Study of the Bacterial Degradation and Detoxification Mechanism of Crystal Violet from Textile Wastewater

Thesis

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BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
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IN

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Submitted By

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Under the supervision of

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ESTABLISHED 1996

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Dedicated to my beloved Family

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CERTIFICATE

This is to certify that the thesis entitled **"Study of the Bacterial Degradation and Detoxification mechanism of Crystal violet from Textile wastewater"** submitted by **Ms. SUJATA** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

The thesis submitted to Babasaheb Bhimrao Ambedkar University, Lucknow satisfies all the requirements as stipulated in the *Doctor of Philosophy (Ph.D.) Regulations – 1999 as amended in 2008/2010/2013* and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

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Supervisor

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Head of Department

STUDENT DECLARATION

This is to certify that the material embodies in the present Ph.D. work entitled **"Study of the Bacterial Degradation and Detoxification mechanism of Crystal violet from Textile wastewater"** is original research work done by me. It has not been submitted in part or full for any other diploma or degree in any other University. In this thesis, matter written, data presented and plagiarism, if any, is the sole responsibility of the student Ms. Sujata. If any allegations/query/question arises regarding the thesis, I, Ms. Sujata, will be solely responsible and answerable.

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Its great fortune that in vast universe, we worked on the omnipotent creature of the almighty God.

Place: Lucknow SUJATA

Date:

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ABBREVIATIONS & SYMBOLS

α	Alpha
~	Approximately
BOD	Biological Oxygen Demand
BSC	Bael Shell Carbon
BA	Bottom Ash
CO_2	Carbon dioxide
COD	Chemical Oxygen Demand
Cm	Centimeter
CR	Congo red
CV	Crystal violet
°C	Degree Celsius
DO	Dissolved Oxygen
DOS	de-oiled soya
DNA	Deoxyribonucleic acid
EC	Electrical Conductivity
△H°	Enthalpy
△S°	Entropy
GC-MS	Gas Chromatography Mass Spectrophotometry
△G°	Gibb's free energy
g	Gram
g/L	Gram per litre
HPLC	High Pressure Liquid Chromatography
H_2O_2	Hydrogen peroxide
HMSB	H_2SO_4 modified sugarcane bagasse
HF	Hen feather
IUPAC	International Union of Pure and Applied Chemistry
K	Kelvin

kDa	Kilo Dalton
kJ/mol	Kilo joule per mole
LiP	Lignin peroxidase
µ	Miu
MG	Malachite green
MB	Methylene blue
MSM	Mineral Salt Medium
M	Molarity
mL	Mililitre
Min	Minute
MnP	Manganese peroxidase
nm	Nanometer
-	Negative
NEQS	National Environmental Quality Standards
NIST	National Institute of Standards and Technology
OD	Optical density
+	Positive
%	Percentage
rpm	Revolution per minute
RNA	Ribonucleic acid
TDS	Total dissolved solids
TS	Total Solids
TSS	Total Suspended Solids
TGW	Treated ginger waste
UV	Ultra violet
U/mL	Unit per milliliter
H_2O	Water
w/v	Weight over volume

CHEMICALS & GLASSWARES

All the chemicals used throughout this research work were of analytical grade and purchased from:

1. **Media** from **Hi-media and Merck Millipore, Mumbai**
2. **Crystal violet dye** from **Spectrochem, Mumbai**
3. **Glasswares** from **Borosil, Mumbai**
4. **Chemical Reagents** from **Merck, Mumbai**
6. **Plasticwares** from **Merck Millipore, Mumbai**

INSTRUMENTS

All the instruments used in this research work are listed below:

1. Autoclave (SMI-102 & Indfos)

2. Centrifuge (Universal-320-R & Hettich, Zentrifugen)

3. Dry Air Oven (LSI-145)

4. Fridge for storing cultures (BFS-345 & Celfrost)

5. GC-MS (Shimadzu Model Number: QP2010S)

6. Laminar Air Flow (AEM-915-H)

7. PCR (Verti-R & Applied Biosystems)

8. Polyacrylamide Gel Electrophoresis unit (GX-SCZ2, Genetix Biotech Asia Pvt Ltd)

9. Spectrophotometer (Evolution 201, Australia)

10. Temperature Controlled Incubator Shaker (LSI-3016R & Labtech)

11. Water Bath (AEM-54003-Q)

Chapter 1

INTRODUCTION

Introduction

The textile dyeing industry came into existence for above 4000 years. Textile dyeing and fermentation of sugar to produce alcohol are the two specialized areas that date back to antiquity. Fabrics dyed with indigo and madder have been found in the tombs of predynastic Egypt. These and a few matters extracted from insects and tropical woods formed the only sources of dyes awaiting the middle of the last century. In prehistoric times, dyes were obtained from natural sources and only important members like kings and priests could possess colored fabrics. Natural coloring agents are mainly of inorganic origin such as clays, earths, minerals, metal salts, and even semi-precious stones, such as malachite or organic dyestuffs (either from animals or plants) (Ackacha et al., 2003).

