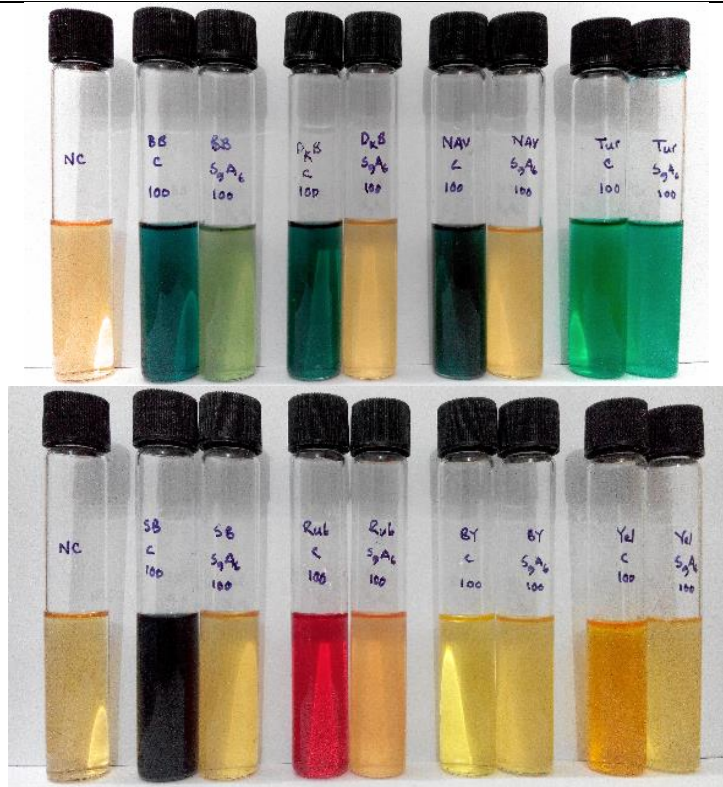
(II) Decolorization by bacterial isolate S9A6

Figure 4.1 Decolorization of eight synthetic experimental dyes by the selected bacterial isolate S1A6 and S9A6 in Nutrient broth containing individual dyes. NC is the dye free abiotic control. Compared to the dye containing abiotic control, visible decolorization was observed in 72 hours of incubation at 37°C and at static condition.

Selection of The Best Isolate for Maximum Decolorization

From above results, it could be observed that the seven different isolates revealed different decolorization percentage of the eight experimental dyes. If we compare the seven bacterial strains among themselves for their decolorization ability, the S9A6 bacterial isolate showed better potential than other two strains as maximum decolorization was achieved with it in six dyes. Thus the isolate S9A6 was used for further investigation. Isolate S1A2 and S1A6 showed the next best ability to decolorize five synthetic dyes.

Effect of Repeated Addition of the Dye Aliquots

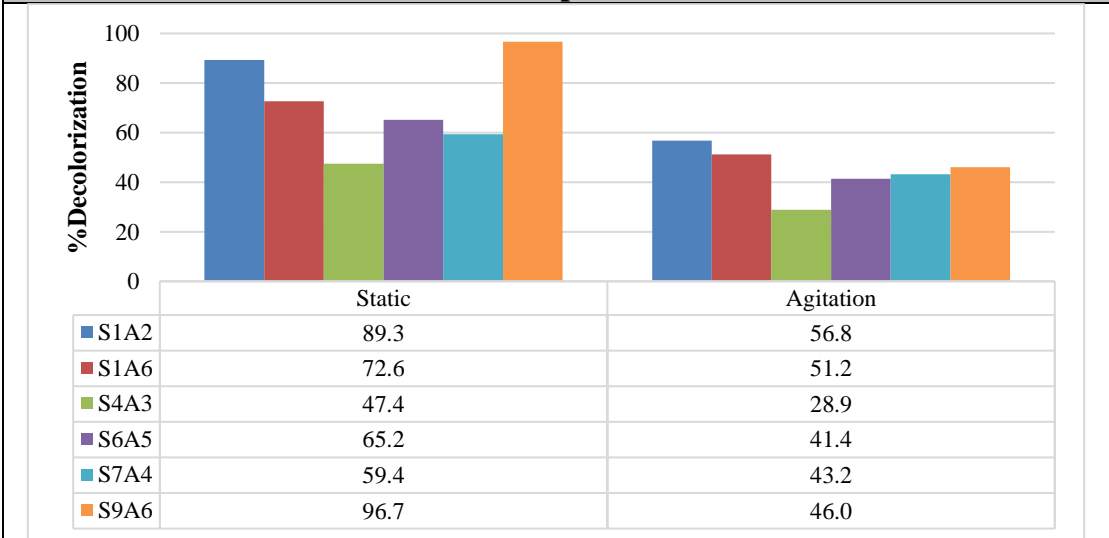This study was carried out to test the ability of highly efficient experimental isolate S9A6 to decolorize repeated additions of Novacron Brilliant Blue C-B, Novacron Dark Blue S-GL, Novacron Navy S-GI, Novacron Super Black-G, Novacron Ruby S-3B, and Novacron Yellow S-3R (100 ppm) into the decolorized nutrient broth (without further addition of supplement). First cycles showed more than 90% decolorization of all the six dyes in 72 hours, however, the fourth cycle took 120 hours for 50% removal of the dye (Chart 4.6). Similar interpretations have been documented previously for the decolorization of reactive dye (Chougule, Jadhav and Jadhav, 2014). The longevity of S9A6 exhibited the ability to decolorize repeated additions, which is important for its commercial application.

	Cycle 1	Cycle 2	Cycle 3	Cycle 4
Novacron Brilliant Blue C-B	95.0	89.4	68.3	51.3
Novacron Dark Blue S-GL	97.4	91.7	70.0	59.0
Novacron Navy S-GI	96.8	89.5	78.8	58.8
Novacron Super Black-G	96.8	88.4	66.3	49.7
Novacron Ruby S-3B	97.6	88.3	73.9	54.3
Novacron Yellow S-3R	97.7	90.5	72.3	61.2

