Degradation of Dyes used in Textile Industries by Bacteria isolated from local textile effluents

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Submitted by
Banjir Sultana
Id no: 15176007
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Departments of Mathematics and Natural Sciences
BRAC University
Dhaka 1212, Bangladesh

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DECLARATION

I declare that the work in the thesis entitled **"Degradation of dyes used in textile industries by bacteria isolated from textile effluents"** has been performed by me under the joint-supervision of Professor Naiyyum Choudhury, former Coordinator of Biotechnology and Microbiology Programmes, MNS Department, BRAC University and Monzur Morshed Ahmed, Research Coordinator & Principal Scientific Officer, Industrial Microbiology Division, IFST, BCSIR in the Department of Industrial Microbiology Lab of the Institute of Food Sciences and Technology at the Bangladesh Council of Scientific and Industrial Research. This is further to certify that research work presented here is original and suitable for the submission for the partial fulfillment of the degree of Master of Science in Biotechnology, BRAC University, Dhaka. The information derived from the literature has been duly acknowledged in the text and list of references provided. No part of this thesis was previously presented for another degree or diploma at any university.

Banjir Sultana
Student ID: 15176007
Certified:

_____

Prof. Dr. Naiyyum Choudhury
Former Coordinator, Biotechnology
and Microbiology Program
Department of Mathematics and
Natural Sciences
BRAC University
Dhaka-1212, Bangladesh

_____

Monzur Morshed Ahmed
Research Coordinator
Bangladesh Council of Scientific and
Industrial Research &
Principal Scientific officer
Institute of Food Science &
Technology,(IFST) BCSIR
Dr. Qudrat-i-Khuda Road
Dhanmondi, Dhaka-1205 Bangladesh

DEDICATED

TO ALL THOSE WHO EVER RAISED THEIR HANDS TO PRAY FOR MY HAPPINESS AND SUCCESS

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Abstract

The effluent discharges from textile processing industries contain a significant amount of unreacted dyes. Release of these dyes into the environment causes toxic effects to the environment. The application of microorganisms for biodegradation of dyes is an attractive method for the wastewater treatments. In present study 10 different bacteria isolated from textile effluent were tested for decolorization ability of some common dyes like Novacron Ruby, Novacron Navy, Novacron Yellow Novacron, Novacron Blue dk and Novacron super black dyes. Based on morphological and biochemical characteristics, the isolates were provisionally identified as *Micrococcus sp, Bacillus sp, Pseudomonas sp and Enterococcus sp*. Out of 10 isolates 3 bacterial isolate found to posses significant decolorization ability and their identification was confirmed by Biolog identification system as *Bacillus badius, Bacillus thurengiensis and Enterococcus faecium*. The optimum condition for decolorization was observed at pH 7, temperature 37˚C, with 10% inocula size and 100mg/l dye concentration under stationary condition. All the 3 isolates showed maximum decolorization after 72 hrs of incubation. Beef extract and sucrose served as best nitrogen and carbon source respectively for the growth of these organisms. Under optimized condition percent decolorization by *Bacillus thurengiensis* of all Novacron dyes was 91.2-98.3%, *Bacillus badius* 89.2-96.4% and *Enterococcus faecium* 85.1-91.3%.Biodegradation of dyes was confirmed through UV-VIS Spectrophotometer. An experiment on growth of agriculturally important bacteria revealed non toxic nature of decolorized products (100 mg /L) as compared to original dye. Gel electrophoresis study showed the presence of low mol wt protein in the bacterium growing under dye stress condition supporting the mechanism of biodegradation.

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List of Abbreviations

˚C : Degree centigrade

hrs : Hours

etc :Etceteras

mg : Milligram

gm : Gram

l :Liter

µg : Microgram

µl : Milliliter

e.g. : Exempli gratia

OD : optical density

et al : And others

pH :Negative logarithm of hydrogen ion concentration

% : Percentage

UV : Ultraviolet

Fig : Figure

SDS : Sodium dodecyl sulphate

PAGE : Poly acrylamide gel electrophoresis

Mol.wt : Molecular weight

Rpm : Rotation per minute

Conc : concentration

λmax :absorption maxima

ppm :parts per million

1. Introduction

Textile industries in Bangladesh are playing an important role offering tremendous opportunities for the economy of Bangladesh. The garments sector accounts for 77% of country's foreign exchange earnings and 50% of its workforce. But unplanned growth of industries led to adverse environmental consequences in an alarming way. The liquid effluents from industries are causing major havoc to the environment, ecology, agriculture, aquaculture and public health since the development of textile industries in the country (Shuchismita Dey & Ashraful. 2015). Among the most concerned environmental pollutions that threatening our biodiversity, water pollution is a major source where effluents from dye based industries serve as principal source of pollution (Cripps C *et al*., 1990).

Dyes are compound that absorb light with wavelength in the visible range, i.e., 400 - 700nm (Van der Zee *et al*., 2003). These are composed of a group of atoms called chromophores which are responsible for the dye color and an electron withdrawing or donating substituents.

Which are referred as auxochromes that cause intensification of the color of the chromophores (Christie, 2001). The most important chromophores are azo (–N=N–), carbonyl (–C=O), nitro (–NO2) and quinoid groups.

Dyestuff can be classified according to chemical structures, physical properties and characteristics related to the application process (e.g., inks, disperse, pigments or vat dyes) but they are generally divided into anionic (including direct, acid and reactive dyes), cationic (basic dyes) and non-ionic (disperse dyes) (Aksu Z & Tezer S. 2015). Approximately, 10,000 different dyes and pigments are used industrially, and 60-70% of dyes are Azo dyes (Carliell *et al*., 1995). Azo dyes are characterized by the presence of one or more azo linkages and aromatic rings with simple application-technique requirements and high water fastness. Also reactive dyes are extensively used because of their favorable characteristics of bright color and low energy consumption during application (Wesenberg D *et al*., 2003)

Many dyes are visible in water at concentrations as low as 1mg l^{-1}. Textile- processing wastewaters, typically with dye content in the range 10-200mg l^{-1} (O'Neill *et al.*, 1999) are therefore usually highly colored and discharge in open waters presents an aesthetic problem. Now a days production rate as well usage ratio of azo dyes has increased dramatically according to consumer's demand. In effluent of textile industries amount of dye has increased to several folds. The main reason behind this is very low rate of fixation of these dyes in fabric dyeing processes. (Boer *et al*.,2004, Easton., 1995). Almost 10% of the dye is lost during dyeing process of fabric and thus comes in the environment through wastewater discharges (O'Neil *et al*., 1999, Khalid *et al.*, 2008 and Pearce *et al*., 2003) Textile industries consume over 7 x 10^5 tons of dyes annually and use up to 1 liter of water per kg of dye processed and thus contributing to one of the largest pollutants of the environment (Mutambanengwe *et al*., 2007).

Wastewater from the textile industries is a complex mixture of many polluting substances ranging from organochlorine-based pesticides to heavy metals associated with dyes or the dyeing processes (Correia, Stephenson and Judd, 1994). The chemical structures of dye molecules are designed to resist fading of textile on exposure to light or chemical attack and they prove to be quite resistant towards microbial degradation; moreover, synthetic dyes are often toxic, carcinogenic and mutagenic to various organisms, being detrimental to human health (Oliveira D.P *et al.*, 2007).

The majority of dyes pose a potential health hazard to all forms of life (Prakash and Solank, 1993). These dyes may cause allergic responses, skin dermatoses, eczema (Su and Horton, 1998), and may affect the liver (Jaskot and Costa, 1994), the lungs, the vasco-circulatory system, the immune system and the reproductive system (Nikulina *et al*., 1995) of experimental animals as well as humans. Dyes with azo bonds nitro- or amino-groups are carcinogenic, causing tumors of liver and urinary bladder in experimental animals (Dipple and Bigger, 1991).

Furthermore color of the dyestuff interrupts the aquatic environment by reducing light penetration, gas solubility and interference of phytoplankton photosynthesis .However, reduction of azo dyes, i.e. cleavage of the dye's azo linkage(s), leads to formation of aromatic amines and several aromatic amines are known mutagens and

carcinogens. In mammals, metabolic activation (reduction) of azo dyes is mainly due to bacterial activity in the anaerobic parts of the lower gastrointestinal tract. Various other organs, especially the liver and the kidneys, can, however, also reduce azo dyes (Zee, 2002). The toxicity of aromatic amines depends on the nature and location of other substituents. As most soluble commercial azo dyestuffs contain one or more sulphonate groups, insight in the potential dangers of sulphonated aromatic amines is particularly important. Sulphonated aromatic amines, in contrast to some of their unsulphonated analogues, have generally no or very low genotoxic and tumorigenic potentials (Jung *et al*., 1992).

The main problem in textile effluent treatment is the presence of dyes released into the effluent during dyeing stage. Textile wastewaters treatment may require the removal of over 99% of the color in samples and for this level of color removal to be maintained with large volumes of rapidly changing effluents. The frequently high volumetric rate, diversity in composition and recalcitrance of industrial effluents in combination with increasingly stringent legislation, make the search for appropriate treatments technologies an important priority (O'Neil *et al.,* 1999)

There are various physical and chemical methods for the treatment of textile wastewater for the removal of dyes such as, fentons reagent, ozonation, photochemical reaction, sodium hypochlorite , electrochemical destruction, activated carbon, silica gel, membrane filtration , ion-exchange , electro kinetic coagulation ,peat etc. Although these methods are used for effluent treatment ,these methods have many disadvantage like sludge generation, formation of by-products ,requires long retention time, not effective for all dyes, requires high concentrations of dissolved oxygen almost expensive, and applicable only in a limited number of cases (Robinson *et al.*,2002b).Many of these treatments allow the removal of contaminants from wastewater, but not their destruction, so dyes are then recovered and need to be disposed or destroyed by incineration. Moreover, some of these treatments require the use of hazardous chemicals compounds (bleach, ozone or peroxide) dangerous for human health and/or for the environment (Knapp J.S *et al.,* 1995).

Bioremediation for the treatment of industrial wastes

The term "Bioremediation" describes the process of contaminant degradation in the environment by biological methods using the metabolic potential of microorganisms to degrade a wide variety of organic compounds (Beydilli M.I *et al*., 1995). Bioremediation can be achieved using two different principles: bioaccumulation also named biosorption and biodegradation.

Biodegradation

The application of microorganisms for the biodegradation of synthetic dyes is an attractive and alternative method for the wastewater treatments. The use of microorganisms for the removal of synthetic dyes from industrial effluents offers considerable advantages. The process is relatively inexpensive; running costs are low and end products of complete mineralization are generally not toxic. Furthermore, it can deal with low concentration of contaminants where the cleanup by physical or chemical methods would not be feasible. As a result of the primary or secondary metabolic activities, a wide variety of microorganisms including bacteria, fungi algae, and yeast are capable of decolorizing different pollutants, comprising dyes (Manu and Chaudhari S.,2002),under different anaerobic or aerobic conditions.

Dye removal by bacterial treatment

Now-a-days several species of gram negative bacteria of different genus such as *Aeromonas, Escherichia, Citrobacter, Pseudomonas,* and *Sphingomonas* have been proved to be capable of decolorize dye solution or simulated effluents (Forgacs E *et al* .,2004) .Also, gram positive bacteria such as *Bacillus, Clostridium, Nocardia, Paenibacillus* and *Streptomyces, Micrococcus* etc have been found to degrade synthetic dyes.

Bacteria capable of dye decolorization, either in pure cultures or in consortia, have been reported Olukanni O.D *et al*., (2009), Alia N*et al*., (2009), Novotný C *et al*., (2010). It has been demonstrated that synergistic metabolic activities of mixed microbial consortium can lead to complete mineralization of azo dyes (Tony B.D *et al*., 2009)

The mechanism of microbial degradation of azo dyes involves the reductive cleavage of azo bonds (-N=N-) with the help of azoreductase under aerobic conditions resulting into the formation of colorless solutions (Chang S.W *et al*., 2001). For the reduction

of azo dyes, reduction to the anion radical occurs by a fast one-electron transfer reaction, followed by a second, slower electron transfer event to produce the stable dianion (Zimmermann *et al.,* 1982).Thus the functional group of azo dye with higher electronic density might be unfavorable to this second electron transfer to form the dianion, leading to low or no capability for decolorization. Due to this reason sulfonated reactive group of azo dyes are normally considered to be more recalcitrant than carboxylated azo dyes.

Present study focused on investigating the feasibility of biological treatment for degradation of azo dyes. The target dyes for this research project were of analytical grade.

Statements of Research problem

Environmental pollution arising from activities of chemicals in textile industries remains a burden in Bangladesh due to inappropriate treatment of effluent and lack of environmental awareness. In many areas like Narayangonj,Tongi,Gazipur ,Mymensingh,Norshindi,Chittagong the textile factories daily discharge million of liters of untreated effluents in the form of wastewater into public drains that eventually empty into rivers. Water pollution by textile effluents affects man and aquatic ecosystem directly and this is due to large variability of the composition of textile wastewaters and chemicals, thus the need for such a research on biodegradation.

Justification

The increasing demand of water and its dwindling supply has made the treatment and reuse of industrial effluents an attractive option. The ability of microorganisms to degrade and metabolize a wide variety of compounds has been recognized and exploited in various bio treatment processes (Khehra *et al.,* 2005) whereas other methods are not effective for azo dyes. .Microbiological treatment methods are attractive due to their cost effectiveness, and diverse metabolic pathways and versatility of microorganisms involved (Singh *et al*., 2004; Pandey *et al.*, 2007).

Aim and objectives include

1) Enrichment of microorganisms present in textile effluent that decolorize dyes (Novacron Ruby, Novacron Yellow, Novacron Orange, Novacron Navy, Novacron Blue Dk)

2) Isolation of bacterial strains from textile effluent and determine the bioremediation potentials of the selected bacterial strain and Identification of dye degrading isolates.

3) Study of feasibility of biodegradation of selected dyes with isolated microbial strains under optimized environmental conditions.

4) Study effect of co-metabolism on decolorization of selected azo dyes in presence of additional organic and inorganic carbon and nitrogen sources.

5) Partial analysis of azoreductase enzyme.

2. Literature Review

The first synthetic dye was discovered by William Henry Perkin, a student at the Royal College of Chemistry. In the beginning of the 20th century synthetic dyestuffs had almost completely supplanted natural dyes (Welham, 2000).The majority of industrial important azo dyes belong to the following classes: Acid dyes, Basic dyes, direct dyes, Disperse dyes, Mordant dyes, Reactive dyes and Solvent dyes. The Acid, Basic, Direct and Reactive azo dyes are ionic dyes (Anliker, Clarke and Moser, 1981).

Dyes are generally characterized in accordance with their ability to absorb the energy of a part of the electromagnetic radiation in which human eyes are sensitive (Rys and Zollinger, 1972).Dyes are mainly classified as anionic, cationic and nonionic (Fu and Virara, cx2001).The chromophores in ionic and nonionic dyes are mostly azo groups or anthraquinione types (Gupta and Suhas 2009). The majority of industrially important azo dyes belong to the following classes: Acid dyes, Basic dyes, Direct dyes, Disperse dyes, Mordant dyes, Reactive dyes and Solvent dyes. The Acid, Basic, Direct and Reactive azo dyes are ionic dyes (Anliker, Clarke and Moser, 1981).

Azo dyes are typically not removed from waste water by conventional waste-water treatment systems (Isik and Sponza, 2005) due to synthetic origin and complex molecular structures. Azo dyes representing sulphonated and unsulphonated compounds not only have a negative aesthetic effect on the wastewater but few of these compounds and their biodegradation products are toxic, carcinogenic and mutagenic (Chung and stevens.,1993).

2.1. Textile industries and effluent

The textile industry is characterized by the large quantity of water consumption, and the varieties of chemicals used Babu *et al.,* (2000). Liquid waste arising from various steps of operations contains substantial pollution load in terms of organic matter and suspended matter. Chemicals may also adhere to these suspended particles. Wastewater is generally hot and alkaline, with a strong smell and color due to the consumption of a variety of dyes and other chemicals in the dyeing processes (Robinson *et al*., 2001). Direct deposition of these effluents into sewage networks,

produce disturbances in biological treatment processes (Babu *et al*., 2000). On the other hand, these types of effluents produce high concentrations of inorganic salts, acids and bases in biological reactors leading to the increase of treatment costs

2.2. Color Removal Techniques

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

Table 01: advantages and disadvantages dye removal techniques

Physical/chemical method	Advantages	Disadvantages
Fentons reagent	Effective decoloration of soluble and insoluble dyes	Sludge generation
Ozonation	Applied in gaseous state, no alteration of volume	Short half life (20 min)
Photochemical	No sludge production	Formation of by-products
Sodium hypochlorite	Initiates and accelerate azo bond cleavage	Release of aromatic amines
Electrochemical destruction	Break-down compounds are non hazardous	High electricity consumption
Activated carbon	Good removal of wide variety of dyes	Very expensive
Peat	Good adsorbent due to cellular Structure	Specific surface area for adsorption are lower than activated carbon
Membrane filtration	Removal all types of dyes	Concentrated sludge production
Silica gel	Effective for basic dye removal	Side reactions prevent commercial application
Ion-exchange	No adsorbent loss due to regeneration	Not effective for all dyes

2.3. Biodegradation

In biodegradation, the original dye structure is destroyed and the pollutant is split into fragments by the microbes, sometimes complete mineralization is achieved, i.e., conversion of xenobiotic compounds into CO_2 ,H_2O and some salts of organic origin. Decolorization of the dye occurs when chromophoric center of the dye is cleaved Kaushik and Malik, (2009).