Water pollution is considered as most threatening environmental pollutions to our biodiversity where effluents from dye-based industries serve as primary source (Sujata and Bharagava, 2016). Past few decades, there has been a startling increase in the pollution of various water bodies primarily due to the industrialization. Textile industries require large volume of water during the process, thereby it release huge volume of effluent. Textile industries are found in most of the countries and their number is increasing day by day. The annual world production of textiles is about 30 million tones, with the consumption of 7,00,000 tonnes of different dyes where up to 15 % of dyes remains unreacted and are directly lost in the effluents (Mani and Bharagava, 2016; Jadhav and Govindwar, 2006; O' Neill et al., 1999; Zollinger, 1987). Dye containing effluents are released from industries include pigment manufacture, textile, printing, dyeing, leather, food and cosmetic industries (Ahmad, 2009; Amini et al., 2008). In India, there are approximately 2324 textile industries including composite and process houses among which major textile industries amounting 1895 i.e. about 81% of total industries are

located at Gujarat, Maharashtra, Punjab and Tamil Nadu which uses different raw materials/resources such as cotton, wool and synthetic fibres (Fig. 1.1).

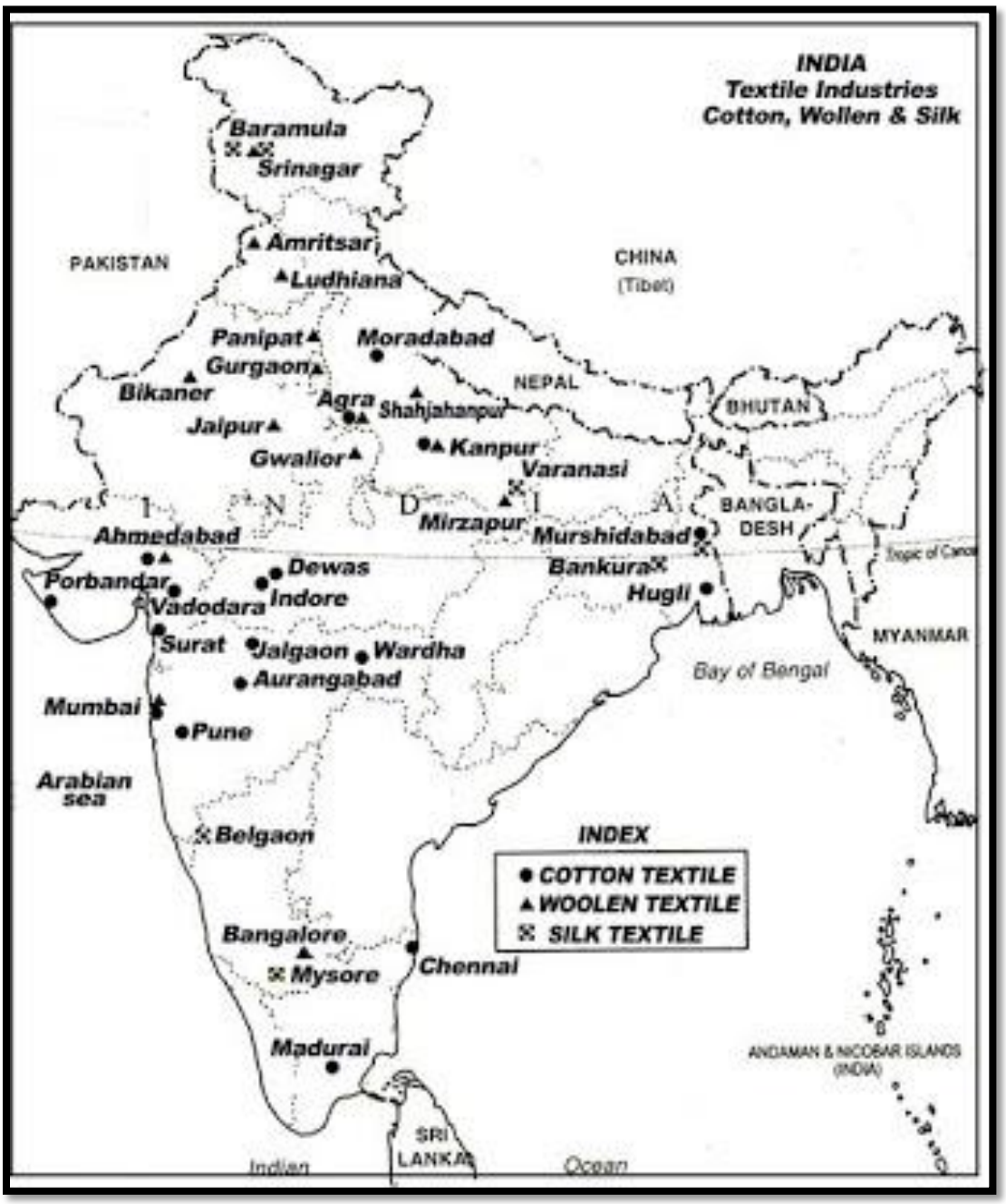


Figure 1.1: Map showing the distribution of textile industries in India

History of dyes

The English word daeg or daeh meaning “color” served as the source of derivation of term “dye”. The accurate period of the instigation of the art of dyeing in the world could not be determined appropriately instead supposed that the dye-vat might have appeared in the olden times. According to the archaeological surveys, dyeing industries were wide spread in India, Egypt and Mesopotamia around 3rd millennium BC. People have been using colorants since the establishment of civilizations, for painting and dyeing of their surroundings, skins and clothes. Until the middle of the 19th century, all colorants applied were of natural origin. Inorganic pigments such as soot, manganese oxide, hematite and ochre have been utilized within living memory. While searching for a cure for malaria, William Henry Perkin discovered the first synthetic dye stuff "Mauve" (aniline, a basic dye), which was a brilliant fuchsia type color, but faded easily.

According to the chemical structure of the chromophoric group (azo dyes, anthraquinonic dyes, xanthene dyes, triphenylmethane dyes, etc), synthetic dyes were specified (Zollinger, 2003). According to the function of their mode of binding to the fibres, dyes were separated as reactive dyes, direct dyes, cationic dyes, etc. Since then, thousands of dyes have been manufactured, which has now become a momentous part of the chemical industry. Nowadays the care of environment has become a major issue which is appealing for the use of natural colors as an environmental friendly alternative to present day practice. The advantages of using natural dyes compounds are the absence of toxicity upon humans, the use of sustainable sources and also fit into the natural pathways of biodegradation of the released dye baths (Sujata and Bharagava, 2016; Kamel et al., 2005; Angelini et al., 1997; Tsatsaroni and Liakopoulou, 1995).