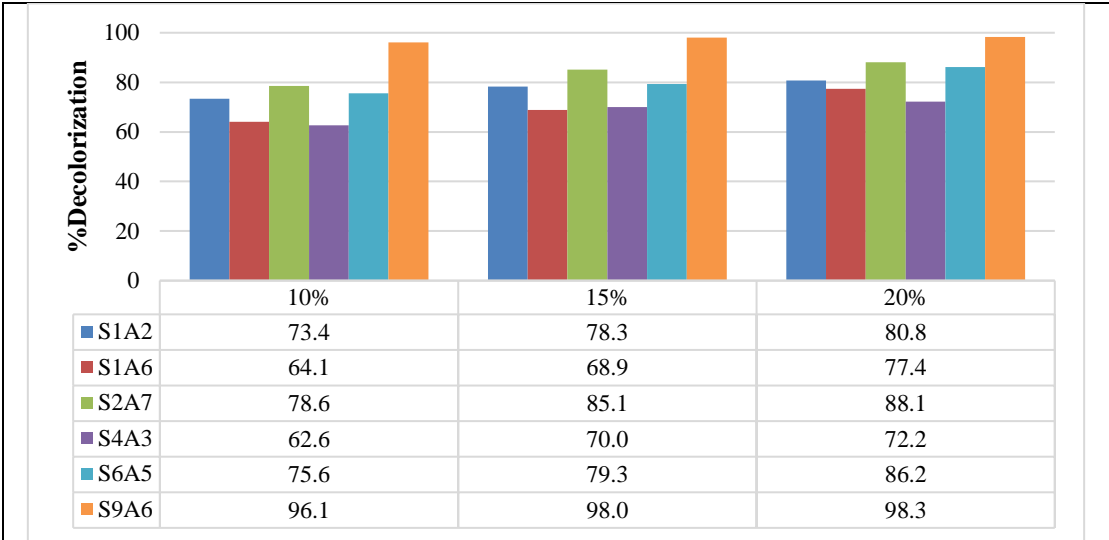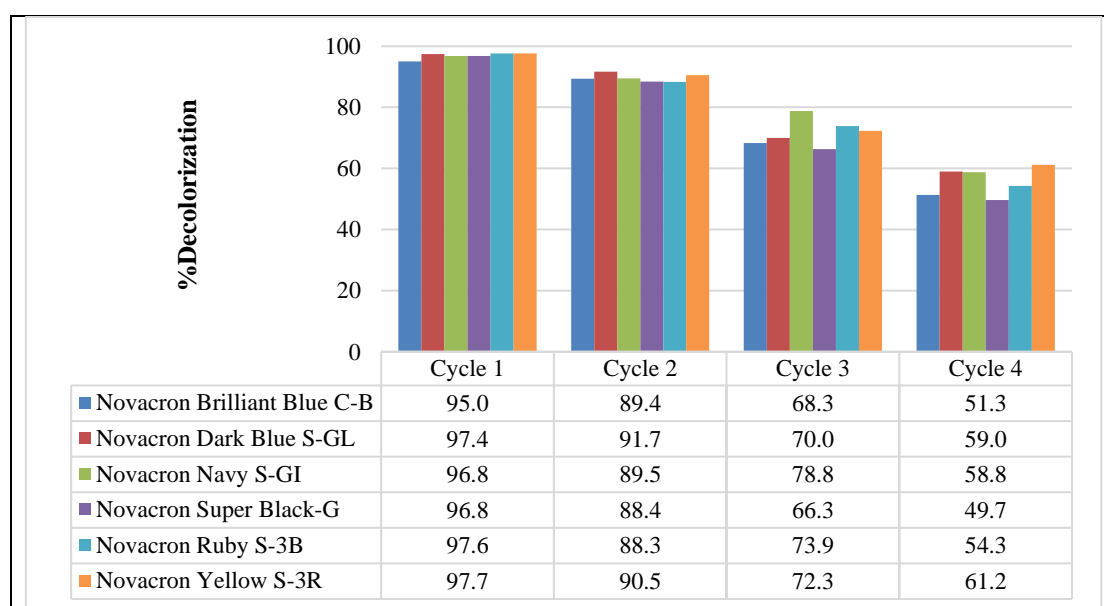Chart 4.6 Effect of continuous addition of dye (100 ppm) on decolorization

Effect of Varying Concentration of Mixed Dyes On Decolorization

Since waste of textile industries consist of a mixture of various dyes, the ability to decolorize the experimental dye mixture by highly efficient bacterial isolate S9A6 was studied. With increasing initial dye mixture concentrations as 100, 200, 300, 400, 500 and 600 ppm %decolorization was observed to be decreased (Chart 4.7).

%Decolorization	100 ppm	200 ppm	300 ppm	400 ppm	500 ppm	600 ppm
%Decolorization	88.6	79.2	71.6	62.7	47.3	10.4
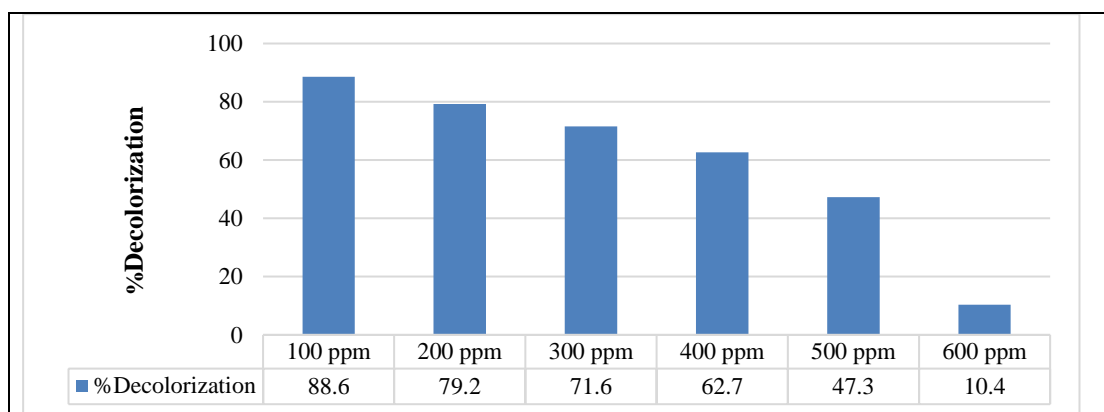
Chart 4.7 Effect of varying concentration of dye mix on decolorization