The rate and extent to which the compound is metabolized in the environment is often determined by the availability of electron acceptors and other nutrients (Chen, 2002). Moreover decolorisation and degradation can also detoxify the effluent effectively without leaving any residues.

In recent years, considerable interest has been generated in studying microbial azo dye degradation (Robinson *et al*., 2001)). Environmental biotechnology relies upon the pollutant degrading capability of naturally occurring microbial consortium in which bacteria plays a central role.

G. McMullan *et al.*, (2001) stated that Wastewater from the textile industry can contain a variety of polluting substances including dyes. Increasingly, environmental legislation is being imposed to control the release of dyes, in particular azo-based compounds, into the environment. The ability of microorganisms to decolorize and metabolize dyes has long been known, and the use of bioremediation based technologies for treating textile wastewater has attracted interest. They observed the ability of microorganisms to decolorize and metabolize dyes. They investigated the mechanisms by which diverse categories of microorganisms, such as the white-rot fungi and aerobic, anaerobic bacteria and bacterial consortia, bring about the degradation of dyestuffs.

2.4. Isolation and screening and identification

Maulin P Shah *et al.*,(2013) isolated, identified and screened a number of bacterial species capable of decolorizing a variety of dyes. They investigated decolorization of dyes and growth of the bacterial species. The strain ETL-1942 decolorized all the selected dyes except Remazol Brilliant Blue R, Reactive Blue H5G, Remazol Turquoise Blue G and Fast Green. In their study, they identified three groups of dyes on the basis of the pattern of dye decolorization by strain ETL-1942. The results

indicated that the strain could grow with all the six dyes tested, but decolorization rates were different for each dye.

PRASAD M.P., *et al* (2013) in their study isolated four microorganisms (one *Pseudomonas* sp. and three *Bacillus* sp.) and assayed their ability to degrade six textile dyes. Optimization of different condition such as pH, temprature and dye concentration for decolorization were carried out. Time for maximum dye degradation was 8 days. The results from present study shows practical application potential of these bacterial species in the biotransformation of various dye effluents that can help to solve the pollution problems caused by textile industries

Elisangel Franciscon *et al*., (2012)described the use of *Brevibacterium sp*. strain VN-15, isolated from an activated sludge process of a textile company, for the sequential decolorization and detoxification of the azo dyes Reactive Yellow 107 (RY107), Reactive Black 5 (RB5), Reactive Red 198 (RR198) and Direct Blue 71 (DB71). Tyrosinase activity was observed during the biotreatment process suggesting the role of this enzyme in the decolorization and degradation process, but no activity was observed for laccase and peroxidase.

Murty Srinivas D *et al*., (2012) subjected bacterial cultures for acclimatization to C.I. Reactive Blue 250 (RB 250), an azo dye in the Bushnell and Haas Broth (BHB). The most promising bacterial isolate was used for further dye degradation studies. The strain showed complete decolorization of the selected dye (RB 250-100 mg/L) within 8 hr in static condition. The optimum pH, temperature, inoculum size and carbon and nitrogen sources for the decolorization were studied at 7.0, 37°C, glucose (0.2 %) and nitrogen (0.5 %) respectively. The biodegradation was monitored by UV-Vis, HPTLC and FTIR analysis. Down Flow Fixed Film Batch Bioreactor was used and response up to 200 mg/L RB 250 dye concentration was obtained.

S.Barathi *et al*., (2012) in their study isolated a total of 40 bacterial isolates from textile effluent contaminated soil samples. Out of 40 bacterial isolates, six potential bacterial strains were chosen based on their decolorization efficiency. Among the six bacterial strains, T11 strain was found to decolorize maximum number of dyes after 120 h incubation at 300ppm concentration. Moreover, the selected bacterial strains were showed resistance to various heavy metals like Cr, Mn, Pb, Zn and Fe. Phytotoxicity test revealed the nontoxic nature of the degraded metabolites. Based on

the biochemical characterization and 16s rDNA gene sequencing analysis, the selected bacterial strain T11 was identified as *Bacillus firmus*

Shailesh R. Dave&Riddhi H. Dave (2009) isolated six morphologically distinct bacterial isolates resistant to 100 ppm AR-119 dye directly from the soil and waste contaminated with azo dyes. The most efficient isolate, which showed decolorisation zone of 44 mm on 100 ppm AR-119 containing plate, was identified as *Bacillus thuringiensis* SRDD. This organism was also able to remove more than 98%, 92%, 95% and 95% color of C.I. Acid brown 14, C.I. Acid black 210, C.I. Acid violet 90 and C.I. Acid yellow 42 azo dyes at 100 ppm concentration in 24 h, respectively.

Susan R *et al*., (2009) stated that several human intestinal microbiotas possess azoreductase activity which plays an important role in the toxicity and mutagenicity of these azo dye compounds. The acpD gene product (AzoEf1) responsible for the azoreductase activity of *Enterococcus faecium*, and azoreductase from *Enterococcus faecalis*, AzoA are intestinal bacterium, was heterologously expressed, purified and characterized AzoEf1 utilized both NADH and NADPH for the reduction of azo dyes, and it contains a leucyl residue at position 104 and threonyl residue at position 19 which differ from AzoA at the active site. Its specific activity was 5095 μM/min/ mg and its catalytic efficiency for Methyl red reduction was lower than AzoA.

Adya Das *et al.*, (2015) isolated bacterial strain identified as *Bacillus pumilus* HKG212 and studied for its potential to decolorize RNB dye. The isolated bacterial strain showed significant decolorizing capacity of more than 95% up to an initial dye concentration of 500 mg/L, within 48 hours and was efficiently tolerant up to a higher concentration of 1500 mg/L, under static condition. They investigated various physicochemical parameters to achieve maximum dye degradation by *Bacillus pumilus* HKG212. The optimal conditions for the decolorizing activity of *Bacillus pumilus* HKG212 were anaerobic culture environment with 10% inoculums and volume and beef extract as a nitrogen source, at pH 8.0, and 30℃. Biodegradation of RNB dye was confirmed through UV-VIS Spectrophotometer, HPLC and FTIR analysis.

2.5. Factors affecting Biodegradation of dyes

Rajeswari K.1 *et al.*, (2012) identified *Lysinibacillus sphaericus* RSV-1 based on biochemical and 16S ribosomal RNA gene sequence study from textile effluent. The strain was utilized for decolorization and degradation of various reactive dyes. Optimization of yeast extract concentration, pH, temperature, salinity and biomass was determined to be 0.5%, 7.0, 30˚C and 20% respectively. Effects of various nitrogen and carbon sources on decolorization of 100 ppm concentration of ten different mixed reactive dyes revealed, yeast extract, soya chunk powder, rice husk extract as best nutritional supplements with 95±0.47, 89±0.94 and 92±1.63 percent decolorization within 2-48 hrs of incubation.

Usha M. S. *et al*., (2011) undertook to study for the statistical optimization of medium components for improved Reactive Red 120 dye and Reactive Black 5 dye degradation by *P. aeruginosa and A.punctata*. Yeast extract, aeration and temperature were identified as significant components influencing Reactive Red 120 dye degradation whereas dye and pH were highly significant on Reactive Black 5 dye degradation. The main factors that had significant positive effects on both the dye degradation were glucose, yeast extract, aeration, inoculums, dye concentration, pH.

H. M. Abdullah Al Masud *et al.*,(2015), isolated five bacterial isolates with the capability of decolorizing textile dyes from textile effluent and identified these as *Bacillus thuringiensis* (Isolate A2, B6), *Bacillus badius* (Isolate B5, B9), *Bacillus aneurinolyticus* (Isolate C2) by different morphological, physiological and biochemical tests. The optimum temperature, pH and inoculum size for the decolorization of three experimental dyes (Novacron Orange FN-R, Novacron Red FN-R, Terasil Green) were found 30 to 35 °C, 7.0 to 8.0, and 10% (v/v) respectively. The present study suggests that the isolated *Bacillus* sp. can be utilized to treat reactive dyes containing waste water

Wynne and Stolz *et al*., (2001) noted that textile effluents are highly colored and saline, contain non-biodegradable compounds, and are high in (BOD, COD). A mixed culture combination of *P. aeruginosa, A. faecalis and P. putida* C15 was found capable of degrading all the dyes most efficiently compared to the other consortia (Omar *et al*., 2009). A temperature of about 29°C - 30°C and pH of 7.2 was reported

to be optimal for degradation and decolorisation of azo dyes(Robinson *et al.*,2001).Previous studies indicated that pH of 7.00 was most suitable for maximum decolorisation of dye effluent (Verma &Madamwar.2002).

2.6. Partial analysis of azorductase

Asha Lata Singh., *et al* (2015) isolated a bacterial strain from textile industry and identified it as *Enterobacter asburiae* by 16s rDNA sequencing.They observed decolorization potential of *Enterobacter asburiae*. Besides, the colorlessness of bacterial cells also indicated that *Enterobacter asburiae* had the capacity to decolorize the textile effluent through biodegradation instead of absorption on the surface. Gel electrophoresis study showed the presence of low mol wt (10.43kDa) protein in the bacterium under dye stress condition supporting the mechanism of biodegradation.

Usman Aftab *et al*., (2011) assessed the ability of *Corynebacterium sp* to decolorize dyes into non-toxic form. Corynebacterium sp could tolerate Reactive Black5 and Yellow15 up to 100mg/l.The maximum growth of bacterium was observed at 37˚C and pH 7. *Corynebacterium sp*. showed high azoreductase activity against Reactive Black5 (68%) and Yellow15 (80%).The presence of extra bands in TLC in decolorized samples indicated the ability of the bacterium to degrade dyes enzymatically and possibilioty of bioremediation of azo dyes into non-toxic product form.

Amar Telke *et al* .,(2008) isolated *Rhizobium radiobacter* MTCC 8161 which was capable of decolorizing various azo, triphenylmethane (TPM), disperse and reactive textile dyes with decolorizing efficiency varying from 80–95%. The induction of various oxidative and reductive enzymes indicated involvement of these enzymes in color removal. Phytotoxicity studies revealed less toxic nature of decolorized products (1000 mg /L) as compared to original dye. FTIR spectroscopy and GC–MS analysis indicated naphthalene diazonium, p-dinitrobenzene and 2- nitroso naphthol as the final products of Reactive Red 141.

Suizhou Ren *et al*., (2006) isolated a broad-spectrum dye-decolorizing bacterium; strain DN322, from activated sludge of a textile printing wastewater treatment plant. The strain was characterized and identified as a member of *Aeromonas hydrophila*.

Decolorization rate was more than 90% within 10 h under aerobic culture condition and Crystal Violet could be used as sole carbon source and energy source for cell growth. The color removal of triphenylmethane dyes was due to a soluble cytosolic enzyme, and the enzyme was an NADH/NADPH-dependent oxygenase; for azo and anthraquinone dyes.

2.7. Environmental concern

O'Neill *et al* .,(1999) stated that dyes are visible in water at concentrations as low as 1 mg /l.Textile processing wastewaters, typically with dye content in the range 10-200 mg /l. are therefore usually highly colored and discharge into open waters presents an aesthetic problem. As dyes are designed to be chemically and photolytically stable, they are highly persistent in natural environments.

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

Maulin P Shahe *et al*., (2013) isolated a novel bacterial strain capable of decolorizing triarylmethane dyes from a textile wastewater treatment plant in Ankleshwar, Gujarat, India. The bacterial isolate was identified as *Pseudomonas* aeruginosa ETL-1 and was shown to decolorize three triarylmethane dyes (50 mg/l) tested within 24 h with color removal in the range of 72% to 96%.Optimization of different decolorization condition. Phytotoxicity studies carried out using Triticum aestivum, and Lens esculenta revealed that the triarylmethane dyes exerted toxic effects on plant growth parameters monitored whereas decolorized dye was less toxic.

3. Materials and Methods

3.1. Design of experiment

This research involved sampling of different areas in Bangladesh. Some physicochemical analyses were carried out at the sites before bioremediation. However, Isolation, characterization of selected bacterial isolates, decolorisation potential of bacterial isolates and characterization of azoreductase was carried out at Industrial Microbiology laboratory, Institute of Food Science and Technology (IFST), Bangladesh Council of Scientific and Industrial Research (BCSIR), Dr. Kudrat-I-Khuda Road, Dhanmondi, Dhaka-1205.

3.2. Sample collection

This research involved sampling from Arty dyeining composite and Consumer nitex; Bhaluka, Mymensingh, South East Textile; Narayangonj, Mega Yarn Dyening and Zaber and Jubayer Textile; Tongi. Samples were in the form of liquid untreated effluent. All the samples were collected in sterile glass-screw cap tubes and preserved at 4˚C in refrigerator.

3.3. Physio-chemical characteristics of effluent samples

The effluent samples, mainly before treatment were tested for its physio-chemical Characteristics like, Color, Odor, pH, temperature.

3.4. Materials

- Dyes: Novacron Ruby, Novacron Brilliant blue, Novacron Yellow, Novacron Super black, Novacron Navy, Novacron Brilliant Yellow, Novacron Turquoise, Novacron Blue DK .All of the dyes were analytical grade and was collected from Swiss Colors Ltd. Bangladesh.
- Chemicals: Tetramethyl-p-phenylenediamine dihydrochloride, 3% hydrogen peroxide,Kovacs reagent, Methyl red, Barritts reagent A and B, Iodine solution, 1N HCl, (NH4)2SO4, Ethanol,2-mercaptoethanol
- Medium: Nutrient Broth, Nutrient Agar, Tryptophan broth, MR-VP broth, Urease broth, Simmons Citrate Agar, Cetrimide Agar, MacConkey Agar, Urea broth, Triple Sugar Iron Agar(TSIA),Nutrient

gelatin medium, Starch Agar medium, Egg Yolk Agar, Carbohydrate Fermentation Broth, PEMBA, DNAase Agar.

3.5. Enrichment and isolation of dye decolorizing bacteria

Total 15 samples were used for isolation of dye decolorizing bacterial cultures by enrichment culture techniques using nutrient broth supplemented with individual dyes a concentration of 100mg/l. The enrichment was carried out in 90 ml nutrient broth medium in 250 ml Erlenmeyer flask by adding 10ml of dye effluent. The culture flasks were incubated on orbital shaker with 120 rpm, 30˚C for 72 hrs. After 72 hrs of incubation (i) 1ml of sample from decolorized flask was (modified H.M.abdullah Al Masud method) serially diluted up to 10^{-7} dilution and plated on the agar medium containing dye and incubate at 37˚C for 24 hrs. (ii) The colony morphology was noted after incubation at 37˚ C for a period of 24 hour. Subculturing the individual colonies to obtain pure culture .The pure cultures of individual bacterial strains were maintained by streaking on nutrient agar slant and stored at 4˚C.

3.6. Secondary Screening of dye decolorizing strains

All of the 32 colonies isolated by enrichment technique were individually inoculated into 10 ml of the Nutrient broth medium with respective dyes with a concentration of 100 mg /l (Novacron Ruby, Novacron Yellow, Novacron super black, Novacron Turquoise, Novacron blue dk, Novacron navy, Novacron Brilliant blue, Novacron Brilliant Yellow).The medium was sterilized through autoclaving. These tubes were inoculated with 500 micro liters of selected organisms and these were incubated on orbital shaker with 120 rpm, 37˚C for 72 hrs and the color change was noted at regular intervals. The organisms that show maximum decolorization within 72 hrs were selected and used for further biodegradation studies. Of the thirty morphologically different strains isolated from the effluent, only ten were found to possess more than 50% decolourization of the respective dye within 48 hrs. These organisms were selected and were maintained for further studies. The selected bacteria were purified by repeated streaking on NA and were stored at -20^{o}C in ependrop containing 20% glycerol.

3.7. Identification of selected bacterial strains

The identification of dye degrading bacterial strains was carried out based on morphological characteristics, Gram staining characteristics, biochemical characteristics and Biolog^{TM} identification system.

3.7.1 Colony characteristics

Colony characteristics of selected bacterial isolates were carried out by streaking of single colony on NA plate and incubate at 37˚C for 24 hrs.The cell shape, size, color, arrangement and other colony characteristics of the isolated bacteria were studied after 24 hrs of incubation and were tabulated.

3.7.2 Staining

The morphological characterizations of the isolates were checked by Gram's reaction. 24 hr old cultures of all of the isolates were used to study Gram reaction. At first smear was prepared from colony and heat the fix the smear. Crystal violet was added on the smear, left for 1 minute and rinsed with water. Then iodine was added and kept for 1 minute, wash with distilled water. After this alcohol was added to remove the excessesive color of primary stain. Thoroughly washed with alcohol until color removal occurs. Then saffranine was added and wait for 30 seconds. Finally wash with water and air dried the slide. Observe microscope under oil immersion lense. Gram positive cells will purple where Gram negative cells stain pink or red.