Synthetic textile dyes are one of the major water and soil pollutants released into the environment by different sources which persists in environment for long period (Nelson and Hites, 1980). These dyes ruthlessly affect all living members of ecosystems by causing severe health hazards in humans and animals such as skin irritation, digestive tract irritation, nausea, vomiting, liver and kidney damage, etc and in plants causes reduced seed germination, root and shoot length and also inhibit the activity of microorganisms contributing soil fertility (Mittal et al., 2010; Senthilkumaar et al., 2006). The variety and quantity of synthetic dyes released into the environment are continuously increasing with the increase in human population and their need for such dyes. Synthetic dye groups such as azo, disperse, acidic, basic triphenylmethane etc. are used in textile dyeing processes in huge quantities, thus releasing large amounts of these dyes into the environment as wastewater (Mani and Bharagava, 2016; Ahmad, 2009; Raffi et al., 1997). Among different classes of synthetic dyes used in textile, dyeing, paper, leather, cosmetic, and food industries, triphenylmethane dyes are the largest and most versatile group of dyes that plays a major role in various industrial applications (Ahmad, 2009; Azmi et al., 1998).

Crystal Violet (CV), (N, N, N^1, N^1, N^{11}, N^{11}-hexa-methyl-para-rosaniline), a triphenylmethane dye, is extensively used in human and veterinary medicine and also as a textile dye in textile processing industries (Azmi et al., 1998; Au et al., 1978). CV is also recognized as gentian violet, an impure form, is a cationic dye and has one dimethylamino group on each phenyl ring (Fig. 1.2). In textile industries, CV is used as a purple dye for coloring cotton and silk fabrics. CV is also used for dyeing nylon, polyacrylonitrile-modified nylon and wool as well as for coloring of plastics, gasoline, varnish, fat, oil and waxes (Daneshvar et al., 2007; Parshetti et al., 2006; Gregory, 1993). In medical solutions, CV is worn as a mutagenic and bacteriostatic agent and also used as an antimicrobial agent for preventing fungal growth in poultry feed

(Mittal et al., 2010; Littlefield et al., 1985). Besides, the medical community, CV is used as a biological stain and is an important ingredient of Gram's stain. The dye is also used as a peripheral membrane disinfectant in humans and is also used as an enhancer for bloody fingerprints (Chakraborty et al., 2011; Mani and Bharagava, 2016).