The increased dye concentrations might be inhibitory to the bacterial enzyme system as it was observed in the previous study. Reduction of color removal capacity of bacteria due to increasing dye concentrations has been reported earlier (Dhanve, Shedbalkar, and Jadhav, 2008).
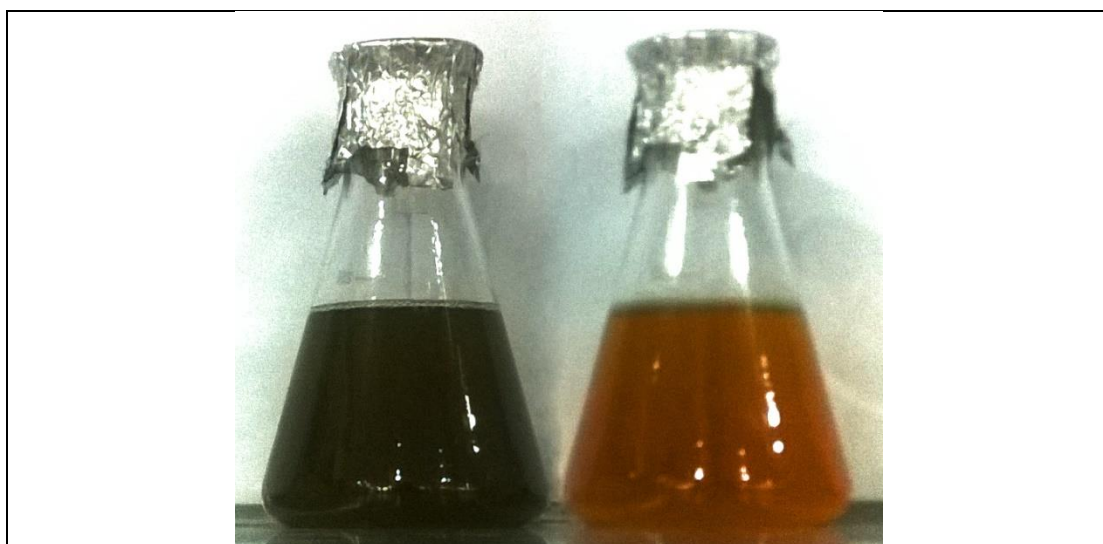
Figure 4.2 Decolorization of mixture of six synthetic dyes by bacterial isolate S9A6 in Nutrient broth. Compared to the dye containing abiotic control, visible decolorization was observed in 72 hours of incubation at 37°C and at static condition.

Morphological Characteristics of Dye Degrading Bacterial Isolates

The seven isolates were streaked on Nutrient agar plates and their distinct colony morphologies were subsequently analyzed. The isolates were also viewed under the microscope after Gram staining. Microscopic observations of seven isolates after Gram staining revealed the presence of three Gram Positive rods, two Gram Negative rods, and one Gram Positive cocci. Isolate S1A2 is rod-shaped Gram variable member of *Brevibacillus* genus. When the bacteria were grown on Nutrient agar plate, there was characteristics coloration of colonies like white, cream tan, greenish, yellow and yellowish-grey. Two isolates S1A2 and S7A4 were found to produce coloration of yellow and yellowish-grey, respectively. Very low greenish pigmentation was observed on Nutrient agar plate containing the isolate S9A6. This greenish pigmentation of this isolate was also observed in Pseudomonas agar. The isolate S9A6 was later identified as *Pseudomonas aeruginosa*. Size of colonies varied from a pinhead to large having a smooth or rough texture with entire (even), curled, undulate margins and circular, round and irregular forms (Table 4.1).

Table 4.1 Microscopic observations on Gram's staining and Colony characteristics in Nutrient agar of seven dye degrading bacterial isolates

DESIGNATION OF ISOLATES	GRAM'S STAINING		COLONY CHARACTERISTICS IN NUTRIENT AGAR MEDIUM						
	Reaction	Morphology	Size	Shape	Edge	Elevation	Color	Texture	Appearance
S1A2	Variable	Rods	Large	Circular	Curled	Flat	Yellowish-gray	Smooth	Dull
S1A6	+ ve	Rods	Large	Circular	Curled	Flat	Creamy	Rough	Dull
S2A7	+ ve	Ovoid cocci	Pinhead	Round	Entire	Raised	Whitish	Smooth	Shiny
S4A3	+ ve	Rods	Small	Irregular	Undulate	Convex	Creamy Tan	Smooth	Shiny
S6A5	- ve	Rods	Small	Circular	Entire	Convex	Transparent white	Smooth	Shiny
S7A4	+ ve	Rods	Small	Circular	Entire	Raised	Yellow	Smooth	Shiny
S9A6	- ve	Rods	Small	Circular	Entire	Convex	Greenish	Smooth	Shiny

BiOLOG GEN III Data Interpretation and Identification of Two Highly Efficient Dye Degrading Bacterial Isolates

Identification of highly efficient two bacterial strains was performed by using BiOLOG GEN III MicroPlate according to the manufacturer's protocol. Figure 4.3 and Figure 4.4 shows BiOLOG GEN III detection results of the 94 phenotypic tests of S1A6 and S9A6, respectively.

(I)

(II)
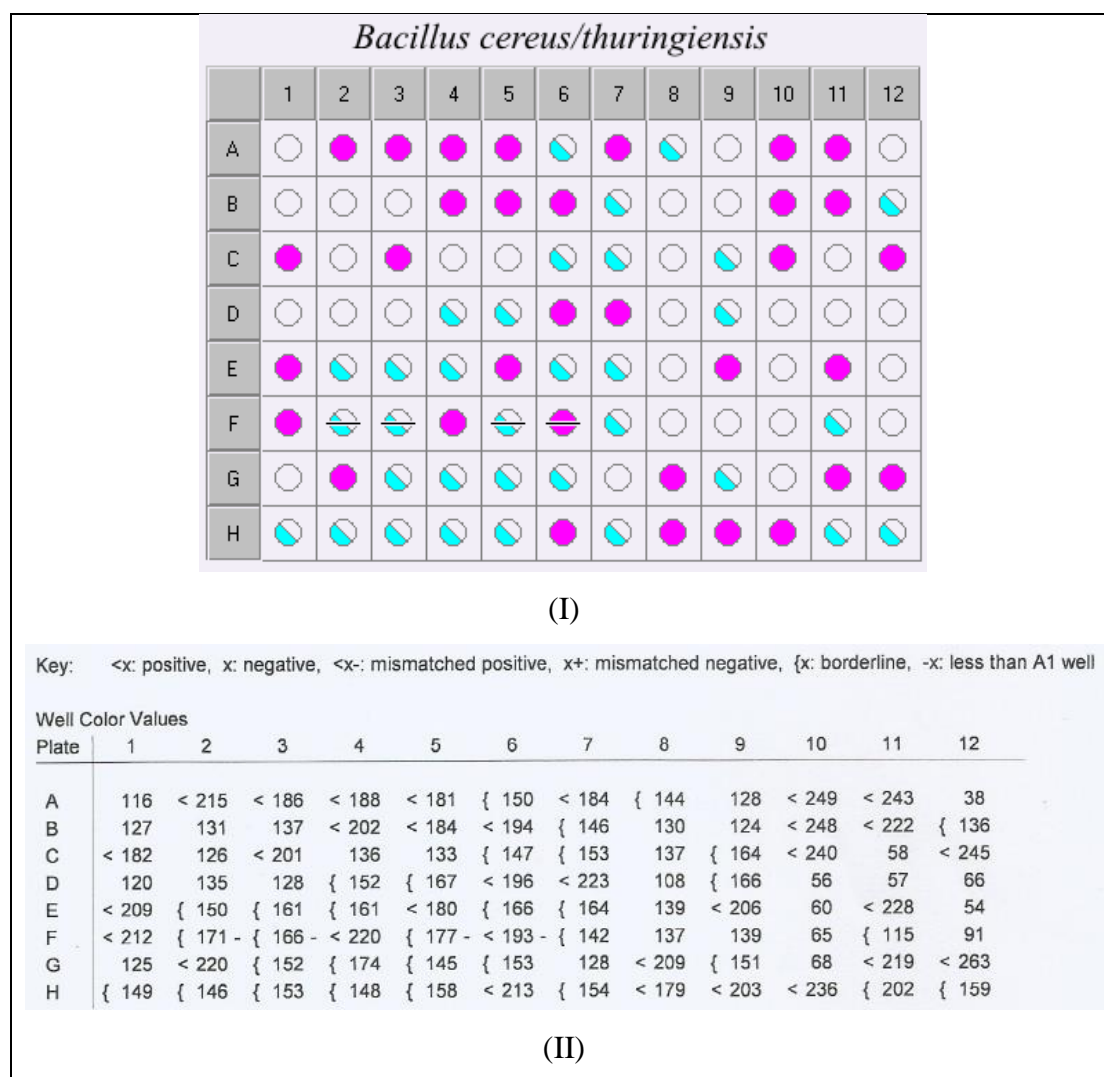
Figure 4.3 Profile of *Bacillus cereus/thuringiensis* (S1A6), identified by GEN III Protocol B.