3.7.3. Growth on different selective media

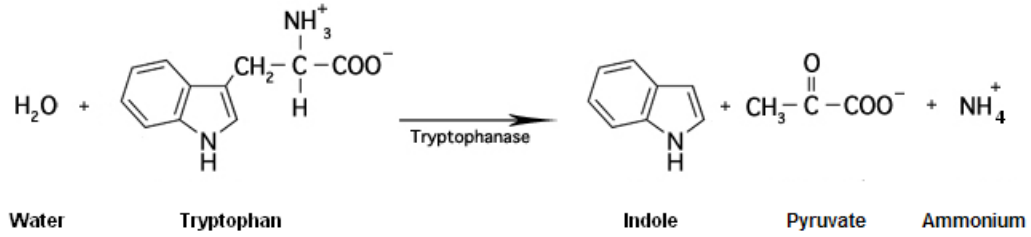
Isolated colonies were grown on different selective media namely Certimide agar, PEMBA, MacConkeys agar and Blood agar. Colonies from pure culture were streaked on to the plate and incubate at 37˚C for 24 hrs. After incubation observe the growth of the colony.

3.7.4 Biochemical Reactions

All required media for biochemical tests were prepared in respective test tubes, flasks and Petri dishes. All biochemical tests were performed with specific requirements for each test as outlined in Bergey's Manual of Determinative Bacteriology ((1989)).

a) Indole Test

Tryptophan is an essential amino acid, which is oxidized by some bacteria resulting in the formation of indole, pyrivic acid and ammonia. The organisms were inoculated and grown in 10 ml of sterilized Tryptophan broth dispersed in test tubes and incubate the tubes at 37^0C for 24hours. *E.coli* ATCC 8739 used as positive control and lab isolates *Pseudomonas* used as negative control. After incubation, add 1 ml of Kovac's reagent to all the tubes. The appearance of cherry red color ring in the surface layer indicated a positive reaction. (VASHIST, *et al*)



b) Methyl Red (MR) Test

The Methyl-Red tests for the ability to perform mixed-acid fermentation. MR-VP broth contains glucose, peptone, and a phosphate buffer. Organisms that perform mixed-acid fermentation produce enough acid to overcome the buffering capacity of the broth, so a decrease in pH results. Organisms that perform other kinds of fermentation cannot overcome the buffering capacity of the broth (H. T. Clarke and W. R. Kirner, 1941).Isolates were inoculated to 10 ml of sterilized MR-VP broth in the test Tubes and incubated for 24 hours at 37^0C. Add 5 drops of Methyl Red indicator was added into each inoculated as well as uninoculated tubes. Appearance of bright red color indicated a positive test.

Glucose+H_2O ⟶ Acids (lactic, acetic, formic acid) CO2+H2 (pH4.4)

↓

Add methyl red indicator

↓

Red color due to high acid conc.

c) Voges-Proskauer Test

VP is a test used to detect acetoin in a bacterial broth culture. The test depends on the digestion of glucose to acetylmethylcarbinol. If glucose is being broken down, it will react with alpha-naphthol (VP reagent #1) and potassium hydroxide (VP reagent #2) to form a red color. Tubes with 10 ml of MR-VP broth were inoculated with each microorganism and 1 ml of Barrit's reagent was added to the tubes. The mixture was shaken vigorously for few minutes and allowed to stand for two hours and positive reaction was given by the appearance of deep rose/ crimson ruby color.

d) Citrate Utilization Test

Citrate test is used to differentiate among enteric bacteria on the basis of their ability to utilize as their soul carbon source. The utilization of citrate depends upon the presence of enzyme "Citrate Permease" produced by organism that helps its transports into the cell. Prepare the citrate slant according to the given composition and dispensed into the test tube and autoclaved at 121˚C for 15lbs.inocciulate the tubes with the given culture and then inoculate at 37^{o}C for 48 hours. Observe the tubes for color change from green to blue.

e) H_2S Production Test

The test is done to show the presence of hydrogen sulphide by sulphide reducing bacteria. H_2S is formed by some bacteria by reduction of sulphure containing amino acids (Cystein), Cysteine and metheonine or through reduction of inorganic sulphure compounds like thiosulphates (S_2O_3-) or sulphates (SO_4 --) or sulphite (SO3--).

TSI agar slant differentiate bacteria on their ability to reduce sulfur to hydrogen sulfide and also on their ability to ferment glucose, lactose and/or sucrose. Prepare the triple sugar iron agar slant. An inoculum from pure culture is transferred aseptically after solidification of the medium and incubates the tubes at 37˚C for 24 hrs and observed for black coloration at the point of stab and gas production (Barton *et al*., 2009).

f) Gelatin Hydrolysis Test

The test was performed to determine capability of bacterial colonies to produce gelatinase enzyme as use gelatin as media source. Degradation of gelatin indicates the presence of gelatinase enzyme (Aneja K. R *et al*., 2003).Prepare the media according

to the given composition and sterilize after pouring into the test tubes. Inoculate the test organism into the sterilized test tubes and left one uninocculated as control. Incubate the tube at 37˚C for 24-48 hrs. After incubation kept the tubes in an ice bath for 30 min. Note down the results depending on the condition whether gelatin is in the liquid state or in the solid form. Liquid form indicate positive result

g) Starch Reduction Test

This test is performed to test the utilization of starch by Bacteria by producing the enzyme Amylase. (De Oliveria, 2007).Amylase is an exoenzyme that hydrolysis starch, a polysaccharides into monosaccharide and disaccharides such as glucose. These mono and disaccharide enters into cell cytoplasm of bacteria through the semi-permeable membrane and their by attacked by endoenzymes. Sterile starch agar medium is poured on to the sterile petriplates and allowed to solidify. The test organism is streaked on the plate and incubated for 48 hrs at 37˚C. The plates are flooded with gram's iodine and excess iodine is drained off. Plates are examined for the zone of clearance around the growth for each organism. Lab isolates *Bacillus subtilis* kept as positive control.

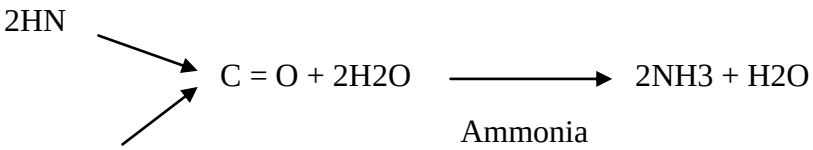
h) Catalase Test

Catalase is a ubiquitous antioxidant enzyme that degrades hydrogen peroxide into water and oxygen. Several pathogens produce catalase in order to defend themselves against attacks by hydrogen peroxide, a weapon commonly used by the host's immune system, in addition to oxidative stress. A few drops of H_2O_2 were kept on a clean glass slide. A loopful of 24 hrs grown colonies of isolated bacterial culture was placed in to the drop and the observations were noted. Bubble production indicate the positive result (Loewen, 1985)

$$2H_2O_2 \xrightarrow{\text{Catalase Enzyme}} 2H_2O_2+O_2$$

i) Urease Test

The test is done if the given organism produces enzyme Urease or not. Urea is a major organic waste production of protein digestion by most vertebrates and is excreted in urine. Some microorganisms have the ability to produce the enzyme urease. Urease is a hydrolytic enzyme, which attack the amide linkage liberating Ammonia.



Prepare and dispense Urea broth (basal medium) into tubes and sterilized. Glucose and phenol red is added to the basal medium and steamed for 1 hr.Add filtered sterilized urea solution and mix all contents well and dispense into sterile test tubes. The test organisms are inoculated and incubated at 37˚C for 24-48 hrs. Colour change is observed.

j) DNase test

DNase Test Agar is used for the differentiation of microorganisms on the basis of deoxyribonuclease activity. DNase test was performed by incubating the isolates for 24 hours at 37˚C on DNase agar, and pouring (~15 ml) of 1 N HCl. Excess acid was removed with a vacuum pipette and clear zones around the bacterial colonies indicated DNase positive colonies.

k) Oxidase Test

Oxidase positive bacteria possess cytochrome oxidase or indophenol oxidase (an iron containing haemoprotein). Both of these catalyse the transport of electrons from donor compounds (NADH) to electron acceptors (usually oxygen). The test reagent, N, N, N', N'-tetramethyl-p-phenylenediamine dihydrochloride acts as an artificial electron acceptor for the enzyme oxidase. The oxidised reagent forms the coloured compound indophenol blue.

Add 2 -3 drops of reagent directly to suspect colonies on an agar plate. Observe for color change within 10 seconds. (Lippincott Wilkins and Williams)

L) Egg yolk test

Lecithinase test is a biochemical test used to identify organisms which liberate phospholipase (lecithinase). Bacterial lecithinase breaks down this lecithin (a normal component of egg yolk) to insoluble diglycerides resulting an opaque halo, surrounding the colony when grown on the egg yolk agar medium

Basal egg yolk media is prepared by dissolving the standard amount in distilled water in which 10% egg yolk is added after autoclaving and cooling the media before dispensing into petridish. Using a direct inoculum from an appropriate organism, perform a four-quadrant streak to well-isolated colonies .incubate anaerobic ally at 35^{o}C. Examine after 48-72 hours. Plates should be kept up to 7 days before regarding them as lipase negative. (Collins and Lyne)

M) Carbohydrate fermentation test

The test was performed to determine capability of bacterial isolate to use as carbohydrates. The fermentation medium was prepared and sterilized with the indicator and Durham's tube has no air bubbles in them. The sugar solution is autoclaved at 10 lbs/Sq inch pressure for 10 minutes and 0.5 ml of the sugar is added to sterile peptone water. The fermentation tubes are inoculated with the test organism. Negative control is maintained for all the sugars. The tubes are inoculated at 37^{o}c for 24 hrs. After incubation to determine capability of bacterial isolates to acid and gas production. Total 10 sugars were used to observe fermentation ability.

3.7.5. Identification by Biolog^{TM} system

Biolog is a latest generation redox chemistry enables testing and identification of Aerobic Gram-negative and Gram-positive bacteria in the same test panel. Gram Stain and other pre-tests are no longer needed. A simple, one minute setup protocol is used for each sample, expanded GENIII database is designed to meet the needs. Biolog's powerful carbon source utilization technology accurately identifies environmental and pathogenic microorganisms by producing a characteristic pattern or "metabolic fingerprint" from discrete test reactions performed within a 96 well microplate. Culture suspensions are tested with a panel of pre-selected assays, then incubated, read and compared to extensive databases of environmental organisms, human pathogens, veterinary pathogens and plant pathogens. The scope of the 96 assay reactions, coupled with sophisticated interpretation software, delivers a high level of accuracy that is comparable to molecular methods simpler and faster than DNA sequencing.

Procedure

Before starting, pre-warm Microplates and IF to room temperature.

1. Culture Organism on Biolog Recommended Agar Media

2. Single colony was taken after 24 hrs; Check the calibration of the turbidimeter (The target cell density must to be in the range of 90-98%T).Prepare the inoculum at the desired turbidity.

3. Inoculate MicroPlate: Pour the cell suspension into the multichannel pipette reservoir. 8 sterile tips securely onto the 8-Channel Repeating Pipett or and fill the tips by drawing up the cell suspension from the reservoir. Fill all wells with precisely 100 µl. Be careful not to carry over chemicals or splash from one well into another. The inoculating fluid will form a soft gel shortly after inoculation. Cover the MicroPlate with its lid.

4. Incubate MicroPlate: Place the MicroPlate into an incubator, for 3 to 36 hours. Incubate at 33° C.

5. Observe and enter the reaction pattern to obtain ID result

3.8. Determination of decolorization efficiency

Ten different colonies those showed better decolorization were finally tested to observe decolorization percentage. Decolorization activity was expressed in terms of percentage decolorization and was determined by monitoring the decrease in absorbance at absorption maxima (λmax). (590 were used). The uninoculated NB supplemented with respective dye was used as reference. The culture suspension was centrifuged at 10,000 rpm for 10 min at 4˚C for removal of the biomass. The degree of decolorization of the tested dye was measured at its maximum absorbance wavelength using supernatant by UV-visible spectrophotometer. The decolorization assay was calculated according to the following formula.

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

Where, A = initial absorbance

B = Observed absorbance

3.9 .Effect of environmental and nutritional conditions for dye decolorization

3 bacterial isolates namely *Enterococcus faecium, Bacillus pumilus and Bacillus thurengiensis* were identified by microscopic examination, growth on selective media, biochemical properties, and Biolog^{TM} identifying software was used to observe influence of different physiochemical condition and nutritional requirement for growth and decolorization of three different dyes namely Novacron Ruby, Novacron Black, and Novacron Blue dk.

3.9.1: Influence of Physiochemical parameters on the process of dye Decolorization

Effect of incubation condition on dye decolorization

To study the effect of incubation condition on decolorization,100 ml of NB medium containing individual dyes (100 mg/l) was inoculated with bacteria and incubated under two culture conditions, namely, static (no shaking), shaking. To maintain shaking condition, culture tubes were kept in rotary shaker running at 120rpm/min. In order to maintain static condition, culture tubes were placed in the incubator at 37˚C and keep for 72 hrs. After72 hrs of incubation at 37^{o}C, aliquot of decolorized medium was centrifuged at 10,000 rpm for 10 min to separate the bacterial cell mass. The clear supernatant was used to measure the decolorization at the absorbance maxima at 590 nm.

Dye Decolorization in respect to time

Nutrient broth containing 100 mg/L of Novacron dyes was prepared and transferred to 100 ml culture bottles. 5% v/v of 24 hrs old microbial cultures was then inoculated to every bottle and incubated under static condition at 37˚C. Aliquot of culture media from respective vials were withdrawn at different time intervals and centrifuged at 10,000rpm for 10 min. Extent of decolorization was monitored by measuring the absorbance of the clear supernatant.

Dye Decolorization at different dye concentration

To study the effect of initial dye concentration on the rate of decolorization 5 % v/v of 24 hrs old microbial cultures was inoculated to NB containing different concentrations of Novacron dyes respectively (100-300 mg/L) and incubate for 72 hrs at 37˚C, aliquot of decolorized medium was taken after 72 hrs and centrifuged at 10,000 rpm for 10 min to separate the bacterial cell mass. The clear supernatant was used to measure the decolorization at the absorbance maxima of the 590 nm

Effect of pH on dye decolorization

To study the effect of pH on decolorization capacity of the isolated strain, pH of the sterile nutrient medium was adjusted to 5, 7, 9, before dye addition. After pH adjustment, sterilized dye medium through autoclaving was added and the medium were inoculated with 5% v/v of inoculum. After 72 hrs of incubation at 37°C under static condition, extent of decolorization was measured as mentioned earlier.

Effect of temperature on dye decolorization

Effect of incubation temperature on decolorization capacity of the isolated bacteria was studied by inoculating dye (100 mg/L) containing nutrient medium with 5% v/v of inoculum and incubating them under different temperature (25°C, 30°C, 37°C, 44°C) in static condition. The extent of decolorization was measured as earlier.

Effect of Inoculum volume on dye decolorization

Sterile dye (100 mg/L) containing nutrient medium were inoculated with varying volume of inoculum (2%, 5%, 10%, 15%, 20%) to study the effect of inoculum volume on decolorization capacity of the bacteria. Dye concentration of the medium was 100 mg/l, ph 7, and incubates in incubator for 72 hrs.

3.9.2. Effect of Carbon and Nitrogen source on dye decolorization

Study of effect of different carbon and nitrogen source on decolorization capacity of the isolated sp namely *Bacillus thurengiensis* was done with NB medium containing 100 mg/L of Novacron Blue dk dye. Different Carbon sources like glucose, sucrose, and mannose were added individually to the culture medium at a concentration of 1g/L. Similarly different Nitrogen sources (Ammonium Sulfate, Peptone, Yeast Extract and Beef Extract) were added to the medium individually and in combination

with carbon source at a concentration of 0.5g/L to study the effect on decolorization process. The medium was inoculated with 5% v/v 24 hrs old cultures incubated in static condition for 72 hrs

3.10. Effect of bacterial consortium on mixed dye

Those colonies that showed better decolorization potential were used for optimization of different nutritional and environmental parameters namely *Enterococcus faecium, Bacillus pumilus and Bacillus thurengiensis* were used to make consortium and checked decolorization potential on mixed dye. Absorbance was taken different time of intervals. Consortia were:

Consortium-1: *Bacillus thurengiensis, Bacillus pumilus, Enterococcus faecium*
Consortium-2: *Bacillus thurengiensis, Bacillus pumilus*
Consortium- 3: *Bacillus thurengiensis, Enterococcus faecium*
Consortium- *4: Bacillus pumilus, Enterococcus faecium*

5%volume of bacterial suspension was added from respective consortium and added to the medium containing dye and incubate for 48 hrs.