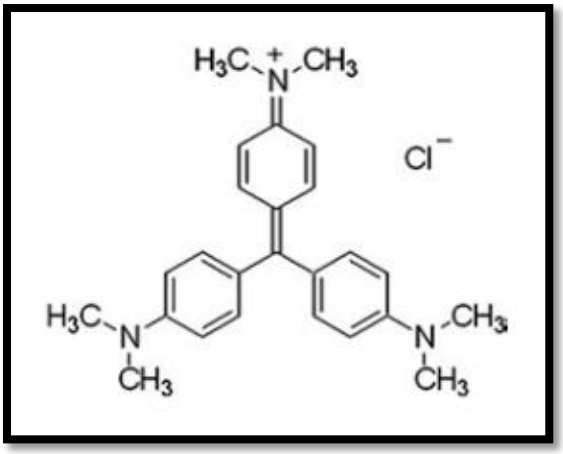


Figure 1.2: Chemical structure of Crystal violet

1.1. Chemical Structure

CV, a typical cationic dye, used as a biological stain, dermatological agent, temporary hair colorant, dyeing cottons, wools and in diverse other profitable textile processes (Shengfang, 2010; Senthilkumaar et al., 2006). CV, also known as hexamethyl pararosaniline chloride, is a basic dye with molecular formula $C_{25}H_{30}N_3Cl$ (Sharma et al., 2011). The IUPAC name of Crystal violet is Tris (4-(dimethylamino) phenyl) methylium chloride of blue-violet color in appearance with 205 °C as melting point and 40 °C as freezing point. It is less soluble in water (1.68 %) and highly soluble in ethanol (13.78 %). CV is found to be even and contrary with strong oxidizing agents, strong acids, combustible and light sensitive. However, the structure and color of crystal violet largely depends on the pH and temperature of medium, which formulates it a valuable acid-base indicator plus an excellent dye (Shah et al., 2013).

The predominant form of CV is the monovalent cation (CV^+) which forms the major structural form and is in solid state as well as in aqueous solution across a broad range of pH values ranging from 1 to 13 (Shah et al., 2013). In addition, the positive charge on central carbon atom of CV is delocalized by the mechanism of resonance of three nitrogen atoms (Sharma et al., 2011). The delocalization of charge across the double bonds in the benzene rings stabilizes the carbonation and is responsible for vibrant purple color of CV dye. In strongly basic solutions, the monovalent cation (CV^+) slowly combines with hydroxide ions and forms an unbiased colorless product (CVOH) (Fig 1.3). Reaction 1

$$CV^+ + OH^- \rightarrow CVOH \qquad \rightarrow \text{Reaction 1}$$

Purple *Colorless*

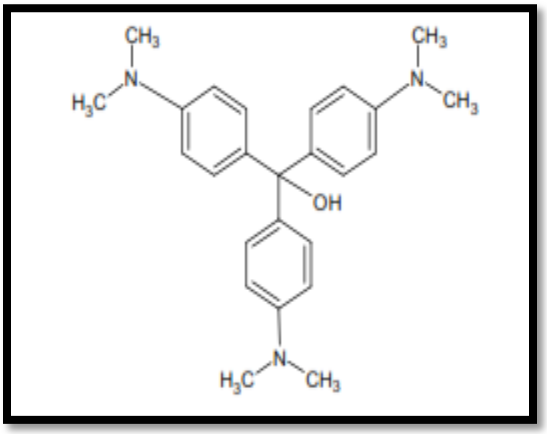


Figure 1.3: Chemical structure of colorless Crystal violet

The rate of this reaction basically depends on the initial concentration of both crystal violet and hydroxide ions which is slower than the classic acid-base proton transfer reactions.

1.2. Production

A number of feasible ways are present for preparing crystal violet dye in laboratory conditions but the original procedure was developed by Caro and Kern (1883), which involves

the chemical reaction of dimethylaniline with phosgene to give 4, 4^1-bis (dimethylamino) benzophenone as an intermediate (Reinhardt and Travis, 2000). Benzophenone was further reacted with dimethylaniline in presence of phosphorus oxychloride and hydrochloric acid.

The dye can also be manufactured by the condensation of formaldehyde and dimethylaniline to give a leuco dye, reduced form of CV as shown in reaction 2 (Gessner and Mayer, 2002; Thetner, 2000).

$$CH_2O + 3\ C_6H_5N\ (CH_3)_2 \rightarrow CH\ (C_6H_4N\ (CH_3)_2)_3 + H_2O \qquad \rightarrow \text{Reaction 2}$$

1.3. Color of dye

When dissolved in water, CV gives blue-violet color with maximum absorbance of 590 nm and an extinction coefficient of 87,000 $M^{-1}cm^{-1}$ (Adams et al., 1914; Cheriaa et al., 2012). The dye color largely depends on the acidity of medium as at a pH of 1.0, the dye is green with absorption maxima at 420 nm and 620 nm, while in a strongly acidic solution (pH of -1) the dye is yellow with absorption maximum at 420 nm.