(I) The BiOLOG GEN III detection results showing the 94 phenotypic tests: 71 Carbon Source Utilization reactions and 23 Chemical Sensitivity reactions by S1A6. The negative control well (A1), the positive control well (A10). The well with a purple color shows the positive reaction, the half-filled light blue circle shows the moderate reaction, while the white (blank) circle shows the negative reaction.

(II) Results with 96 well color values obtained from BiOLOG GEN III tests for S1A6.

48
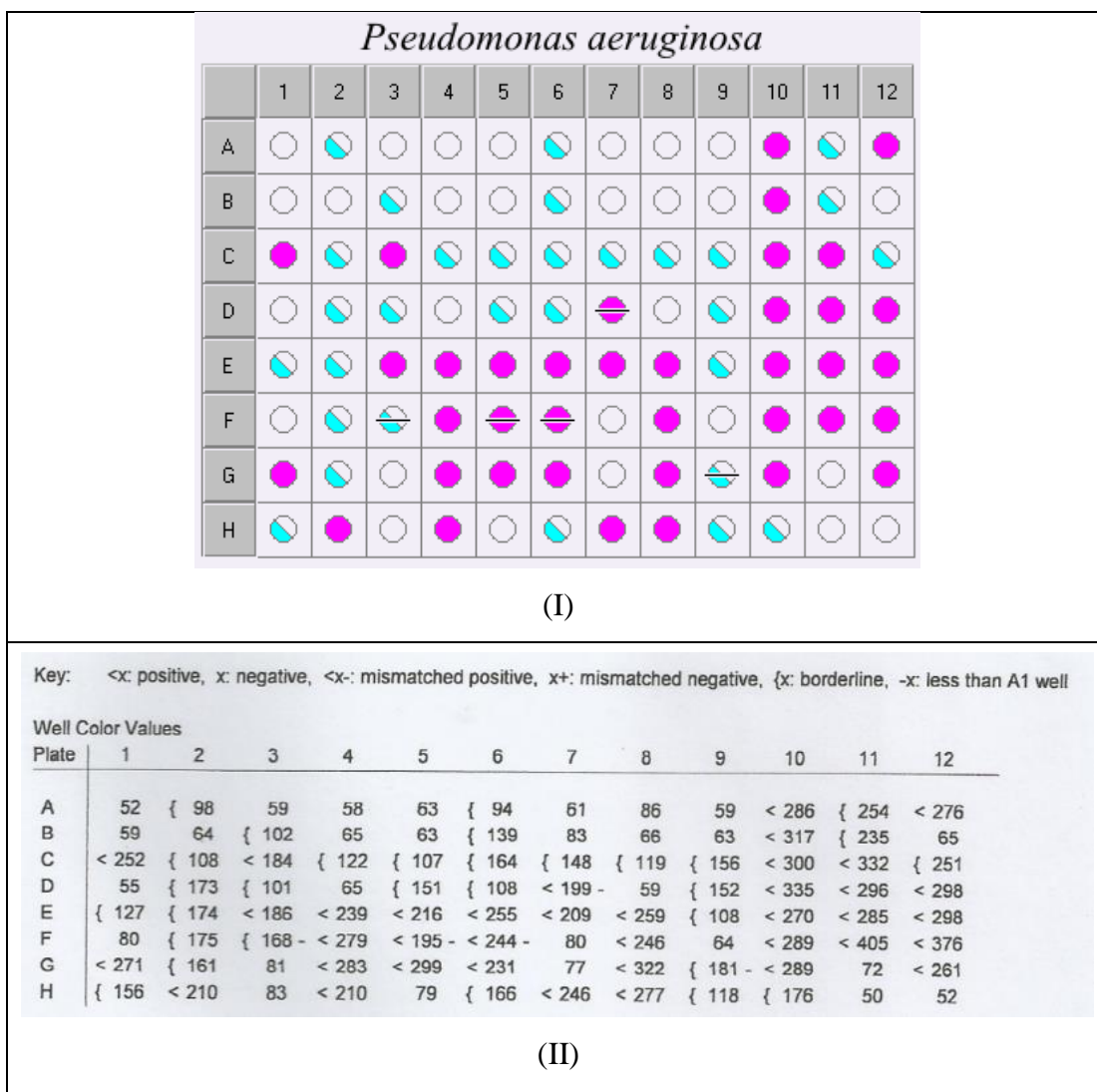
Figure 4.4 Profile of *Pseudomonas aeruginosa* (S9A6), identified by GEN III Protocol A.