3.11. Partial analysis of azo reductase enzyme

3.11.1 Protein extraction:

1.5 ml of decolorized broth was taken in eppendrop

Centrifuge at 10000 rpm for 15 minutes

Then pellets were introduced in 1M solution of sodium phosphate buffer (ph 7.0)

Pelleted bacterial cell mass was reintroduced into same buffer and centrifuge

This process repeats five times

The disruption of bacterial cell was carried out with the help of probe sonicator (10mins at 60 HZ)

The homogenate thus obtained was centrifuged at 10000g for 30 minutes and the supernatant was used for gel electrophoresis

3.11.2. SDS-PAGE analysis

For 5 ml stacking gel

Composition	Amount
H2O	2.975ml
0.5 M Tris-HCl,pH6.8	1.25ml
10% SDS	0.05ml
30% Bis-acrylamide solution(w/v)	0.67ml
10% ammonium per sulfate	0.05ml
TEMED	0.005ml

For 10ml separating gel

Acrylamide percentage	6%	8%	10%	12%	15%
H2O	5.2ml	4.6ml	3.7ml	3.2ml	2.2ml
Bis-acrylamide solution(w/v)	2ml	2.6ml	3.4ml	4ml	5ml
10% SDS(w/v)	0.1ml	0.1ml	0.1ml	0.1ml	0.1ml
1.5M Tris(pH=8.8)	2.6ml	2.6ml	2.6ml	2.6ml	2.6ml
10% ammonium per sulfate(w/v)	100µl	100µl	100µl	100µl	100µl
TEMED	10 µl	10 µl	10 µl	10 µl	10 µl

Sample preparation

- The protein sample (10 µl) was mixed with sample loading buffer at a ratio of 1:1 and was then boiled in water bath for 3 mins.

- During boiling precaution was taken so that the sample does not bump up. Then 5 µl tracing dye (0.1% Bromophenol blue) was added to the boiled mixture.

1. Pour Polyacrylamide Gel

- Assemble glass plate sandwich
- Pour separating solution into gel sandwich using a Pasteur Allow gel to polymerize 30-60 minutes at room temp or until interface appears.
- Pour stacking gel solution on top of separating gel
- Insert comb into stacking gel taking care to avoid forming bubbles on the ends of the teeth
- Allow gel to polymerize 15-30 minutes or until ready

2. Loading sample:

- Comb was removed from the gel then 20 µl of prepared sample pipette into the well .Marker was added in a well.

3. Clamp gel onto electrophoresis tank

- Carefully remove binding clips and the comb from gel
- Place gel / glass plate sandwich into electrophoresis core.
- Place the core assembly into the running tank.
- Add 1X Electrophoresis buffer to the core. Buffer should be added to the top of the assembly.

4. Running gel

After setting into tank gel was run at 80V, 100mA and 20W until dye front reaches the bottom of the gel.

5. Staining of gel to visualize protein bands

- Invert plate with gel into the staining solution and gently allow gel to "float" off of plate into solution

- Cover with plastic wrap and gently agitate gel on gel rocker for 3hrs minutes (longer will increase sensitivity but requires a longer destaining period)

6. Destaining

Remove staining solution and rinse gel with ddH2O to remove excess stain. Add destaining solution and agitate on gel rocker for overnight

3.12. Decolorization through crude extract of isolates

Crude extract obtained from isolates namely *Enterococcus faecium, Bacillus pumilus and Bacillus thurengiensis* .Then 500 µl lysate of each of each isolates added into 10ml of medium containing dye then incubate at 37˚C.Dye medium without crude extract kept as control. After 24 hrs measured the OD of the medium.

3.13. Effect of decolorized effluent on beneficial bacteria

The biodegraded products of isolates were tested for their toxic effect on agriculturally important soil bacterial flora. *Bacillus cereus* was inoculated into nutrient agar 3 wells were made in the respective media containing plate and filled with each decolorized broth. Plates were incubated 37˚C for 24 hrs. Zone of inhibition surround the well represented index of toxicity. Untreated dye medium kept as control.

4. Results

4.1. Physio-chemical characterization of collected samples

All of 15 samples were in liquid form, normal to unpleasant odor and acidic to basic pH. Table 02 represents Physio-chemical characterization of collected samples.

Table 02: Characteristics of samples collected from different industries.

SL.	Nature of sample	Color	Odor	Temperature	pH
Sample 1	Liquid	Dark blue	Unpleasant	30	7.6
Sample 2	Liquid	Greenish	Normal	26	8.2
Sample 3	Liquid	Light yellowish	Normal	30	7.9
Sample 4	Liquid	Light yellowish	Unpleasant	24	8.6
Sample 5	Liquid	Reddish	Normal	33	7.8
Sample 6	Liquid	Blackish	Pungent	28	9.2
Sample 7	Liquid	Light green	Normal	30	7.5
Sample 8	Liquid	Light brown	Unpleasant	28	7.6
Sample 9	Liquid	Bluish	Unpleasant	31	8.2
Sample 10	Liquid	Reddish	Normal	26	7.5
Sample 11	Liquid	Dark blue	Normal	30	9.4
Sample 12	Liquid	Blackish	Pungent	30	8.6
Sample 13	Liquid	Light green	Normal	28	7.6
Sample 14	Liquid	Light green	Normal	31	7.8
Sample 15	Liquid	Blackish	Unpleasant	28	7.4

4.2. Isolation and screening of bacterial isolates for decolorization of dye samples

Enrichment of liquid effluent sample collected from waste disposal site of different textile industries have decolorization ability led to the isolation of 32 morphologically distinct isolates. To observe the ability of decolorization of dyes (Novacron Ruby, Novacron Yellow, and Novacron super black, and Novacron blue dk Novacron navy) all the isolates were tested individually at concentration of 100 mg/l of dyes. Out of 32 colonies only 10 different colonies showed better decolorization efficiency of all five dyes.

4.3.1. Morphological characteristics of 10 isolates

Isolates those showed better decolorization potential were further grown on Nutrient agar plate and showed different pigmentation like white, dirty white, yellow orange etc. Size of the colonies varies from pin head, small to large. Texture of the colony was Shiny and Shiny smooth form. Gram staining result indicated that out10 isolates 6 were Gram positive Rods, 2 were Gram positive Cocci and 2 were Gram negative small rods (table 03).

Table 03: Colony and staining characteristics of isolates

Isolates	**Size**	**Shape**	**Margin**	**Elevation**	**Surface Texture**	**Opacity**	**Pigmentation**	**Gram staining**	**Shape (microscopic)**	Size**(micros copic)**
C-1	Pinhead	Ovoid	Entire	Convex	Shiny, smooth	Translucent	Yellow-Orange	Gm+ve	Ovoid	Small
C-2	Small	Irregular	Wavy	Raised	Shiny, smooth	Opaque	Absent	Gm +ve	Rod	Small
C-3	Large	Irregular	Curled	Flat	Smooth	Opaque	Absent	Gm+ve	Rod	long
C-4	Large	Irregular	Curled	Flat	Smooth	Opaque	Absent	Gm+ve	Rod	long
C-6	Pinhead	Round	Entire	Raised	Smooth	Translucent	Absent	Gm+ve	Round	Small
C-7	Large	Irregular	Undulated	Flat	smooth	Opaque	Absent	Gm+ve	Rod	long
C-9	Large	Irregular	Undulated	Flat	smooth	Opaque	Absent	Gm-ve	Rod	Medium
C-11	Pinhead	Circular	Entire	Convex	Shiny, smooth	Translucent	Yellow-orange	Gm-ve	Rod	Small
C-12	Small	Irregular	Curled	Flat	Smooth	Opaque	Absent	Gm+ve	Rod	Long
C-13	Small	Irregular	Curled	Flat	Smooth	Opaque	Absent	Gm+ve	Rod	Long

4.3.5: Growth on selective media:

All bacterial colonies were grown different selective media for their selectivity.

Out of 10 isolates C-1 and C-11 was grown on Cetrimide agar and C-3, C-4, C-7, C-12 and C-13 were grown on PEMBA (Polymyxin Pyruvate Egg Yolk Mannitol Bromothymol Blue Agar Base). C-2 and C-6 was grown on MacConkeys agar while no colonies showed hemolysis on Blood agar media.

4.3.5. Biochemical characteristics:

For further identification of bacterial isolates Oxidase,Catalase, Indole, MR,VP, Citrate Utilization Test, H_2S Production Test, Gelatin liquefaction Test, Starch Reduction test, Urease Test, DNase test, Egg yolk test were conducted for all of 10 isolates. Table 04 showed the findings of the biochemical test result. Fig: 01 also showed biochemical test result.

Table 04: Biochemical characteristics of isolates

	C-1	**C-2**	**C-3**	**C-4**	**C-6**	**C-7**	**C-9**	**C-11**	**C-12**	**C-13**
Oxidase	+	+	-	+	-	+	+	+	-	+
Catalase	+	+	+	+	-	+	+	+	+	+
MR	-	+	+	+	+	-	+	-	+	+
VP	-	-	-	+	+	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-	-	-
H_2S	-	-	+	-	-	-	-	-	-	-
Citrate utilization	-	-	-	-	-	-	-	+	-	-
Urease	-	-	-	-	-	-	-	-	-	-
Gelatin liquefaction	-	-	+	+	+	+	+	-	+	+
Starch reduction	+	-	+	-	-	-	-	-	+	+
Eggyolk test	-	-	-	+	-	-	-	-	+	-
DNase test	-	-	-	-	-	-	-	-	-	-

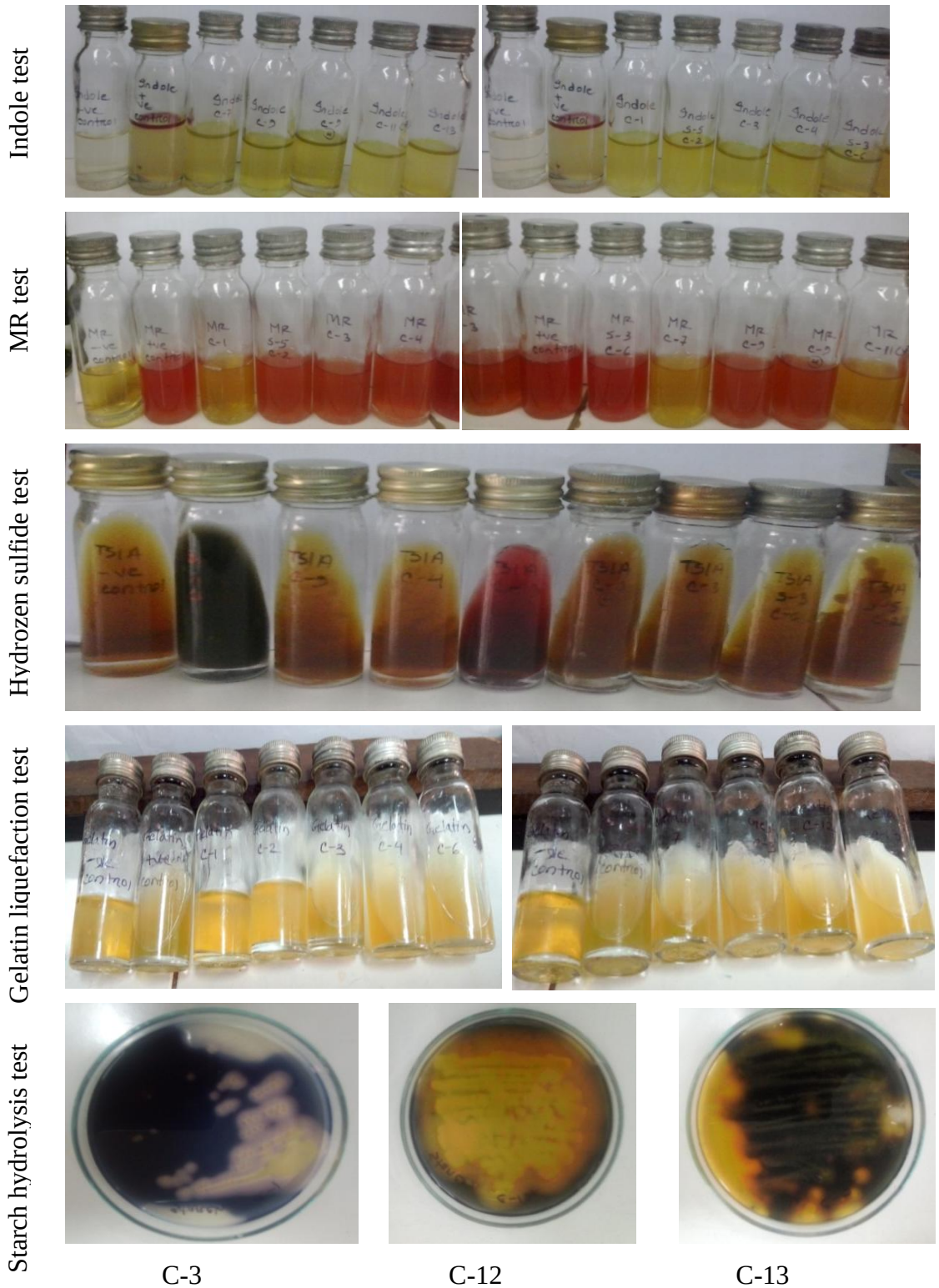


Fig 01: Indole test, MR test, Hydrogen sulphide test, Gelatin liquefaction test, Starch hydrolysis test for all of isolates

Carbohydrate fermentation test

Glucose,Sucrose,Lactose,Galactose,Ribose,Maltose,Xylose,Arabinose,Melibiose,Manitol sugars were tested for obsrbation of carbohydration fermentation capacity.Some isolates can ferment sugar others did not ferment sugar (Table-05).

Table 05 : Sugar fermentation capabilities of isolates

Isolates	C-1	C-2	C-3	C-4	C-6	C-7	C-9	C-11	C-12	C-13
Glucose	-	+	-	-	+	-	-	-	-	-
Sucrose	+	-	-	-	+	+	-	-	-	+
Lactose	-	-	-	-	+	-	-	-	-	-
Galactose	-	+	-	+	+	-	+	-	-	-
Ribose	-	-	+	+	-	-	-	+	+	-
Maltose	-	-	-	+	-	-	-	+	-	-
Xylose	-	+	-	-	df	df	-	-	-	-
Arabinose	-	+	-	+	+	-	-	-	df	-
Melibiose	-	-	-	-	+	-	-	-	-	-
Manitol	-	-	-	-	+	-	-	-	-	-
	Micrococcus spp	***Bacillus spp***	***Bacillus spp***	***Bacillus spp***	***Enterococcus spp***	***Bacillus spp***	***Pseudomonas spp***	***Pseudomonas spp***	***Bacillus spp***	***Bacillus spp***

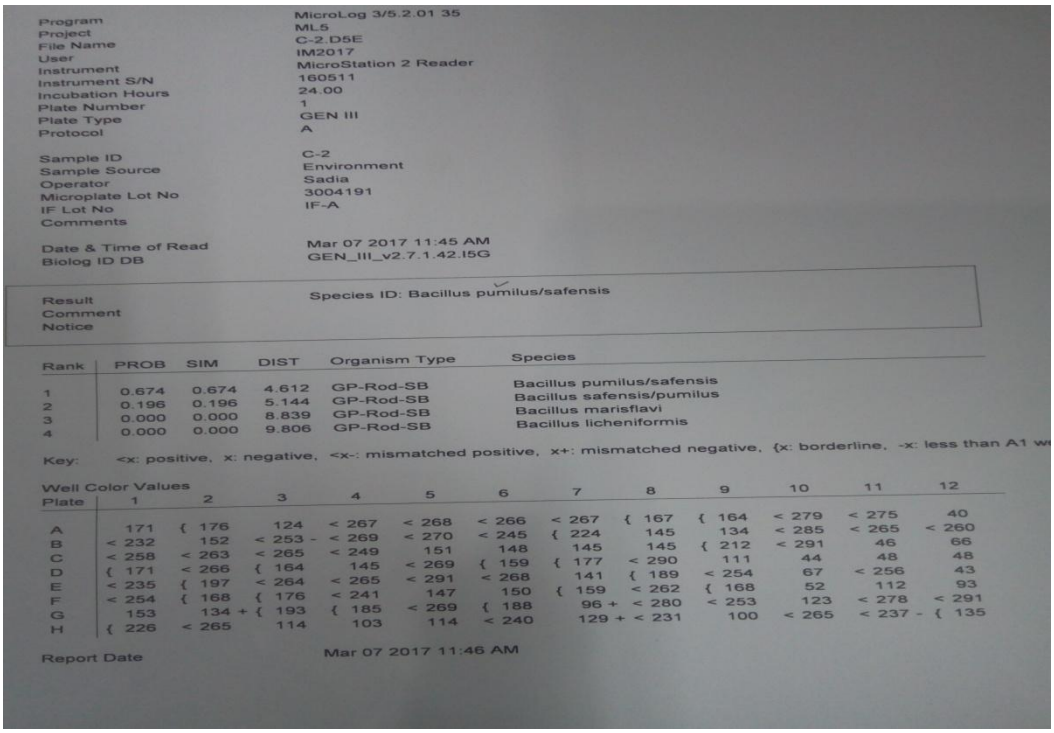
“+” frementation positive, “-” fermentation negative, “df ”delayed fermentation

4.3.6. Identified by Biolog™ system

Based on the decolorization ability it was observed that 3 isolates namely C-2,C-4,C-6(Presumptively identified as Bacillus spp,Bacillus spp and Enterococcus spp) were able to decolorize all of five test dye better than other isolates.The isolates were further subjected to identification by Biolog system.