Different charge on dye molecules results in different colors of dye. In yellow form, all three nitrogen atoms carry a positive charge, of which two are protonated, while in green form two of nitrogen atoms are positively charged, but at neutral pH both the extra protons are lost to the solution leaving behind one positively charged nitrogen atom.

1.4. Sources of CV contamination in environment

Industries such as textile, leather, paint, acrylic, cosmetics, plastics, pharmaceutical manufacturers, etc., use dyes as coloring agent which consumes substantial volumes of water during the processing of products. CV is not only used in coloring plastics, gasoline, varnish, fat, oil and waxes but also used for dyeing nylon, polyacrylonitrile-modified nylon and wool (Daneshvar et al., 2007; Parshetti et al., 2006; Gregory, 1993). As a result of which, a substantial

quantity of colored effluent is generated and discharged into the environment. During the manufacturing and processing of products, approximately 12 % of estimated synthetic dyes are lost in the wastewater. In medical solutions, CV is used as a mutagenic and bacteriostatic agent, as a antimicrobial agent to prevent the fungal growth in poultry feed and also as a biological ingredient in Gram's staining. In humans, CV dye is used as an external skin disinfectant and in enhancing the bloody fingerprints. The high concentrations of CV dye from these sources contaminate/pollute the environment when discharged into water and soil environment without adequate treatments which causes serious environmental and health problems in surrounding areas (Akar et al., 2009; Kiran et al., 2009). Besides these, CV is also one of the major sources of esthetic pollution and eutrophication in environment (Tsai et al., 2004).

1.5. Toxic effects of CV

CV is reported to be highly toxic to mammalian cells as well as to aquatic flora and fauna. It can cause moderate eye irritation, conjunctiva or permanent injury to cornea and causes skin irritation or digestive tract irritation, if absorbed through skin in harmful amount. But in extreme cases, may lead to respiratory and kidney failure (Mittal et al., 2010; Saeed et al., 2010; Ahmed, 2009). In water ecosystems, hampers the sunlight penetration and photosynthetic activity of aquatic flora by reducing dissolved oxygen (Ajao et al., 2011; Cunningham and Siago, 2001)

With various kinds of toxic recalcitrant organic compounds, the CV containing wastewater released from different industries has high BOD and COD values which decreases the significant amount of dissolved oxygen in aquatic environment whereas in terrestrial environment, it adversely affects seed germination as well as root and shoot length (Kagalkar et al., 2010; Rajamohan and Karthikeyan, 2004). Parshetti et al. (2011) have studied the effect of

CV on seed germination, root and shoot length of *Sorghum bicolor*, *Triticum aestivum*, *Vigna radiata* and *Lens culinaris* and found that undegraded CV inhibited 50 %, 60 %, 70 % and 100 % of seed germination in *Sorghum bicolor, Triticum aestivum, Vigna radiata* and *Lens culinaris,* respectively along with significant reduction in root and shoot length. Along with the coloration dilemma, the toxic and carcinogenic nature of CV is another concern. There is no such universally useful method available to treat the dye wastes because of the complex chemical structures of these dyes. Therefore, CV may be regarded as biohazard substance.

Hence, the primary objective of this study was to develop a bacterial consortium for the effective degradation and decolorization of Crystal violet dye, while this study has been divided into seven (7) different chapters.

In objective 1st, the textile wastewater collected from Kanpur industrial area was brought to laboratory for physico-chemical analysis. Further, the effluent was used for the isolation of potent bacteria capable of the effective degradation and decolorization of CV dye. Three most potent morphologically distinct and biochemically tested bacteria were finally opted for advance studies and characterized by 16S rRNA gene sequencing analysis. In objective 2nd, all the three isolated bacteria were used for the development of bacterial consortium.

In objective 3rd, different nutritional and environmental parameters were optimized for the maximum decolorization and degradation of Crystal violet dye. The optimization of CV dye decolorization was done by varying one factor at a time and all the decolorization experiments were performed in triplicates. The effects of different environmental parameters such as temperature (25-45 °C), pH (5-9), inoculum size (1-5 mL), different nutritional parameters such as carbon sources (glucose, sucrose, starch, maltose and fructose) and nitrogen sources (yeast extract, peptone, urea, sodium nitrate and ammonium sulphate) on decolorization were

studied/monitored in flasks containing 100 mL of autoclaved modified MSM-CV amended medium inoculated with fresh culture and incubated under shaking flask conditions. In objective 4th, the decolorization of CV by the developed bacterial consortium was monitored at finally obtained results of all the environmental and nutritional parameters.