(I) The BiOLOG GEN III detection results showing the 94 phenotypic tests: 71 Carbon Source Utilization reactions and 23 Chemical Sensitivity reactions by S9A6. The negative control well (A1), the positive control well (A10). The well with a purple color shows the positive reaction, the half-filled light blue circle shows the moderate reaction, while the white (blank) circle shows the negative reaction.

(II) Results with 96 well color values obtained from BiOLOG GEN III tests for S9A6 strain investigated in this study.

Carbon Source Utilization Characteristics of S1A6 and S9A6

The seventy-one Carbon Source Utilization Assays of the BiOLOG GEN III identification system are the basic test processes used to identify microbes based on the chemicals they can utilize. The well labeled as A1 is the negative control well and the color densities in wells of the Carbon Source Utilization Assays in columns 1-9 are referenced against it. All wells visually similar to the A1 well were scored as "negative" (-) and all wells with a noticeable purple color (more than well A1) were scored as

"positive" (+). Wells with extremely faint color, or with small purple flecks or clumps were scored as "borderline" (+/-). Mismatched positive and mismatched negative results were denoted as "MP" and "MN", respectively. Table 4.2 tabulates the Carbon Source Utilization profiles of S1A6 and S9A6 dye degrading bacterial strains investigated in this study.

Table 4.2 Carbon Source Utilization characteristics of S1A6 and S9A6 in BiOLOG GEN III MicroPlates

Substrates for Carbon Source Utilization Assays	Chemical formula	S1A6	S9A6
1. Dextrin	$C_6H_{10}O_5$	+	+/-
2. D-Maltose	$C_{12}H_{22}O_{11}$	+	-
3. D-Trehalose	$C_{12}H_{22}O_{11}$	+	-
4. D-Cellobiose	$C_{12}H_{22}O_{11}$	+	-
5. Gentiobiose	$C_{12}H_{22}O_{11}$	+/-	+/-
6. Sucrose	$C_{12}H_{22}O_{11}$	+	-
7. D-Turanose	$C_{12}H_{22}O_{11}$	+/-	-
8. Stachyose	$C_{24}H_{42}O_{21}$	-	-
9. D-Raffinose	$C_{18}H_{32}O_{16}$	-	-
10. α-D-Lactose	$C_{12}H_{22}O_{11}$	-	-
11. D-Melibiose	$C_{12}H_{22}O_{11}$	-	+/-
12. β-Methyl-D-Glucoside	$C_7H_{14}O_6$	+	-
13. D-Salicin	$C_{13}H_{18}O_7$	+	-
14. N-Acetyl-D-Glucosamine	$C_8H_{15}NO_6$	+	+/-
15. N-Acetyl-β-D-Mannosamine	$C_8H_{15}NO_6$	+/-	-
16. N-Acetyl-D-Galactosamine	$C_8H_{15}NO_6$	-	-
17. N-Acetyl Neuraminic Acid	$C_{11}H_{19}NO_9$	-	-
18. α-D-Glucose	$C_6H_{12}O_6$	+	+
19. D-Mannose	$C_6H_{12}O_6$	-	+/-
20. D-Fructose	$C_6H_{12}O_6$	+	+
21. D-Galactose	$C_6H_{12}O_6$	-	+/-
22. 3-Methyl Glucose	$C_7H_{14}O_6$	-	+/-

Substrates for Carbon Source Utilization Assays	Chemical formula	S1A6	S9A6
23. D-Fucose	$C_6H_{12}O_5$	+/-	+/-
24. L-Fucose	$C_6H_{12}O_5$	+/-	+/-
25. L-Rhamnose	$C_6H_{12}O_5$	-	+/-
26. Inosine	$C_{10}H_{12}N_4O_5$	+/-	+/-
27. D-Sorbitol	$C_6H_{14}O_6$	-	-
28. D-Mannitol	$C_6H_{14}O_6$	-	+/-
29. D-Arabitol	$C_5H_{12}O_5$	-	+/-
30. myo-Inositol	$C_6H_{12}O_6$	+/-	-
31. Glycerol	$C_3H_8O_3$	+/-	+/-
32. D-Glucose-6-PO4	$C_6H_{13}O_9P$	+	+/-
33. D-Fructose-6-PO4	$C_6H_{13}O_9P$	+	MP
34. D-Aspartic Acid	$C_4H_7NO_4$	-	-
35. D-Serine	$C_3H_7NO_3$	+/-	+/-
36. Gelatin		+	+/-
37. Glycyl-L-Proline	$C_7H_{12}N_2O_3$	+/-	+/-
38. L-Alanine	$C_3H_7NO_2$	+/-	+
39. L-Arginine	$C_6H_{14}N_4O_2$	+/-	+
40. L-Aspartic Acid	$C_4H_7NO_4$	+	+
41. L-Glutamic Acid	$C_5H_9NO_4$	+/-	+
42. L-Histidine	$C_6H_9N_3O_2$	+/-	+
43. L-Pyroglutamic Acid	$C_5H_7NO_3$	-	+
44. L-Serine	$C_3H_7NO_3$	+	+/-
45. Pectin	$C_6H_{10}O_7$	+	-
46. D-Galacturonic Acid	$C_6H_{10}O_7$	MP′	+/-
47. L-Galactonic Acid-γ-Lactone	$C_6H_{10}O_6$	MP′	MP′
48. D-Gluconic Acid	$C_6H_{12}O_7$	+	+
49. D-Glucuronic Acid	$C_6H_{10}O_7$	MP′	MP
50. Glucuronamide	$C_6H_{11}NO_6$	MP	MP
51. Mucic Acid	$C_6H_{10}O_8$	+/-	-

Substrates for Carbon Source Utilization Assays	Chemical formula	S1A6	S9A6
52. Quinic Acid	$C_7H_{12}O_6$	-	+
53. D-Saccharic Acid	$C_6H_{10}O_8$	-	-
54. *p*-Hydroxy-Phenylacetic Acid	$C_8H_8O_3$	-	+
55. Methyl Pyruvate	$C_4H_6O_3$	+	+/-
56. D-Lactic Acid Methyl Ester	$C_4H_8O_3$	+/-	-
57. L-Lactic Acid	$C_3H_6O_3$	+/-	+
58. Citric Acid	$C_6H_8O_7$	+/-	+
59. α-Keto-Glutaric Acid	$C_5H_8O_5$	+/-	+
60. D-Malic Acid	$C_4H_6O_5$	-	-
61. L-Malic Acid	$C_4H_6O_5$	+	+
62. Bromo-Succinic Acid	$C_4H_5O_4Br$	+/-	MP′
63. Tween 40		+/-	+/-
64. γ-Amino Butyric Acid	$C_4H_9NO_2$	+/-	+
65. α-Hydroxy-Butyric Acid	$C_4H_8O_3$	+/-	-
66. β-Hydroxy-D, L-Butyric Acid	$C_4H_8O_3$	+/-	+
67. α-Keto-Butyric Acid	$C_4H_6O_3$	+/-	-
68. Acetoacetic Acid	$C_4H_6O_3$	+	+/-
69. Propionic Acid	$C_6H_6O_2$	+/-	+
70. Acetic Acid	$C_2H_4O_2$	+	+
71. Formic Acid	CH_2O_2	+	+/-