Result indicated that C-2 colony was identify as *Bacillus pumillus*,C-4 colony was identify as *Bacillus thurengiensis and* C-6 colony was identify as *Enterococcus faecium.*Fig 02 showed the result of Biolog identification system.

Bacillus pumilus



Program	MicroLog 3/5.2.01 35
Project	ML5
File Name	C-2.D5E
User	IM2017
Instrument	MicroStation 2 Reader
Instrument S/N	160511
Incubation Hours	24.00
Plate Number	1
Plate Type	GEN III
Protocol	A
Sample ID	C-2
Sample Source	Environment
Operator	Sadia
Microplate Lot No	3004191
IF Lot No	IF-A
Comments	
Date & Time of Read	Mar 07 2017 11:45 AM
Biolog ID DB	GEN_III_v2.7.1.42.I5G
Result	Species ID: Bacillus pumilus/safensis
Comment	
Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.674	0.674	4.612	GP-Rod-SB	Bacillus pumilus/safensis
2	0.196	0.196	5.144	GP-Rod-SB	Bacillus safensis/pumilus
3	0.000	0.000	8.839	GP-Rod-SB	Bacillus marisflavi
4	0.000	0.000	9.806	GP-Rod-SB	Bacillus licheniformis

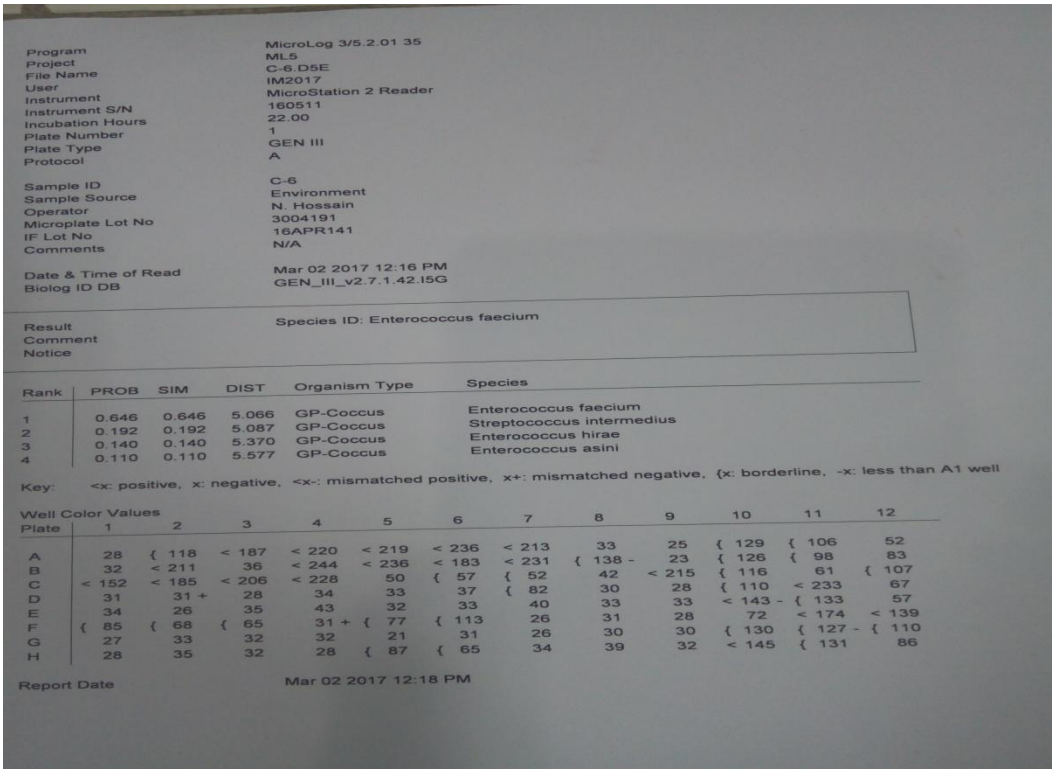
Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 w

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	171	{ 176	124	< 267	< 268	< 266	< 267	{ 167	{ 164	< 279	< 275	40
B	< 232	152	< 253 -	< 269	< 270	< 245	{ 224	145	134	< 285	< 265	< 260
C	< 258	< 263	< 265	< 249	151	148	145	145	{ 212	< 291	46	66
D	{ 171	< 266	{ 164	145	< 269	{ 159	{ 177	< 290	111	44	48	48
E	< 235	{ 197	< 264	< 265	< 291	< 268	141	{ 189	< 254	67	< 256	43
F	< 254	{ 168	{ 176	< 241	147	150	{ 159	< 262	{ 168	52	112	93
G	153	134 +	{ 193	{ 185	< 269	{ 188	96 +	< 280	< 253	123	< 278	< 291
H	{ 226	< 265	114	103	114	< 240	129 +	< 231	100	< 265	< 237 -	{ 135

Report Date Mar 07 2017 11:46 AM

Enterococcus faecium



Program	MicroLog 3/5.2.01 35
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File Name	C-6.D5E
User	IM2017
Instrument	MicroStation 2 Reader
Instrument S/N	160511
Incubation Hours	22.00
Plate Number	1
Plate Type	GEN III
Protocol	A
Sample ID	C-6
Sample Source	Environment
Operator	N. Hossain
Microplate Lot No	3004191
IF Lot No	16APR141
Comments	N/A
Date & Time of Read	Mar 02 2017 12:16 PM
Biolog ID DB	GEN_III_v2.7.1.42.I5G
Result	Species ID: Enterococcus faecium
Comment	
Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.646	0.646	5.066	GP-Coccus	Enterococcus faecium
2	0.192	0.192	5.087	GP-Coccus	Streptococcus intermedius
3	0.140	0.140	5.370	GP-Coccus	Enterococcus hirae
4	0.110	0.110	5.577	GP-Coccus	Enterococcus asini

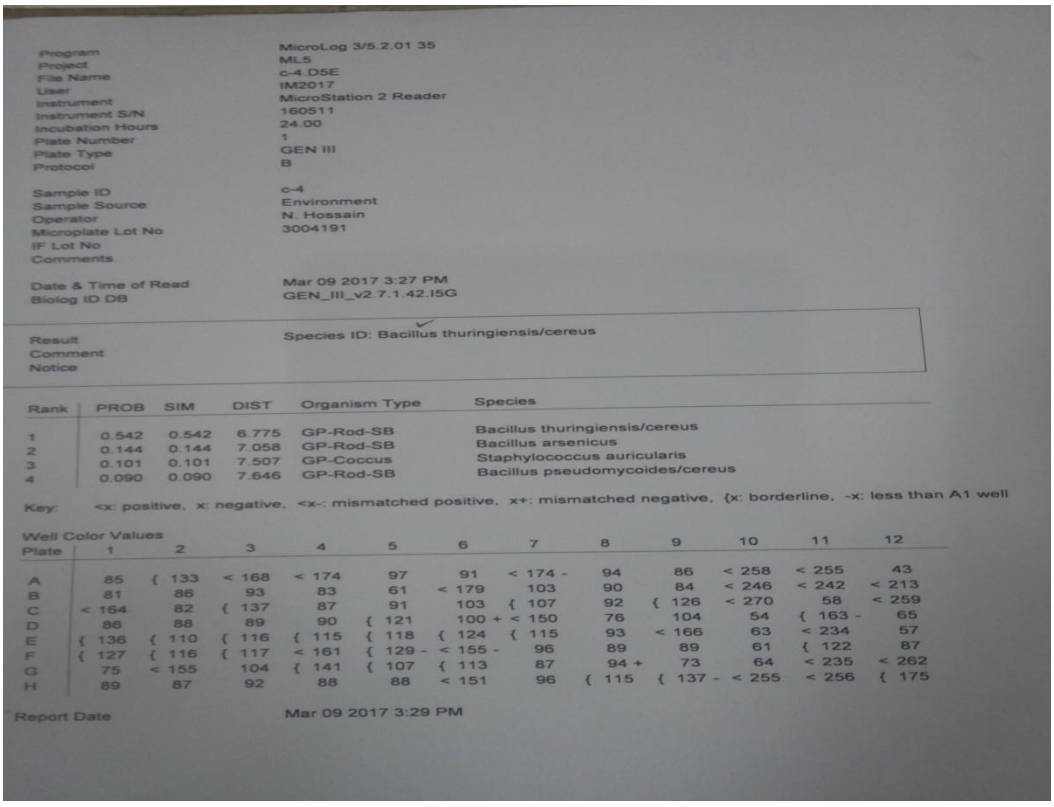
Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	28	{ 118	< 187	< 220	< 219	< 236	< 213	33	25	{ 129	{ 106	52
B	32	< 211	36	< 244	< 236	< 183	< 231	{ 138 -	23	{ 126	{ 98	83
C	< 152	< 185	< 206	< 228	50	{ 57	{ 52	42	< 215	{ 116	61	{ 107
D	31	31 +	28	34	33	37	{ 82	30	28	{ 110	< 233	67
E	34	26	35	43	32	33	40	33	33	< 143 -	{ 133	57
F	{ 85	{ 68	{ 65	31 +	{ 77	{ 113	26	31	28	72	< 174	< 139
G	27	33	32	32	21	31	26	30	30	{ 130	{ 127 -	{ 110
H	28	35	32	28	{ 87	{ 65	34	39	32	< 145	{ 131	86

Report Date Mar 02 2017 12:18 PM

Bacillus thuregiensis



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Project	ML5
File Name	c-4.D5E
User	IM2017
Instrument	MicroStation 2 Reader
Instrument S/N	160511
Incubation Hours	24.00
Plate Number	1
Plate Type	GEN III
Protocol	B
Sample ID	c-4
Sample Source	Environment
Operator	N. Hossain
Microplate Lot No	3004191
IF Lot No	
Comments	
Date & Time of Read	Mar 09 2017 3:27 PM
Biolog ID DB	GEN_III_v2.7.1.42.I5G
Result	Species ID: Bacillus thuringiensis/cereus
Comment	
Notice	

Rank	PROB	SIM	DIST	Organism Type	Species
1	0.542	0.542	6.775	GP-Rod-SB	Bacillus thuringiensis/cereus
2	0.144	0.144	7.058	GP-Rod-SB	Bacillus arsenicus
3	0.101	0.101	7.507	GP-Coccus	Staphylococcus auricularis
4	0.090	0.090	7.646	GP-Rod-SB	Bacillus pseudomycoides/cereus

Key: <x: positive, x: negative, <x-: mismatched positive, x+: mismatched negative, {x: borderline, -x: less than A1 well

Well Color Values

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	85	{ 133	< 168	< 174	97	91	< 174 -	94	86	< 258	< 255	43
B	81	86	93	83	61	< 179	103	90	84	< 246	< 242	< 213
C	< 164	82	{ 137	87	91	103	{ 107	92	{ 126	< 270	58	< 259
D	86	88	89	90	{ 121	100 +	< 150	76	104	54	{ 163 -	65
E	{ 136	{ 110	{ 116	{ 115	{ 118	{ 124	{ 115	93	< 166	63	< 234	57
F	{ 127	{ 116	{ 117	< 161	{ 129 -	< 155 -	96	89	89	61	{ 122	87
G	75	< 155	104	{ 141	{ 107	{ 113	87	94 +	73	64	< 235	< 262
H	89	87	92	88	88	< 151	96	{ 115	{ 137 -	< 255	< 256	{ 175

Report Date Mar 09 2017 3:29 PM

Fig 02: Identification of C-2, C-4, C-6 by Biolog system. C-2 *Bacillus pumilus*, C-4 *Bacillus thurengiensis*, C-6 *Enterococcus faecium*

4.4. Determination of decolorization efficiency

Those isolates namely C-1, C-2, C-3, C-4, C-6, C-7, C-9, C-11, C-12, C-13 were further assayed for decolorization efficiency. Decolorization efficiency of all 10 isolates were studied and the results were showed in table 06 and visualized in fig 03.The result indicated that decolorization ability was ranging from 68.2-98.3% to respective dyes. Decolorization of Novacron Ruby dye by all isolates ranging from 70.3-93.2%,Novacron Navy 76.3-96.4%, Novacron yellow 72.3-95.3%,Novacron Blue dk 73.3-98.3%, Novacron black 68.2-91%. Among the all isolates colony C-4 showed better decolorization efficiency (91.2-98.3%) of all dyes.

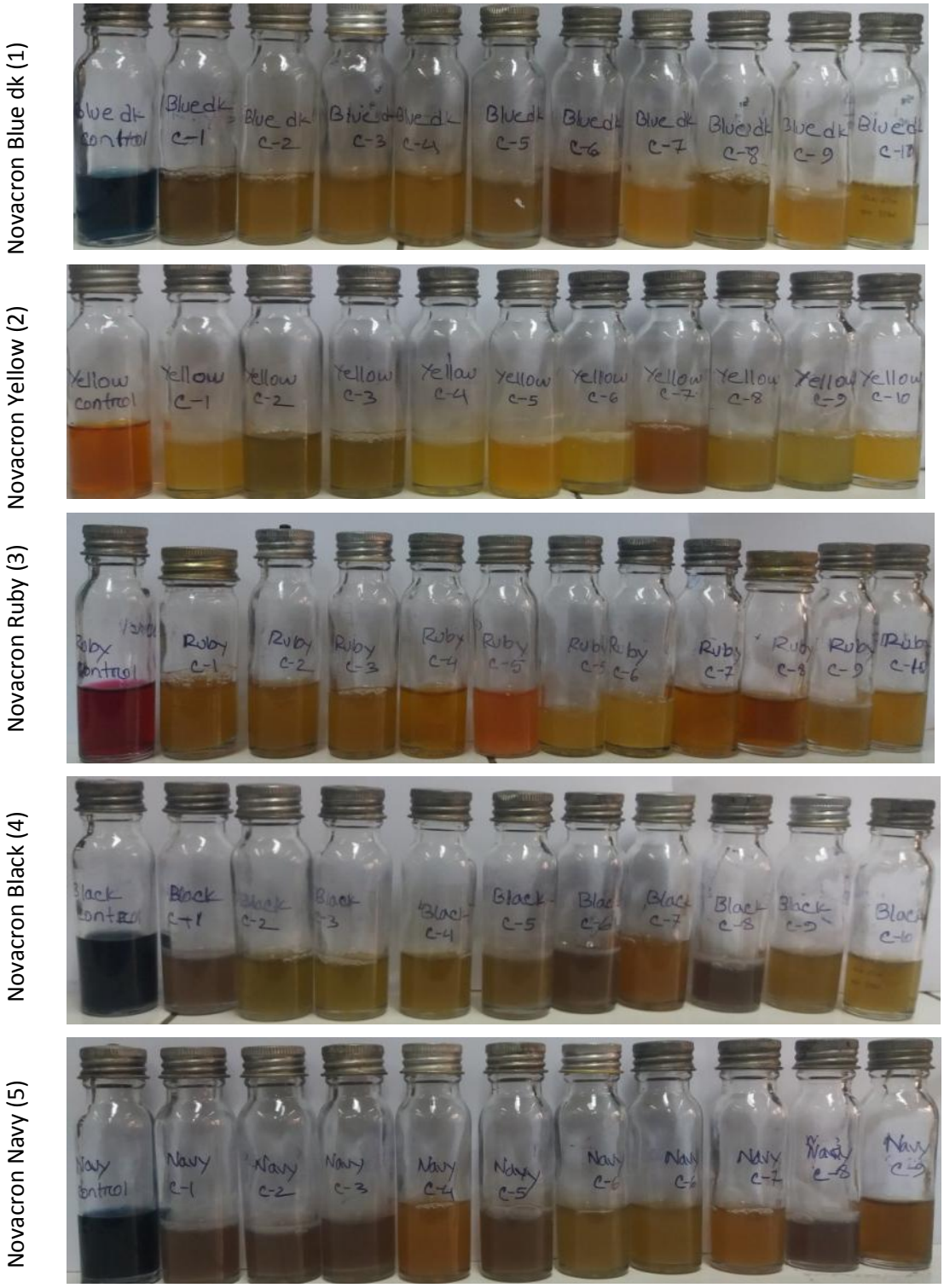


Fig: 03: Decolorization of (1) Novacron blue dk, (2) Novacron Yellow, (3) Novacron Ruby,(4) Novacron Black ,(5) Novacron Navy by all of 10 isolates

Table 06: Decolorization percentage achieved by single isolates

Isolates	Novacron Ruby	Novacron Navy	Novacron Yellow	Novacron Blue dk	Novacron Black
Colony-1	70.3	76.4	72.4	73.3	68.2
Colony-2	91.5	96.4	95.4	94.5	89.2
Colony-3	82.4	78.5	75.3	80.4	79.1
Colony-4	93.2	95.4	97.3	98.3	91.2
Colony-6	87.4	89.2	90.2	91.3	85.1
Colony-7	86.4	85.3	88.2	90.4	82.3
Colony-9	73.7	75.3	78.4	79.2	71.5
Colony-11	88.4	86.2	84.3	81.2	81.4
Colony-12	78.4	77.2	72.3	81.3	76.2
Colony-13	77.3	85.4	79.3	83.2	82.4