In objective 5th, the characterization of ligninolytic enzymes namely laccase (E.C. 1.10.3.2) and Lignin Peroxidase (E.C. 1.11.1.14) was assessed. Further, the enzymes were subjected for protein profiling through SDS-PAGE analysis. In objective 6th, the characterization of textile wastewater and metabolites obtained after bacterial degradation of wastewater as well as metabolic products obtained by bacterial degradation of Crystal violet dye were done through GC/MS/MS analysis. Finally in objective 7th, toxicity experiments were done to analyze the rate of toxicity reductions of Crystal violet dye before and after bacterial treatment. Further, to know the state of art, each part of this thesis is reviewed thoroughly and complete information is discussed in review of literature chapter with following objectives.

Objectives of the study:

1. **Isolation, screening and characterization of bacteria capable of the decolorization of CV**
    - *1.1. Collection of dye wastewater sample and its physicochemical analysis*
    - *1.2. Isolation, screening and purification of CV degrading bacterial strains*
    - *1.3. Decolorization study*
    - *1.4. Characterization of isolated CV decolorizing bacterial strains*
        - *1.4.1. Morphological and biochemical characterization*
        - *1.4.2. Genomic DNA preparation, PCR amplification and 16S rRNA gene sequencing analysis*

2. **Development of a bacterial consortium for the optimum degradation and decolorization of CV**

   2.1. Development of bacterial consortium

   2.2. Dye Decolorization experiments

   2.3. Decolorization of industrial effluent

3. **Optimization of nutritional and environmental parameters for the optimum degradation and decolorization of CV**

   3.1. Effect of static and shaking condition

   3.2. *Effect of physicochemical parameters*

   3.3. Effect of carbon and nitrogen sources

   3.4. Effect of different inoculum concentrations on CV decolorization

   3.5. Effect of initial dye (CV) concentrations

   3.6. Effect of repeated addition of (CV) dye aliquots

4. **Degradation and decolorization of CV by developed bacterial consortium at optimized nutritional and environmental conditions.**

   4.1. Effect of optimized environmental and nutritional conditions on degradation and decolorization of CV

5. **Detection and characterization of enzymes responsible for degradation and decolorization of CV.**

   5.1. Preparation of cell free extract

   5.2. Enzyme assays

   5.3. SDS-PAGE preparation

6. **Characterization of metabolic products by HPLC/GC-MS/LC-MS-MS analysis**

6.1. Characterization of dye from textile wastewater and their metabolic products after degradation by the developed bacterial consortium

6.1.1. Liquid-Liquid extraction

6.1.2. GC-MS analysis

6.2. Characterization of metabolic products of degraded Crystal violet dye by bacterial consortium

6.2.1. Liquid-liquid extraction

6.2.2. GC-MS analysis

7. Toxicity assessment of crystal violet before and after bacterial treatment process

7.1. Phytotoxicity study

7.2. Animal toxicity study

Chapter 2

REVIEW OF LITERATURES

Review of Literature

CV is reported as a recalcitrant dye molecule that persists in the environment for a long period and has toxic effects on aquatic as well as terrestrial life (Azmi et al., 1998; Au et al., 1978). In *vitro* investigations have revealed that CV acts as a mitotic poison, potent carcinogen, potent clastogene and promotes tumor growth in some species of fish (Fan et al., 2009; Cho et al., 2003; Au et al., 1978). Hence, CV is regarded as a biohazard substance. The dye is also found to cause moderate eye irritation, painful sensitization to light, permanent injury to cornea and conjunctiva, since the product contains a cationic dye, which is highly toxic to mammalian cells. Nevertheless, in extreme cases it may lead to respiratory and kidney failures also (Mani and Bharagava, 2016; Ahmad, 2009; Amini and Younesi, 2009; Azargohar and Dalai, 2005). Triphenylmethane dyes are one of the most widely used dermatological agents. Earlier, CV was widely used for the treatment of pinworms through oral route and in topical applications in humans and domestic animals. It has been shown to be effective in controlling the fungal growth under varying conditions and therefore was added to poultry feed to control fungal growth exposing human population directly or indirectly to CV through its extensive medicinal and commercial use (Willian et al., 1978; Kumar and Ahmad, 2011).

The dark colored wastewater from different industries containing CV significantly affect the photosynthetic activity of aquatic plants because of reduced sunlight penetration and may also be toxic to some other aquatic life due to the presence of aromatics, metals, and chlorides etc. (Gill et al., 2002; Liu et al., 2004). The thin layer of discharged dyes formed over the surface of a receiving water body ultimately reduces the photosynthetic activities and dissolved oxygen content in water whereas in agricultural soil, it inhibits the seed germination and growth of crop plants (Kalyani et al., 2008). In recent years, interest in environmental control of dyes has

increased, due to their toxic and genotoxic effects on living organisms as these consists of known carcinogens, such as benzidine and other aromatic compounds. Due to its adverse effects on human health, CV has been listed as hazardous chemical or material and its use has been prohibited in aquaculture and food industry.