Chemical Sensitivity Profiles of S1A6 and S9A6

The twenty-three Chemical Sensitivity Assays of the BiOLOG GEN III identification system are the basic test processes used to identify microbes based on the sensitivity to chemicals. The color concentrations in wells of the Chemical Sensitivity Assays in columns 10-12 are referenced against the positive control well, A10. All wells exhibiting significant sensitivity to the inhibitory chemical, with less than half the color of the A10 well were scored as "negative" (-) for growth. All other wells showing normal or near normal purple color (similar to well A10) were considered "positive" (+). Uncertain interpretations were scored as "borderline" (+/-). Mismatched positive

and mismatched negative results were denoted as "MP" and "MN", respectively. Table 4.3 shows the Chemical Sensitivity profiles of S1A6 and S9A6 dye degrading bacterial isolates.

Table 4.3 Chemical Sensitivity profiles of S1A6 and S9A6 in BiOLOG GEN III MicroPlates

Chemical Sensitivity Assays	S1A6	S9A6
1. pH 6	+	+/-
2. pH 5	-	+
3. 1% NaCl	+	+
4. 4% NaCl	+	+/-
5. 8% NaCl	+/-	-
6. 1% Sodium Lactate	+	+
7. Fusidic Acid	-	+
8. D-Serine	+	+/-
9. Troleandomycin	-	+
10. Rifamycin SV	-	+
11. Minocycline	-	+
12. Lincomycin	-	+
13. Guanidine hydrochloride	+	+
14. Niaproof 4	-	+
15. Vancomycin	-	+
16. Tetrazolium Violet	+/-	+
17. Tetrazolium Blue	-	+
18. Nalidixic Acid	-	+
19. Lithium Chloride	+	-
20. Potassium Tellurite	+	+
21. Aztreonam	+	+/-
22. Sodium Butyrate	+/-	-
23. Sodium Bromate	+/-	-

Determination of ID

The S9A6 isolate was perfectly identified by using the 94 phenotypic tests on BiOLOG GEN III MicroPlate as *Pseudomonas aeruginosa* with 93% probability (PROB), 0.617 similarity (SIM) index and 4.916 distance (DIST). The ID is accepted as correct only if the difference (delta) in distance (DIST) between the first and second listed is around or higher of 1 (Figure 4.5). The delta in DIST for *P. aeruginosa* is 2.254 which is a very good identification. However, the difference in DIST for S1A6 is 0.409 and the bacterial ID displayed as *Bacillus cereus/thuringiensis* with 69.7% probability and 0.697 similarity index.

	PROB	SIM	DIST	Organism Type	Species
==>1	0.697	0.697	4.322	GP-RodSB	Bacillus cereus/thuringiensis
2	0.210	0.210	4.731	GP-RodSB	Bacillus thuringiensis/cereus
3	0.026	0.026	5.996	GP-RodSB	Bacillus pseudomycoides/cereus
4	0.026	0.026	5.997	GP-RodSB	Bacillus weihenstephanensis/cereus

Species ID: Bacillus cereus/thuringiensis

S1A6: *Bacillus cereus/thuringiensis*

	PROB	SIM	DIST	Organism Type	Species
==>1	0.935	0.617	4.916	GN-NEnt	Pseudomonas aeruginosa
2	0.028	0.014	7.170	GN-NEnt	Pseudomonas syringae pv maculicola
3	0.021	0.011	7.354	GN-NEnt	Pseudomonas fulva
4	0.017	0.008	7.496	GN-NEnt	Pseudomonas citronellolis

Species ID: Pseudomonas aeruginosa

S9A6: *Pseudomonas aeruginosa*

Figure 4.5 The probability (PROB), similarity (SIM) and distance (DIST) values as reported and displayed by the MicroStation/MicroLog 3 software.

Discussion

The textile effluents generated and released from the textile industries containing synthetic dyes are highly colored and for that reason, they are visually identifiable. Researchers have been trying to develop cost-effective methods for the treatment of these textile dyes in the textile effluents. There are several methods for the treatment of textile effluents for the removal of dyes. The treatment methods can be broadly categorized into physical, chemical and biological methods. These treatment methods have been comprehensively reviewed by numerous researchers. The biological wastewater treatment gives reliable results, generates less sludge and is more environment-friendly treatment compared to physicochemical treatment (Robinson *et al.*, 2001; Kharub, 2012).

Bacteria with efficient dye degrading potential of textile dyes were isolated from textile effluent and soil collected from local handloom fabrics and dye wastewater disposal areas. It was expected that spots near handloom fabric mills contaminated with textile dyes harbor some microbes which are capable to live with higher toxic levels of contamination. Pure bacterial strains were then isolated from the microbial community of the collected samples to find out the most promising bacterial strains with higher dye degradative activity. Isolation of bacteria from soil and effluent samples were carried out by spread plate technique and enrichment technique, respectively. Screening tests using all the eight dyes under study assessed the potential of thirty-four bacterial isolates for decolorizing these eight different textile dyes under static conditions in liquid nutrient broth. Out of these strains, seven efficient bacterial strains were isolated and purified. The growth characteristics were investigated in Nutrient Agar media. Microscopic observations after Gram staining revealed the presence of three Gram Positive rods, two Gram Negative rods, and one Gram Positive cocci. Isolate S1A2 is rod-shaped Gram variable member of the *Brevibacillus* genus. All of these seven strains were then examined to decolorize the eight synthetic textile dyes singly in Nutrient broth medium. In this experiment, two bacterial strains (S1A6 and S9A6) exhibiting maximum decolorization potential were selected for identification using BiOLOG identification system.

The BiOLOG identification result call is based on two criteria: match and separation. The distance value is a measure of how many mismatches there are between the test pattern and the reference pattern in the BiOLOG database. The "match" parameter denotes how closely the test pattern agrees with the reference pattern. Good matches will have small distance (DIST) values. Separation is the difference in distance between the first choice in the database and all the rest of the choices. The separation and the distance are multiplied to get an overall ID value called "similarity (SIM)," which is the likelihood that the microbial ID is correct based on data available in the database. The probability (PROB) value is the possibility that the test sample in the species identified, given that the test sample does not actually belong to a species not in the database.