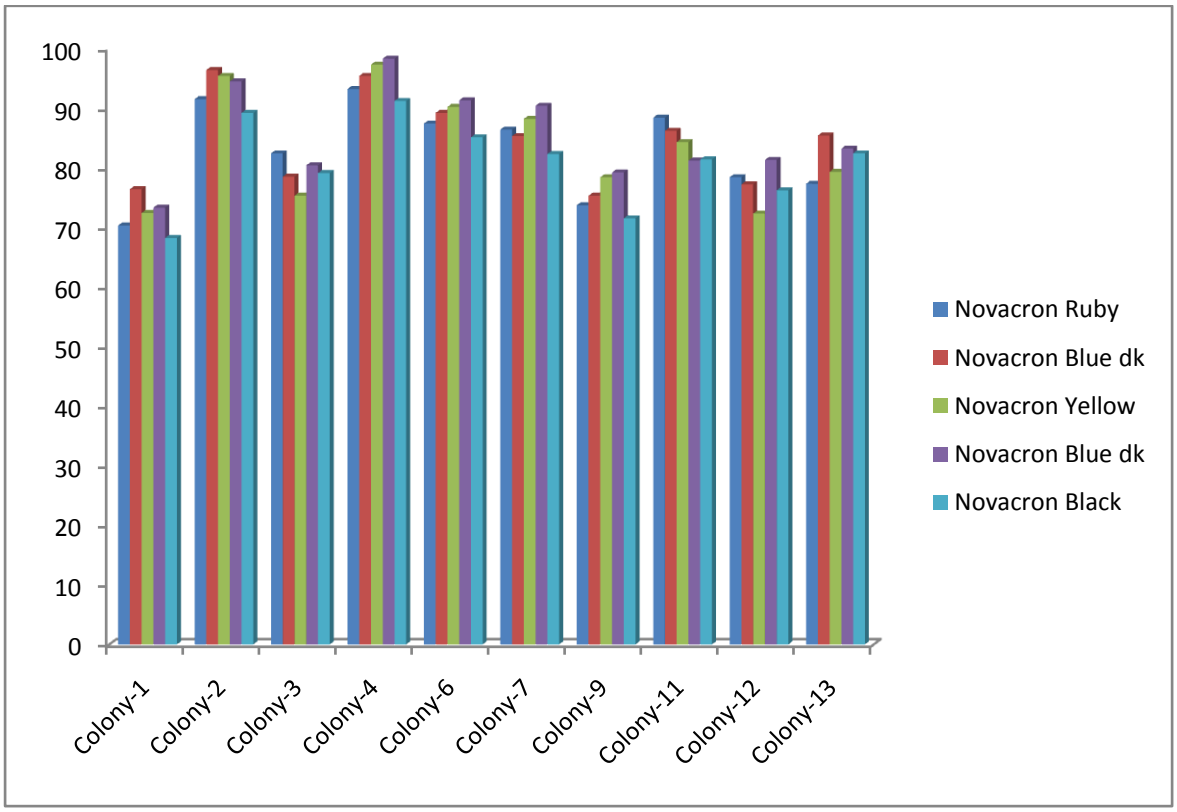


Fig: 04: Decolorization percentage achieved by single isolates

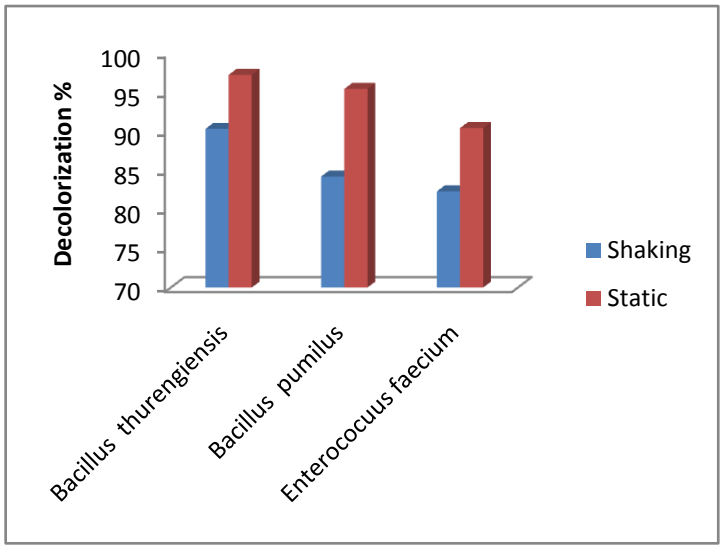
4.4. Effect of environmental and nutritional conditions for dye decolorization

4.4.1 Incubation condition

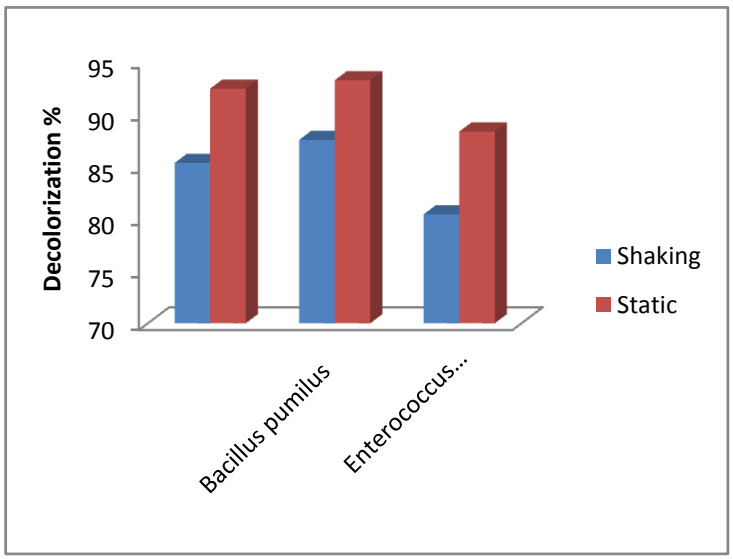
All three bacterial strains namely *Enterococcus faecium, Bacillus pumilus and Bacillus thurengiensis* were able to degrade all three namely Novacron Ruby, Novacron Black, and Novacron Blue dk. ranging from 80.1-97.2% Depending on the bacterial strain and dye structured decolorization efficiency of all bacterial sp *Enterococcus faecium, Bacillus pumilus and Bacillus thurengiensis* was better under static condition as compared to shaking condition. Decolorization under static and shaking condition by was *Bacillus pumilus* 95.4 %and 87.5%, *Bacillus thurengiensis* 97.2% and 90.3%, *Enterococcus faecium* 90.4% and 83.1% after72 hrs of incubation time (fig:05).

4.4.2. Incubation time

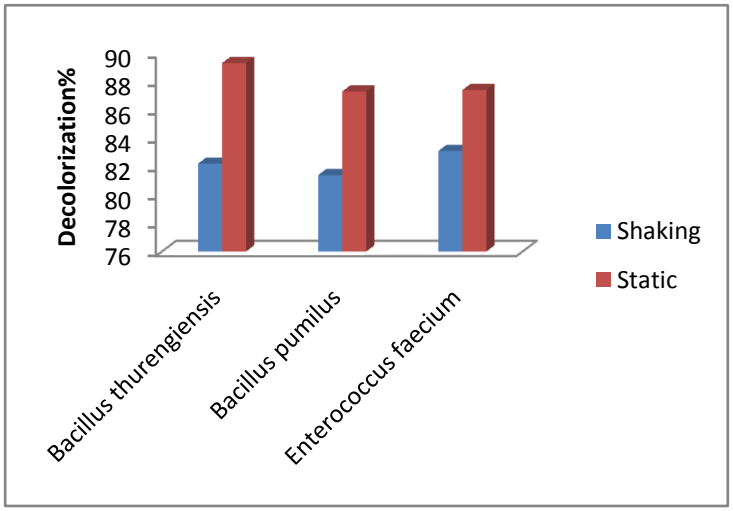
Decolorization of all three dyes by bacterial isolates occurs within 72 hrs. But Novacron ruby degraded readily by all of isolates than other dyes. Initially up to 08 hrs of incubation, no noticeable decolorization was observed. As evident after 72 hrs very little change in decolorization percentage could be noticed. Since, maximum decolorization of the dye was noticed after 72 hrs, this optimum time period was used for the subsequent experiments. Decolorization% of all isolates and all dyes given below (fig: 06)



Novacron Ruby

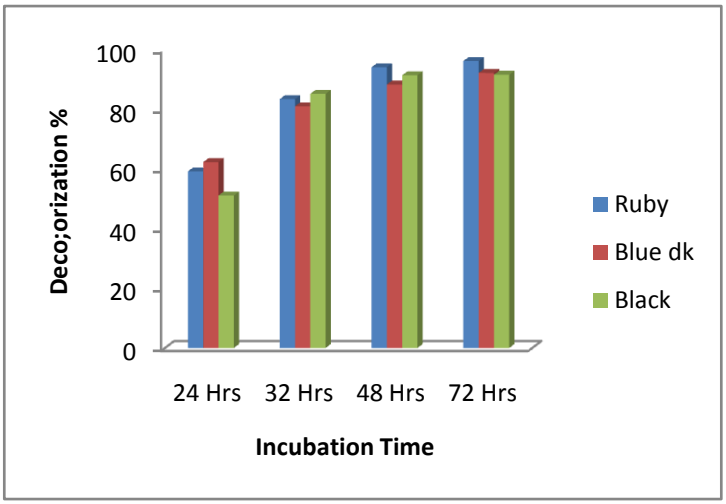


Novacron Blue dk

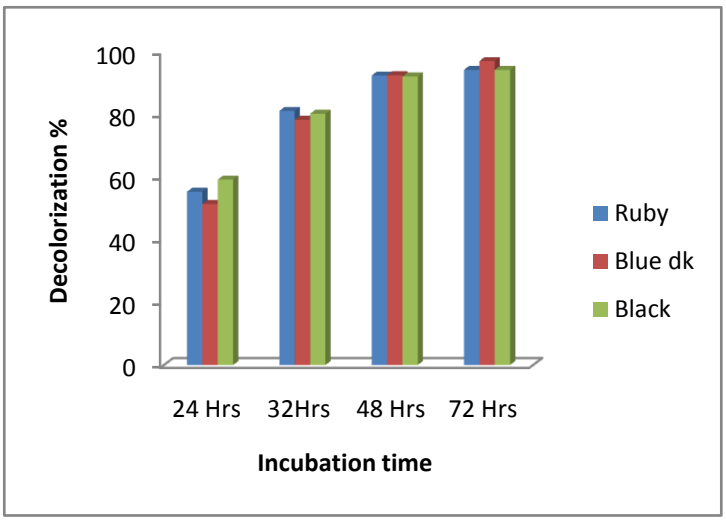


Novacron Super Black

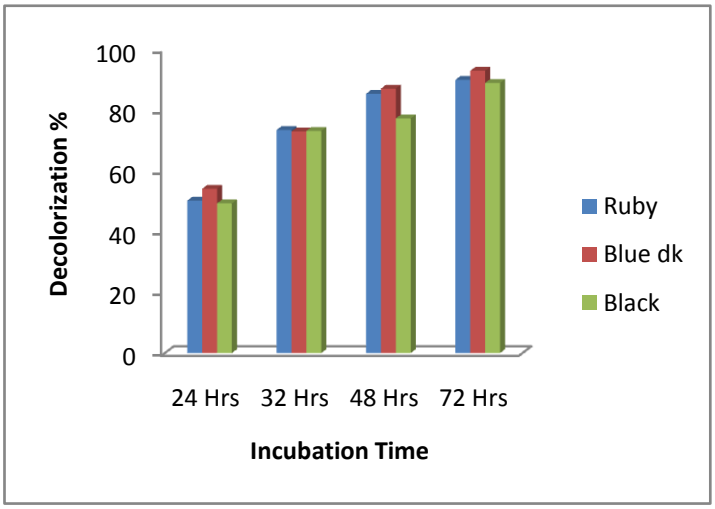
Fig 05: Decolorization % at different incubation condition



Bacillus thurengiensis



Bacillus pumillus



Enterococcus faecium

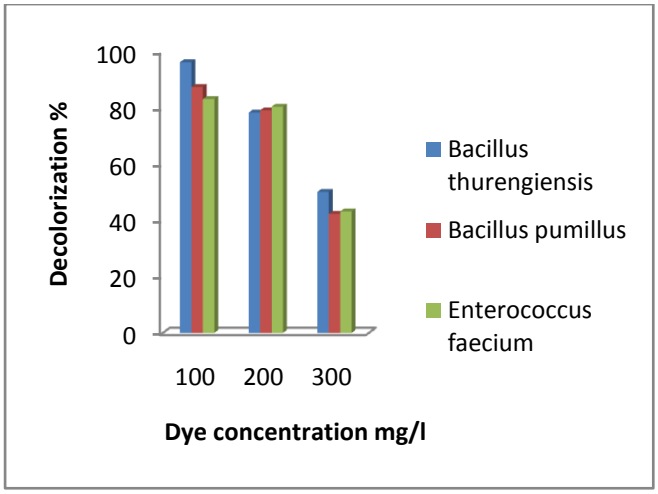
Fig 06: Decolorization % at different incubation time

4.4.3: Decolorization at different dye concentration

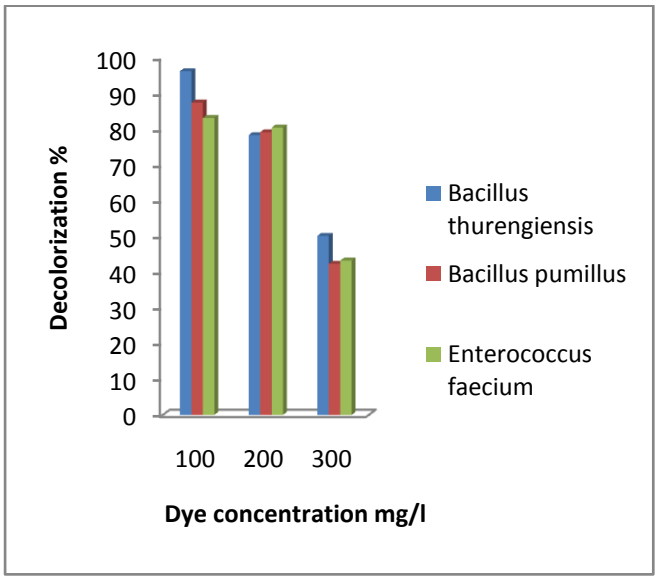
Study of the effect of initial dye concentration on the efficiency of color removal of the isolated strain revealed that, the bacterial strain is capable of decolorizing an acceptable high concentration of color. Decolorization with different initial dye concentration ranging from 100 mg/L to 300 mg/L was studied. All the selected three bacterial strains were able to degrade more than one dye with ranging from 52.5-96.2% depending on the bacterial strains and dyes. All three isolates showed maximum decolorization at a concentration 100mg/l of dye (fig 07). But further increase of dye concentration isolates showed decrease rate of decolorization.

4.4.4: Effect of pH

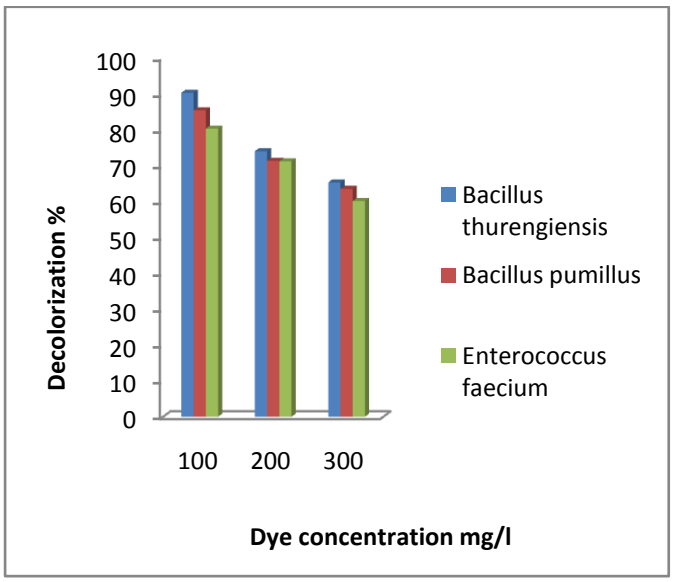
It was noticed in study that initial pH had a great influence on the decolourization of textile dyes. The dye removal was in the range of (54.4-97.3%) by bacterial isolates when studied at different pH values ranging from 5-9 at constant concentration of dye i.e. 100mg/l and temperature was 37°C. Removal of dye was maximum at P^H 7(Fig 08).Lowest decolorization observed at pH 5.