2.1. Recent techniques for analysis of Crystal violet dye

Globally a huge quantity of dye effluents is produced from various industries including tannery, paint, paper and textile industries, etc. These dye molecules are quiet stable and its removal is not an easy task. Researchers employed various technology to develop a promising and significant one by which the removal can be done. To remove dye from wastewater, the physico-chemical methods like adsorption, chemical precipitation, flocculation, photolysis, chemical oxidation and reduction, electro-chemical treatment and ion-pair extraction were extensively used, but these methods are insufficient for the complete removal of CV from industrial wastewaters and also produce large quantity of secondary pollutants. However, biological methods are observed as cost-effective and eco-friendly for the treatment of industrial wastewaters, but these methods also has certain limitations. Many scientists are trying to solve the problem of decolorization of colored textile effluent by using various chemicals and microorganisms as a variety of fungi and bacterial strains, which they found to be effective to decolorize Crystal violet dye.

The removal of color is often more imperative than the removal of soluble colorless organic substances from wastewaters, which usually contribute major fraction of BOD. However, it is difficult to degrade and detoxify dyes because of their synthetic origin and complex aromatic molecular structures. A wide array of treatment technique has been developed for the removal of synthetic dyes from water and wastewaters to reduce their impact on the

environment, which are grouped into three major categories: Physical, chemical and biological treatment methods as shown in Table 2.1.

Table 2.1: Advantages and disadvantages of physical, chemical and biological treatment methods used for the treatment of dye wastewaters

Processes	Advantages	Disadvantages	References
Coagulation-Flocculation	Elimination of insoluble dyes	Production of sludge blocking filter	Aguilar et al., 2005; Golob & Ojstrsek, 2005
Adsorption on activated carbon	Suspended solids and organic substances well reduced.	Cost of activated carbon	Slokar & Majcen Le Marechal, 1998
Electrochemical Processes	Capacity of adaptation to different volumes and pollution loads	Iron hydroxide sludge	Carneiro et al., 2005
Reverse osmosis	Removal of all mineral salts, hydrolyzes reactive dyes and chemical auxiliaries	High pressure	Sadrghayeni et al., 1998; Treffry-Goatley et al., 1983; Tinghui et al., 1983
Nanofiltration	Separation of organic compounds of low molecular weight and divalent ions from Monovalent salts.	-	Chakraborty et al., 2003
Ultra filtration & Microfiltration	Low pressure	Insufficient quality of the treated wastewater	Sadrghayeni et al., 1998; Watters et al., 1991
Fenton's Reagent	Effective decolorization of both soluble and insoluble dyes	Sludge production	Hao et al., 2000; Nesheiwat & Swanson, 2000
Ozonation	Applied in gaseous state: no alteration of volume	Short half-life (20 min) of O_3	Muthukumar et al., 2005; Aplin & Wait, 2000
Photochemical	No sludge production	Formation of by-products	Gogate & Pandit, 2004; Forgacs et al., 2004
NaOCl	Initiates and accelerates azo bond cleavage	Release of aromatic amines	Robinson et al., 2001
Stabilization pond	High reduction of solids, BOD and pathogens	Sludge requires proper removal and treatment	Sperling and Chernicharo, 2005
Aerated lagoon	High reduction of BOD and pathogens	Sludge /effluent require further treatment and/or appropriate discharge	Crites and Tchobanoglous, 1998
Trickling filter	Efficient nitrification (ammonium oxidation)	Risk of clogging, depending on pre- and primary treatment	US EPA 2000
Activated sludge	High reduction of BOD and pathogens (up to 99 %)	Sludge/effluent require further treatment and/or appropriate discharge	Crites and Tchobanoglous, 1998
Anaerobic digestion	Digested slurry therefore provides organic fertilization	Heavy metals cannot be destroyed	Cowley and Wase, 1981

2.1.1. Physical Treatment Methods

2.1.1.1. Adsorption method

Due to the higher efficiency for removal of pollutants, adsorption technique has gained much favor than other treatment methods. Adsorption produces economically feasible and high quality product. In recent years, adsorption technique for the treatment of wastewater has become more popular due to its efficiency towards removal of non-biodegradable pollutants. It is a process by which ions or molecules present in one phase (usually gas or liquid) tend to accumulate and concentrate on the surface of another phase (usually solid). Physical adsorption occurs when weak interspecies bonds exist between the adsorbate and adsorbent, while chemical adsorption occurs when strong interspecies bonds are present between the adsorbate and adsorbent due to an exchange of electrons (Bizuneh, 2012). Decolorization occurs mainly of two mechanisms i.e. adsorption and ion-exchange which are influenced by various physico-chemical factors such as pH, temperature, nature of dye, contact time, particle size, sorbent interaction and sorbent surface area (Kumar et al., 1998; Shah et al., 2013). The role of adsorption in water treatment processes and development of new adsorbents has been discussed by Suzuki, (1997). Following are the investigators who evaluated and modernize the treatment systems and role modeling of the findings plays in their development.