The BiOLOG identification result call for S1A6 isolate was *Bacillus cereus/thuringiensis* with 69.7% probability (PROB), 0.697 similarity (SIM) index and 4.322 distance (DIST) value. The S9A6 isolate was identified as *Pseudomonas aeruginosa* with 93% PROB, 0.617 SIM index and 4.916 DIST value. The ID is accepted as trustable only if the difference (delta) in distance (DIST) between the first and second listed is around or higher of 1. The delta in DIST for *P. aeruginosa* is 2.254 and it is a very good identification. But the difference in DIST for *Bacillus cereus/thuringiensis* is 0.409. Actually, the BiOLOG GEN III phenotyping system currently does not reliably identify *Bacillus cereus* or *Bacillus thuringiensis* (Cote *et al.*, 2014). The limitation of BiOLOG system is expected since the members of the *Bacillus cereus* family are almost indistinguishable in terms of their genetic similarities (Helgason *et al.*, 2000). Hence, the BiOLOG identification system was considered to provide a preliminary screen for *Bacillus cereus sensu lato* group and required confirmatory tests. The *Bacillus cereus sensu lato* includes three main species: *B. thuringiensis*, *B. cereus*, *B. anthracis,* and three less-known and less-understood species: *B. mycoides*, *B. pseudomycoides*, *and B. weihenstephanensis* (Okinaka and Keim, 2016). Sophisticated method, for example, multi-REP-PCR fingerprinting can be used to distinguish the species of these closely related bacteria (Cherif et al., 2007). However, as reported by Yan *et al.* (2011), a preliminary test for free protein toxin crystals or parasporal inclusion bodies within the exosporium of *B. thuringiensis* can be performed. *Bacillus cereus* and other members of the *B. cereus sensu lato* group do not produce protein toxin crystals.

The isolate that showed maximum decolorization potential of six synthetic dyes in Nutrient broth media is *Pseudomonas aeruginosa*. *Bacillus cereus/thuringiensis* showed the next best ability to decolorize five synthetic dyes. The decolorization obtained in this study were expressed in percentages with *Bacillus cereus/thuringiensis* having up to 86.2% (Novacron Navy S-GI), 77.4% (Novacron Dark Blue S-GL), 73.6% (Novacron Brilliant Blue C-B), 72.6% (Novacron Ruby S-3B), 70.2% (Novacron Super Black-G), however, *Pseudomonas aeruginosa* had 98.5% (Novacron Super Black-G), 98.4% (Novacron Yellow S-3R), 98.3% ((Novacron Dark Blue S-GL), 97.4% (Novacron Navy S-GI), 96.7% (Novacron Ruby S-3B) and 94.2% (Novacron Brilliant Blue C-B) for 100 ppm of dye when incubated at 37°C for 72 hours.

In the experiments with different dye concentration, the decolorization percentage was decreased with an increase in dye concentration. The reason is that the higher concentration of dye inhibits nucleic acid biosynthesis and cell growth (Chen *et al.*, 2003). Maximum decolorization was observed for Novacron Super Black-G 98.5% at 100 ppm dye concentration by *Pseudomonas aeruginosa*. The %decolorization by all the experimental bacteria was strongly inhibited at 500 ppm dye in the Nutrient broth except for *Pseudomonas aeruginosa*. At a higher level of dye concentration, a decrease in decolorization can be due to substrate inhibition effect as a result of saturation in the enzymatic system. Khehra *et al.*, (2005) also suggested that the decrease in decolorization efficiency might be due to the toxic effect of dyes.

Temperature is one of the most critical parameters for dye decolorization. According to Çetin and Dönmez (2006), the decolorization percentage increases with increase in temperature. Similar results were observed in this study from temperature 25°C to 37°C. However, at 42°C, the decolorization ability was reduced for all the experimental microorganisms. This might have occurred due to the adverse effect of temperature on cell viability and the enzymatic activities. Lower temperature also affects microbial activity and decolorization

pH of the dye-broth media was found to affect dye decolorization. The experimental isolate *Pseudomonas aeruginosa* showed maximum decolorization at pH 7. As pH gradually reduced or increased from near neutral, decolorization declined. The requirement of neutral pH for optimum growth had been reported in several studies. As

per Evans and Furlong (2003), the optimum pH for color removal is often the neutral pH value or a slightly alkaline pH value and the rate of color removal tends to decrease rapidly at strongly acid or strongly alkaline pH values.

The decolorization decreased to almost half when experimental conditions were changed from static to shaking keeping all the factors same. Several reports revealed that agitation might enhance the competition between abundant oxygen and the azo compounds for reduced electron carriers under aerobic conditions (Chang *et al.*, 2001) and consequently decreased the decolorization process (Khalid, Arshad and Crowley, 2008). Chen *et al.*, 1999 and Khehra *et al.*, 2005 reported that agitation and vigorous aeration should be avoided to achieve an effective color removal. In this experiment, the isolate *Pseudomonas aeruginosa* showed the similar result when grown on dye-Nutrient broth. It was also observed that as the seed culture concentration was increased, there was an increase in dye removal rate. A similar pattern was reported by Ayed *et al.*, (2009) where dye removal capacity increased significantly with the increase in initial biomass.

Since waste of textile industries consist of a mixture of various dyes, the ability to decolorize the experimental dye mixture by *Pseudomonas aeruginosa* was studied. With increasing initial dye mixture concentrations as 100, 200, 300, 400, 500 and 600 ppm %decolorization was observed to be decreased. *Pseudomonas aeruginosa* showed more than 90% decolorization of all the six dyes in 72 hours in the first cycle, however, the fourth cycle took 120 hours for 50% removal of the dye. Similar interpretations have been documented previously for the decolorization of reactive dye (Chougule, Jadhav and Jadhav, 2014). The longevity of *Pseudomonas aeruginosa* exhibited the ability to decolorize repeated additions, which is important for its commercial application.

Conclusions and Recommendations

Decolorization of textile dyeing effluent is a challenging task in dye wastewater treatment. The results of this study suggest that some dyes viz. Novacron Brilliant Blue C-B, Novacron Dark Blue S-GL, Novacron Navy S-GI, Novacron Ruby S-3B, Novacron Super Black-G and Novacron Yellow S-3R can be efficiently degraded by *Pseudomonas aeruginosa* isolated from textile effluent. The isolated bacterial strain showed almost complete decolorizing activity of 100 ppm dye under 72 hours of the study period. *Bacillus cereus/thuringiensis* showed the next best ability to decolorize five synthetic dyes. It is thus possible as reported elsewhere to develop bioremediation system different textile dyes based on local bacterial isolates.