Novacron Ruby

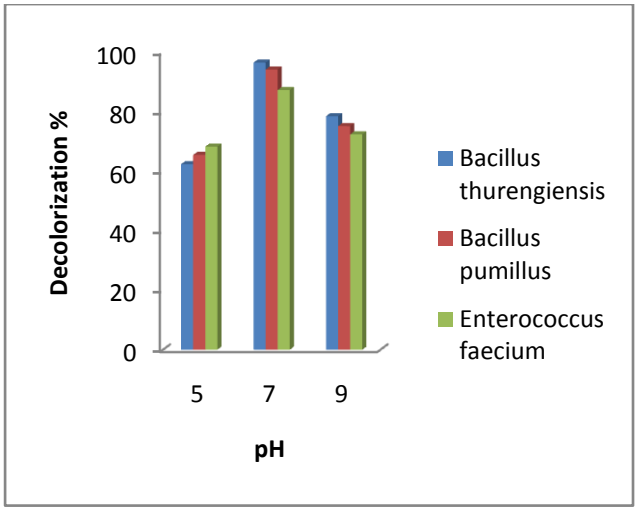


Novacron Blue dk

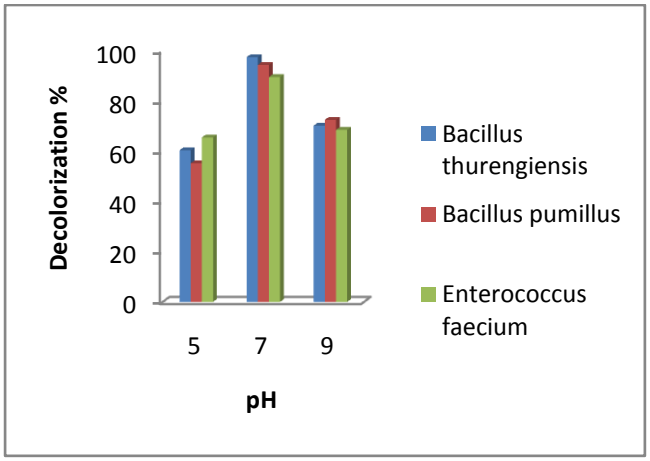


Novacron Super Black

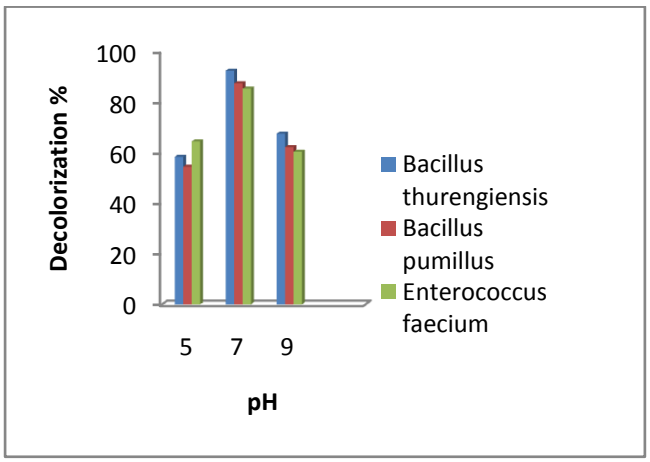
Fig 07: Decolorization % at different dye concentration



Novacron Ruby



Novacron Blue dk



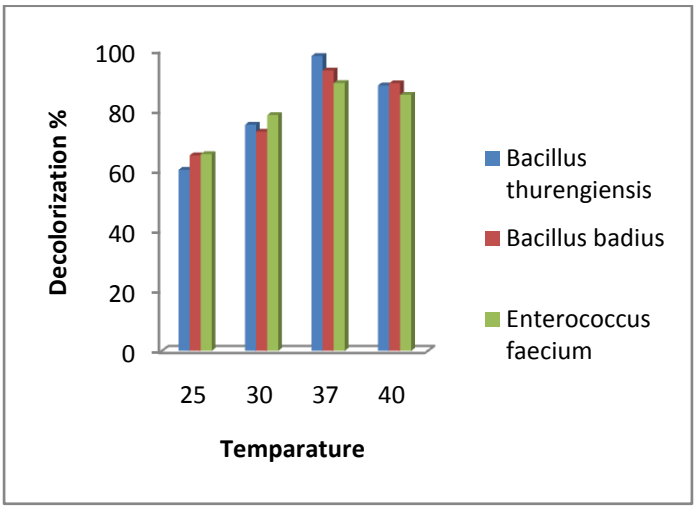
Novacron Black

Fig 08: Decolorization % at different pH

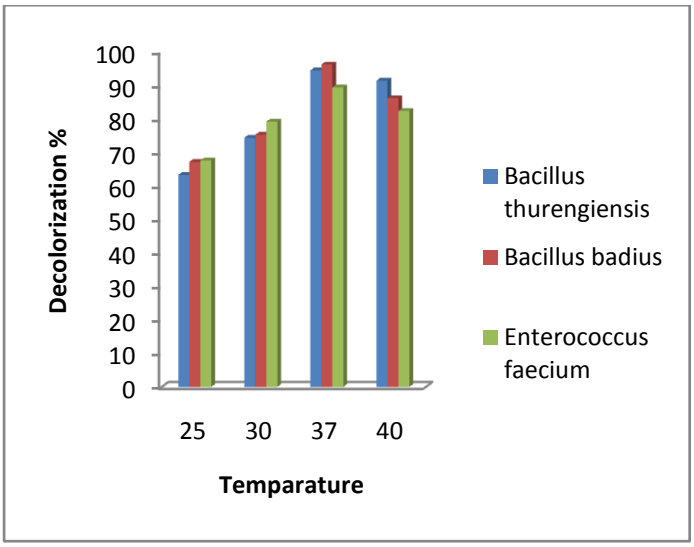
4.4.5: Effect of temperature

In order to determine the optimum temperature, decolorization assay were performed over 25-44^{0}C temperature range. Figure 09 shows that the decolorization rate increases with increase in temperature from 25-37^{o}C. At 44^{o}C, the decolorization

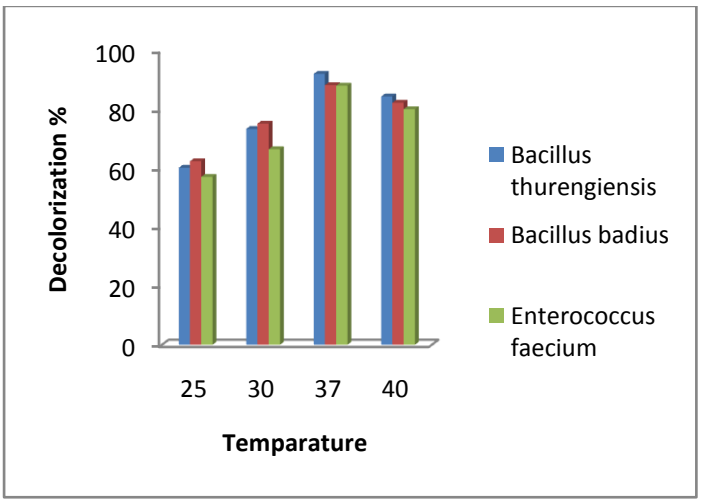
ability was sharply reduced. Highest decolorization 98.2% was noticed at 37^0C.So, 37^0C temperature was optimum for growth of bacteria and decolorization of all dyes



Novacron Ruby



Novacron Blue dk

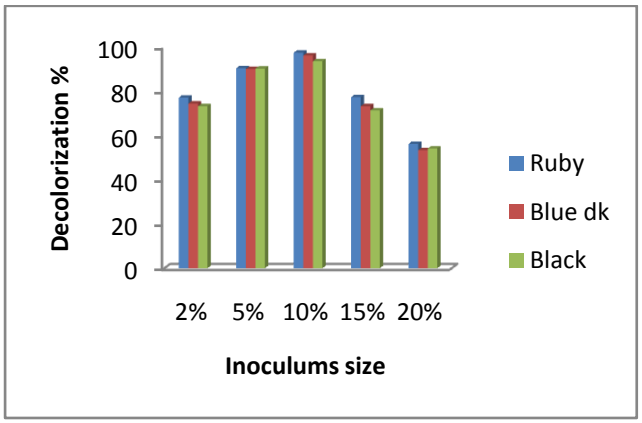


Novacron Black

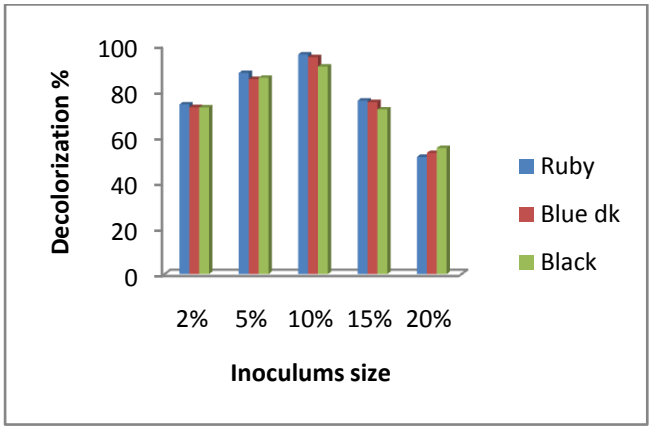
Fig 09: Declolorization % at different incubation temperature

4.4.6. Effect of inoculums size

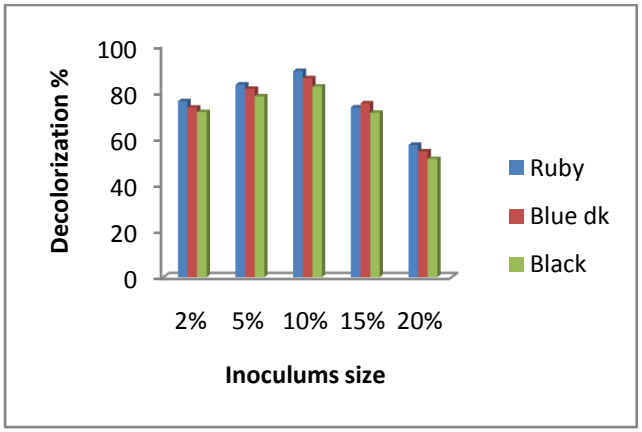
Figure 10 depicts the change in the extent of dye decolorization in response to varying inoculums size. After 72 hrs of incubation at 37°C in shaking condition, 77.1% decolorization was achieved in 2% v/v of bacterial inoculums. With an increase in inoculums size, the extent of decolorization also increased and 97.4% decolorization was achieved in 10% v/v of inoculums. A further increase in volume of inoculums resulted in decreased decolorization efficiency of the bacteria.



Bacillus thurengiensis



Bacillus pumillus



Enterococcus faecium

Fig 10: Decolorization at different inoculums size

4.4.7: Influence of Nutritional Parameters on Dye Decolorization by

The efficacy of *Bacillus thurengiensis* to decolorize Novacron Blue dk dye in presence of additional carbon (1%) and nitrogen sources (0.5%) was tested in order to obtain efficient and faster decolorization. The maximum percentage of decolorization was observed with beef extract (94.21%), while peptone and yeast extract showed a moderate decolorization value of 91.5% and 82.27% respectively. (Fig:11). Negligible decolorization was observed for (NH4)2SO4 as supplements of nitrogen source. Effect of carbon source was evaluated with and without beef extract as a nitrogen source. Presence of sucrose, dextrose and mannose as carbon source without beef extract showed 85.34 %, 83.55%, 85.43%

On addition of beef extract along with sucrose, dextrose and mannose, the decolorization percentage was increased to 97.34%, 95.01% and 97.03% respectively.

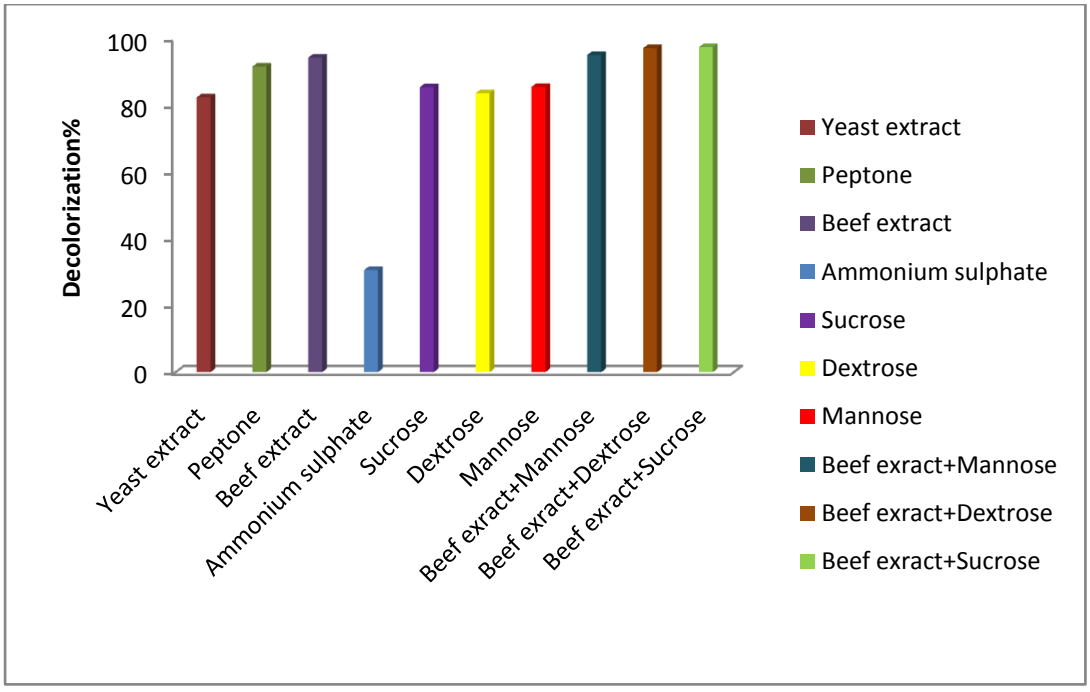


Fig 11: Effect of various carbon and nitrogen source on dye decolorization

4.5. Consortium effect on mixed dye

Different consortium was made by 03 bacteria namely *Bacillus thurengiensis, Bacillus pumilus, and Enterococcus faecium.* OD was taken different time intervals. Significant amount of decolorization was observed within 16 hrs and maximum decolorization obtained after 60 hrs of incubation. Highest decolorization % occurred by consortium -3 (96.3%) and minimum were obtained by consortium-4. (89.2%)

Table 07: Decolorization effect of consortium

Consortium	16hrs	24hrs	42hrs	60hrs
1	64.5%	73.1%	78.3%	86.4%
2	58%	68.2%	76.3%	89.2%
3	70.2%	78.4%	89%	96.3%
4	59.4%	66.3%	77.2%	83%

4.6. Partial analysis of azo reductase enzyme

The bacterial cultures were centrifuged after treating dye effluent for 72 hrs, the bacterial Cell pellets were lysed with the help of sonicator and again centrifuged. The supernatant was used to run SDS to know the kinds of protein present in the bacteria .Relative mol.wt of protein were determined by SDS-PAGE (10% gel) and was compared with the standard protein marker .The mol.wt of protein present in the bacteria were identified as *Bacillus pumillus* 15,50,75,150,225 kda; *Bacillus thurengiensis* 25,35,75,150 and *Enterococcus faecium* 75,150,225 kda.

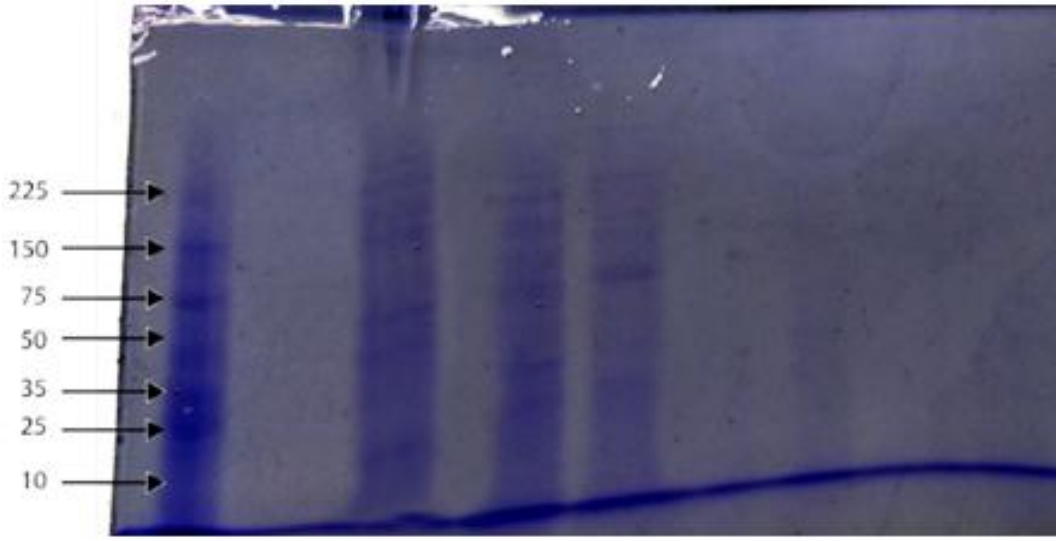


Fig 12: gel electrophoresis result of isolates. Lane1=Marker, Lane2=Blank, Lane3=*Bacillus pumillus*, Lane 4=*Bacillus thurengiensis*, Lane 5=*Enterococcus faecium*

4.7. Decolorization through crude extract of isolates

Crude extract of each isolates were subjected to inoculate in dye containing medium and after incubation 48hrs OD were taken where extract of *Bacillus thurengiensis* showed better decolorization potential (94.3%) than *Bacillus pumilus* (92.1%) and *Enterococcus faecium*(89.4%.)