Recommendations for future work

i. More extensive screening may be undertaken for isolation of potential dye degrading organisms from more locations of both contaminated soil and wastewater as well.

ii. Further improvement of selected potential isolate through genetic manipulation.

iii. Development of mixed culture system involving potential dye degrading organisms for synergistic effects on dye degrading capability. A consortium of microorganisms can enhance dye decolorization processes by providing different enzyme classes and pathways. If used in combination with other dye decolorizing bacteria, this bacterium is expected to provide success in biodegradation of more hazardous dyes.

iv. Sophisticated method, for example, multi-REP-PCR fingerprinting can be used to distinguish the species of these closely related bacterial strains. Plasmid profiling can also be accomplished which can reveal much information related to the genes associated with biodegradation pathway enzymes.

v. The physical parameters such as dye concentration, temperature, pH, agitation, seed culture concentration play an important role in decolorization efficiency. The ability of the isolated *Pseudomonas aeruginosa* to adapt, tolerate and decolorize experimental dyes at high concentration gives it an advantage for treatment of textile industry. Pilot-scale study with these experimental bacteria

must be performed because the experimental condition in a laboratory does not represent the true performance of the bacteria in a polluted environment.

vi. The decolorization of a real effluent from a textile industry should be evaluated in order to examine the effectiveness of the bacteria to a more complex system. This study could also be extended to other classes of dyes often present in wastewater from other dye utilizing industries, for instance, tanning (leather), paint, paper, and ink industries.

vii. The monitoring of UV-Vis spectra in the present study did not provide any information about products formed as a result of dye decolorization. In order to detect dye metabolites formed after dye decolorization, Gas Chromatography-Mass Spectrometry (GC-MS) analysis can be performed. GC-MS analysis of decolorized sample provides precise information about molecular weight and structure of an unknown compound. Furthermore, other biodegradation analysis, for instance, High-Performance Liquid Chromatography (HPLC) and Fourier-Transform Infrared Spectroscopy (FTIR) along with GC-MS, dye degrading mechanism of selected experimental bacteria can be investigated.

viii. Immobilization of whole bacterial cells provides distinct stability over free cells. Different and efficient immobilization supports and techniques should also be examined to develop immobilized bacterial dye decolorization. Dye decolorizing enzyme immobilization studies for biotransformation of dyes can also be performed.

It can be concluded that the newly isolated native bacteria, *Pseudomonas aeruginosa*, have promising potential to degrade multiple synthetic textile dyes. Therefore, it is a prime candidate that could effectively be used as an alternative to the physical and chemical process used for textile effluent treatment.

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Appendix A : Media and Reagents

- **Nutrient Agar (Difco, USA)**

Ingredients	Amount
Beef Extract	3.0 g
Peptone	5.0 g
Agar	15.0 g
Distilled Water	1000 ml
Dissolved in distilled water by stirring with gentle heating and then sterilized by autoclaving at 121°C, 15 psi pressure for 15 minutes.	

- **Nutrient Broth (Oxoid, England)**

Ingredients	Amount
'Lab-Lemco' Powder	1.0 g
Yeast Extract	2.0 g
Peptone	5.0 g
Sodium Chloride	5.0 g
Distilled Water	1000 ml
Dissolved in distilled water by stirring then sterilized by autoclaving at 15 psi pressure (121°C) for 15 minutes.	

- **Pseudomonas Agar**

Ingredients	Amount
Casein Enzymic Hydrolysate	10.0
Proteose Peptone	10.0
Dipotassium Phosphate	1.50
Magnesium Sulphate	1.50
Agar	15.0
Distilled Water	1000 ml
Dissolved in distilled water by stirring with gentle heating and then sterilized by autoclaving at 121°C, 15 psi pressure for 15 minutes.	

- **Ringer's solution**

Ingredients	Amount
Sodium Chloride	8.50 g
Potassium Chloride	0.20 g
Calcium Chloride	0.20 g
Sodium Bicarbonate	0.01 g
Distilled Water	1000 ml
Sterilized by autoclaving at 15 psi pressure (121°C) for 15 minutes.	

- **Gram's Crystal Violet**

Ingredients		Amount
Solution A	Crystal Violet	2.0 g
	Ethyl Alcohol, 95%	20.0 ml
Solution B	Ammonium Oxalate	0.8 g
	Distilled Water	80.0 ml
Solution A and Solution B were mixed and filtered into sterile reagent bottle.		

- **Gram's Iodine**

Ingredients	Amount
Iodine Crystals	1.0 g
Potassium Iodide	2.0 g
Distilled Water	300.0 ml
Solution was stored into a sterile brown reagent bottle at room temperature.	

- **Gram's Decolorizer**

Ingredients	Amount
Pure Ethyl Alcohol	95.0 ml
Distilled Water	5.0 ml

- **Gram's Safranin, 0.5% w/v**

Ingredients	Amount
Safranin O	0.5 g
Ethyl Alcohol, 95%	100.0 ml

Appendix B : Equipment and Apparatus

List of equipment and apparatus used are listed below:

Name	Manufacturer/Model	Origin
8-Channel Repeating Pipette	VistaLab Technologies, Ovation	USA
Autoclave	Sturdy, SA-300VF	Taiwan
Biological Safety Cabinet	LabTech, LCB-1803B-A2	Korea
Electric balance	METTLER TOLEDO, B154-S	Switzerland
GEN III Microplates	BiOLOG	USA
Hot Air Oven	Binder ED23	Germany
Incubator	DAIHAN Scientific, WIR-150	Korea
Microcentrifuge	Hettich, MIKRO 120	Germany
Micropipettes	Eppendorf Research Plus	Germany
Microscope	OLYMPUS, EX41	Japan
MicroStation Reader	BiOLOG Inc., ELx808BLG	USA
Nylon Syringe Filter	RanDisc	India
Paraffin wrapping film	Fisher Scientific, Parafilm M	UK
Plastic spreader	PRO-LAB Diagnostics	USA
pH meter	HACH, Sension3	USA
Refrigerator	Siemens, KG56NA01NE/04	Turkey
Shaking Incubator	GFL, 3031	Germany
Turbidimeter	BiOLOG, 21907	USA
UV-Vis Spectrophotometer	PG Instruments, T60	UK
Water distillation unit	GFL, 2004	Germany