4.8: Effect of decolorized effluent on beneficial bacteria

No zone of inhibition observed the wells containing decolorized dye water indicated that the biodegraded or decolorized product were not harmful to beneficial soil bacteria.

The main problem in textile industry is the presence of dyes released into the effluent during dyeing stage thus necessitating proper effluent treatment for removal of dyes in the effluent. There are various physical and chemical methods for the treatment of textile wastewater for the removal of dye but the application of microorganisms for the biodegradation of synthetic dyes is an attractive and alternative environment friendlily method for the wastewater treatments.

5.1. Isolation and screening of dye decolorizing bacteria

Final screening of the microorganisms in liquid media with incorporated dyes resulted in isolation of 10 bacterial isolates capable of degrading various dyes with some isolates exhibiting high capability of degrading a wide spectrum of dyes. Similar kind of screening process was reported by Neelambar *et al*., (2013).

The difference in decolorization pattern is due to the dissimilarity in specificities, structure and complexity, particularly on the nature and position of substituent in the aromatic rings and the interaction with azo bond with different dyes as reported by many authors (Sani and Benerjee, 1999); Radha and Raghupati, (2005); Vijaykumar and Vaishampayan, (2007).

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

5.2. Identification of isolates

Identification of bacterial isolates were usually based on growth and morphological characteristics, Gram staining result, biochemical identification and Growth on selective media and Biolog identification system. Analysis of growth characteristics on selective media, staining reaction, and different biochemical tests were carried out by Bergey’s Manual of Determinative Bacteriology and microrau identification

software suggested that the isolates were presumptive of C-1 was *Microccou spp,* C-2 *Bacillus pumilus*,C-3 *Bacillus spp,*C-4 *Bacillus thurengiensis,* C-6 *Enterococcus faecium,*C-7 *Bacillus spp,*C-9 *Pseudomonas spp* ,C-11 *Pseudomonas spp* ,C-12 *Bacillus spp,*C-13 *Bacillus spp* and their characteristics was similar with the result of following.

Rajani V *et al*., (2015) and Qurratulane Bari *et al*., (2014) identified *Micrococcus luteus***.** Their result matched with result of the present study. A. G. O'DONNEL *et al.,* (1999) isolated and identified *Bacillus pumilus and* Md. Fakruddin *et al*., (2012) characterized *Bacillus thurengiensis* and their result supports the result of present study. Archana A *et al.,* (2011) and T. Marimuthu *et al* (2013) and identified *Bacillus sp, pseudomonas sp, Acinetobacter, Legionella* and *Staphylococcus*.Similar results were reported by L. A. DEVRIESE *et al*., (1987) on their study when they characterized and Identified of *Enterococcus* Species.

5.3.1. Influence of Physiochemical parameters on the process of dye Decolorization

Effect of Incubation condition

Under static condition decolorization of Novacron Ruby, Blue dk and Black dyes by all three sp. of bacteria was better as compared to shaking condition. The reason for decreased decolorization at shaking condition could be due to the competition in oxidation of reduced electron carriers with either oxygen or azo group as electron acceptor (Mabrouk and Yusef.,2000); Dawkar *et al*.,2010). Under shaking condition, the aerobic respiration of the strain might dominate the utilization of NADH and inhibit azoreductase for obtaining electrons from NADH to decolorize azo dyes (Stolz.,2001; Chang *et al*.,2004). It is a well known fact that decolorizatuion performance of some bacteria is better in presence of low oxygen content. The result was similar with that Bhuktar j.j and V.Manwar; (2011).

Effect of Incubation time

No decolorization occurs within 08 hrs probably indicating the lag period for acclimatization to culture environment. Novacron black dye takes more time as

compared to other dyes. This may be due to the structure and complex nature of textile dyes. Some azo dyes are more resistant to removal by bacterial cells and this may be attributed to their structural differences (Adya Das *et al*., 2015).

As evident, beyond 48 hrs very little change in decolorization percentage could be noticed. This may be due to the culture approaching the death phase that reduced the enzyme activity. Since, maximum decolorization of the dye was noticed after72 hrs it considered as optimum time. Similar studies have been reported in various literatures (Ponraj *et al.,* 2011); Praveen Kumar and Bhat Sumangala (2012) where with increase in time the rate of decolorization increased and after a certain point of time it showed no further decolorization (S.Barathi *et al*.,2015)

Effect of pH

Decolorization of all three dyes was optimum at pH 7.0. With different pH dye decolorization efficiency varies. However, the decolorization rate decreases under extremely alkaline pH and under acidic pH.At optimum pH surface of the biomass gets negatively charged which enhanced the binding of positively charged dye. Below the optimum pH, H+ ions compete effectively with dye cations, causing a decrease in color removal efficiency. At alkaline pH, the azo bonds will be deprotonated to negatively charged compounds and this results in obstruction of azo dye decolorization. (Hsueh, C.C *et al*., 2007).Similar study was done by Mehta *et al.*, (2015).

Effect of temperature

Temperature beyond 37˚C had an adverse effect on both growth and decolorization. Similar effect was noticed by Mehta *et al*., (2015).This could be owing to a greater production of enzymes and maximal growth conditions of the bacterial culture for its dye decolonization ability. Decolorizing activity was significantly suppressed at 44˚C, which might be due to the loss of cell viability or deactivation of the enzymes responsible for decolorization at 44˚C (Cetin and Donmez, 2006); Panswad and Luangdilok, (2000).

Effect of dye concentration

This present study clearly showed that, as the dye concentration was increasing, decolorization efficiency was decreased. Similar studies have been reported (Chang *et al*., 2001); Shah *et al*., 2013) in literatures where a decrease in the efficiency of decolorization was observed with increase in initial dye concentration. With subsequent increase in dye concentration toxic effect of dye and its metabolites became dominant, leading to inhibition in decolorization. The decrease in the efficiency of color removal with increase in concentration of dye can be due to toxic effect of dye and inadequate amount of biomass to uptake this higher concentration of dye and the lack of ability of the enzyme to recognize the substrate efficiently at the very low concentrations (Jadhav *et al*., 2007; Pearce *et al*., 2003).

Effect of inocula size

With the increase of inocula size (2-20%) gradually decolorization efficiency also increased (77.1-97.4%) up to certain limit. Further increasing causes decreased decolorization. Bhatt *et al* (2012) reported similar result as early depletion of nutrient occurs as the inoculum volume increased and hence biological process of decolorization involving microorganisms require an optimum amount of microbial cells.

5.3.2. Effect of Carbon and Nitrogen source on dye decolorization

The results indicate that presence of Nitrogen source in media has a significant effect on the rate of decolorization, where as the presence of different carbon sources seems to be less effective to promote the decolorization of Novacron Blue dk dye by the bacterial sp *Bacillus*. Similar results were reported by Saratale *et al*., (2009) where using *Micrococcus glutamicus* and *Trichosporon beigelii* NCIM-3326 bacterial strain. These researchers observed the effect of nitrogen sources to supersede the effect of carbon sources under shaking conditions. Dye being a complex carbon ring is less preferred than a supplementary carbon source by the organisms. Hence the dye degradation potential of the organism decreases in presence of supplementary carbon source. In addition to that organic nitrogen (Adya Das.,2015) sources acts as the source of electron donor to reduce the azo dyes by microorganisms (Hu, 1994).

5.4 Consortium effect on mixed dye

The efficiency of the decolorization process depends on the survival, adaptability and activities of enzymes produced by microorganisms present in the mixed cultures (Cripps *et al*., 1990); Senan & Abraham.,2004).Decolorization percentages by different consortium were different and consortium containing two isolates were better than consortium of three isolates. This is possibly because broader enzymatic capacity is achieved and the formation of toxic intermediate metabolites is counteracted by the selection of these dead end products formed mainly by co metabolism processes. (Rashid Mahmood *et al*., 2015)

5.5. SDS-PAGE

Electrophoretic pattern of enzyme from 3 isolates showed different band size. Purified azo reductase enzyme on SDS-PAGE with a molecular weight of approximately 61.6KDa, 21.1KDa, 60KDa, 58KDa have also been reported (Maier *et al*., 2004).

5.6. Decolorization of dye through crude extract of isolates

Dye decolorization by microorganisms may take place in two ways: either by adsorption or degradation. Sorption is frequently the first step of biodegradation process. Visual changes of the biomass color can suggest what is the way of decolorization (E. ZABŁOCKA-GODLEWSKA *et al).*In this study there was no visual color change of cell mass and decolorization by crude extract can described as dye decolorization was through intra or extracellular enzyme.

5.7. Effect of decolorized effluent on beneficial bacteria

Dye water after treatment was not harmful to beneficial bacteria whereas untreated dyes were harmful to beneficial bacteria. The toxic effect of untreated Malachite green, Brilliant green, fast green, ethylene blue and Congo red and removal of their toxicity after biological treatment has been reported by Mali *et al*., (2000)

Conclusion

Textile effluent is a good source or growth of dye decolorizing bacterial population. Although Bioremediation/degradation is a challenging process to both the textile industry and the wastewater treatment analysts, the results of this study and those reported in the literature suggest a great potential for bacteria to be used to remove pollutants from textile effluents. In the present study a total of 32 isolates were collected from the waste effluent and discharge.10 different colonies showed decolorization ability at screening level and were further investigated for their characterization, based on Gram's reaction, cell morphology, and colony characteristics, growth patterns in nutrient broth, growth patterns in selective media and biochemical tests. Three important bacterial species were identified as *Enterococcus faecium, Bacillus pumilus* and *Bacillus thurengiensis* through Biolog identification system.

Various aspects of environmental and cultural parameters were studied for decolorization of Novacron Ruby, Novacron Black, and Novacron Blue dk by all the three isolates. The suitable initial pH for decolorization of all dyes was in the range of 5-9 under static condition. Optimum temperature for decolorization was 37˚C. The results on the effect of size of inoculum indicated that under static condition 10% v/v was ideal for rapid decolorization .Optimum time for maximum decolorization was 72 hrs by all the three isolates. Under optimized condition bacterial isolates can degrade dyes up to 100mg/l of dye while further increase of dye concentration causes decrease in efficiency of decolorization

The efficacy of Bacillus thurengiensis to decolorize Novacron Blue dk dye in presence of additional carbon (1%) and nitrogen sources (0.5%) was tested in order to obtain efficient and faster decolorization. The maximum percentage of decolorization was observed with beef extract (96.16%)and negligible decolorization was observed for (NH4)2SO4 as supplements of nitrogen source. whereas the presence of different carbon sources seems to be less effective to promote the decolorization of Novacron Blue dk dye by the bacterial sp Bacillus. This experiment revealed that decolorization ability of isolates was affected by source and amount of nutrition in the medium.

Decolorization % was better in consortium than pure culture and different consortia were different and consortia containing two isolates were better than consortia of three isolates. This may occur due to favourable interaction of enzyme for or degradation in the combination of two selected isolates..SDS-PAGE analysis of selected isolates showed broad range of band on electrophoretic pattern. Study for observation of harmful effect of non treated dyes revealed that degraded product was not toxic to agriculturally important bacteria.

The study concludes with isolated bacteria Bacillus *badius, Bacillus thurengiensis, Enterococcus faecium* could effectively be used as an alternative to physical and chemical processes used for textile effluent treatment. Further study on azoreductase enzyme activity of isolates may reveal the mechanism for dye degradation and thus facilitate possible for biotechnological application of treating dye containing waste water.

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Appendix 1

Medium composition

Nutrient broth (pH 7.2)

Peptone	5g
Sodium chloride	5g
Beef extract	1.5g
Yeast extracts	1.5g
Distilled water	1000ml

Carbohydrate broth (pH 7.3)

Peptone	10g
Carbohydrate*	5g
Sodium chloride	5g
Phenol red	0.018g
Distilled water	1000ml

(* a specific carbohydrate was added)

MR-VP broth (pH 6.9)

Peptone	7g
Dextrose	5g
Potassium phosphate	5g
Distilled water	1000ml

Simmon's citrate agar (pH 6.9)

Ammonium dihydrogen phosphate	1g
Dipotassium phosphate	1g
Sodium chloride	5g
Sodium citrate	2g
Magnesium sulphate	0.2 g
Agar	15g
Bromo thymol blue	0.08g
Distilled water	1000ml

Blood agar (pH 7.3)

Beef heart infusion	500g
Tryptose	10g
Sodium chloride	5g
Agar	15g
Distilled water	1000ml

The above ingredients were dissolved and autoclaved .sterile blood agar base cooled to 45-50˚C.Aseptically 50ml of sterile defibrinated blood was added, mixed thoroughly and dispensed into sterile plates while liquid.

MacConkeys agar (pH 7.1)

Peptic digest of animal tissue	17g
Proteose peptone	3g
Lactose	10g
Bile salts mixture	1.5g
Sodium chloride	5g
Neutral red	0.03
Agar	15g
Distilled water	1000ml

Nutrient gelatin (pH 6.8)

Peptone	5g
Beef extract	3g
Gelatin	120g
Distilled water	1000ml

Starch agar (pH 7.0)

Peptone	5g
Beef extract	3g
Starch (soluble)	2g
Agar	15g
Distilled water	1000ml

Egg-yolk medium (pH7.4)

Pancreatic Digest of casein	10g
Beef extract	3g
Yeast extract	1g
Sodium chloride	5g
Aagr	15g
Egg yolk Emulsion	100ml

Urea broth (pH 6.8)

Na2HPO4	9.5g
KH2PO4	9.1g
Yeast extracts	0.1g
Phenol red	0.01g
Urea	20g
Distilled water	1000ml

Appendix ii

Reagents

Methyl Red solution

Ingredient	Amount
Methyl Red	5g
Ethanol (95%)	75ml
DW	25ml

Kovac's Reagent

Ingredient	Amount
Para-dimethyl amino benzaldehyde	5gm
Butyl Alcohol	75ml
HCL	25ml

IM Tris-HCL (pH 8.0)

Ingredient	Amount
Tris-base	121.1gm
DW	1000ml

Tris-base dissolved into distilled water then pH adjusted by adding concentrated HCl.

PBS

Ingredient	Amount
NaCl	8.0gm
KCl	0.2 gm
Na2HPO4	1.44gm
KH2PO4	2.0gm
DW	1000ml

Dissolved all of ingredients into distilled water then pH adjusted into 7.4 by adding concentrated HCL

Reagents for SDS-PAGE

30% acrylamide-bisacrylamide solution

Ingredient	Amount
Acrylamide	29.0g
Bis-acrylamide	1.0g
DW	100ml

10% ammonium per sulphate (APS)

Ingredient	Amount
APS	1g
DW	10ml

Sample loading Buffer

Ingredient	Amount
0.5M Tris-Cl	10ml
10% SDS	10ml
2-mercaptoethanol	1ml
Glecerol	10ml
Distilled water	19ml

0.1% BMB (Bromophenol blue solution) or tracking dye

Ingredient	Amount
Bromophenol blue	0.1g
DW	100ml

Electrophoresis buffer

Ingredient	Amount
Tris	3.0g
Glycine	14.4g
10%SDS	10ml
DW	1000ml

Stacking/Upper gel buffer (pH 6.8)

Ingredient	Amount
Tris base	6.1g
SDS	0.4g
DW	100ml

Dissolved Tris into DW. Then pH adjusted into 6.8 by adding concentrated HCL after that dissolved SDS.

Separating /lower gel buffer(pH 8.8)

Ingredient	Amount
Tris base	18.17g
SDS	0.4g
DW	100ml

Dissolved Tris in DW. Then pH adjusted into 6.8 by adding concentrated HCL after that dissolved SDS**.**

Destaining solution

Ingredient	Amount
Glacial acetic acid	10ml
DW	90ml

Staining solution

Ingredient	**Amount**
Coomassie brilliant blue G250	0.20g
10% acetic acid	100ml

Appendix iii

Instrument

Instruments name	Model no.	Country
Autoclave	Model;Mc-40w,ALP Co.Ltd	Japan
Electronic Microscope	Olympus BX 41	Japan
Electric balance	Denver Instrument company,AA-160	
Freeze	Singer	
Incubator	BE200,Memmert Gmbh+Co	KG
Hot water bath	Memmert	Germany
Incubator	Memmert	England
Laminar air flow	Holten	Japan
Microwave oven	Rangs	
pH meter		Bangladesh
Centrifuge machine	Mikro 120	Germany
Orbital shaker	GFL 3031	Germany
Refrigerator	General,ER-141F	Japan
Small weighting	AA-160,Dever Instrument CO	USA
Spectrophotometer	Model T60U	
Vortex-1	WiseMix	Korea
SDS-PAGE	Bio-Rad	USA

Sl No.	Name of the glassware
1	Measuring cylinders
2	Beaker
3	Spatula
4	McCartney
5	Screw cap test tube
6	Erlenmeyer flask
7	Eppendrop
8	Micropipettes (5µl,10 µl,100 µl,1000 µl)
9	Disposable tips
10	Pipette
11	Inoculating needle, Inoculating loop,Spreader
12	Peridishes
13	Slide