Ahmad and Kumar, (2010a) investigated adsorption of malachite green (MG) from aqueous solution onto treated ginger waste (TGW) by batch and column methods. The adsorption of MG was studied at various aspects such as initial dye concentration, pH, temperature and contact time and maximum adsorption was found at pH9. For describing the MG adsorption equilibrium, Langmuir and Freundlich isotherms was employed. The monolayer adsorption capacities were found to be 84.03, 163.9 and 188.6 mg/g at 30, 40 and 50 °C,

respectively. The values of thermodynamic parameters like $\triangle G°$, $\triangle H°$ and $\triangle S°$ indicated that adsorption was spontaneous and endothermic in nature. The pseudo second order kinetic model fitted well in correlation to the experimental results. The mechanism of adsorption was determined by Rechienberg's equation which indicated film diffusion to be the major mode of adsorption.

Ahmad and Kumar, (2010b) also investigated the potential use of bael shell carbon (BSC) as an adsorbent for the removal of Congo red (CR) dye from aqueous solution through various operational parameters such as dye concentration, pH, temperature and contact time. The adsorption kinetics was modeled by first-order reversible kinetics, pseudo-first-order kinetics, and pseudo-second-order kinetics. The dye uptake process obeyed the pseudo-second-order kinetic expression at pH 5.7, 7 and 8 whereas the pseudo-first-order kinetic model was fitted well at pH 9. Langmuir, Freundlich and Temkin adsorption models were applied to fit adsorption equilibrium data but the best-fitted data was obtained with Freundlich model. Thermodynamic study showed that adsorption of CR onto BSC was endothermic in nature and favorable with the positive $\triangle H°$ value of 13.613 kJ/mol.

Chakraborty et al., (2012a) investigated the removal of hazardous Crystal Violet (CV) dye from aqueous solutions through batch adsorption process, which was carried out using H_2SO_4 modified sugarcane bagasse (HMSB). The effects of various parameters such as pH, temperature, initial solution and adsorbent dose were also investigated on the adsorption process. The equilibrium dye uptake was well described by Langmuir isotherm model while the pseudo-second-order kinetic model showed good agreement with the experimental kinetic data. Gibb's free energy change ($\triangle G°$) was spontaneous for all interactions, and the adsorption process

exhibited endothermic enthalpy values. Results suggested HMSB as an effective adsorbent for the removal of CV from wastewater.

Chakraborty et al., (2012b) also investigated the biosorption potential of hen feathers (HFs) for removing hazardous textile dyes, namely Congo red (CR) and crystal violet (CV), from their aqueous solutions in batch and dynamic flow modes of operation. The effects of various biosorption parameters such as pH, temperature, initial dye concentration, feed flow rate and bed height and used Langmuir isotherm model to well describe biosorption equilibrium data. Kinetic studies at different temperatures showed that the rate of biosorption followed the pseudo second-order kinetics well. A thermodynamic study showed that biosorption of both CR and CV was spontaneous and endothermic. Breakthrough time increased with increase in bed height but decreased with increase in flow rate. The Thomas model showed good agreement with the dynamic flow experimental data and the overall results suggested the high-quality applicability of HFs as an efficient biosorbent for removal of carcinogenic textile dyes from aqueous media.

Chakraborty et al., (2012c) investigated the probability of utilizing fish scales as a new biosorbent for removal of textile dyes, namely crystal violet (CV) and methylene blue (MB), from their aqueous solutions in a batch system. The effects of initial solution, pH (2-10), contact time (0-180 min), biosorbent dose (0.5-5 g) and temperature (293-313 K) were studied. The Langmuir isotherm model showed excellent fit to the equilibrium biosorption data of both CV and MB. The maximum dye biosorption capacity of fish scale was calculated as 74.39 and 58.67 mg g^{-1} for CV and MB, respectively at 313 K. Biosorption phenomena of CV and MB by fish scales followed pseudo-second-order kinetics. Activation energy calculated by using the Arrhenius equation suggested the chemisorptions nature of the biosorption processes. A thermodynamic evaluation indicated the spontaneous and endothermic nature of biosorption of

the textile dyes. And finally they concluded fish scales to be an inexpensive and effective biosorbent for the removal of dyes from aqueous solutions.

Li, (2010) studied the dynamic removal of CV by Semi-IPN hydrogels constituted of poly (acrylic acid-acryl-amide-methacrylate) and amylase. They evaluated the adsorption capacity, kinetic and isotherm of CV onto hydrogels and found that the sorption process agreed very well with the Langmuir model and the adsorption of CV depended on length of the side chain, amylose content and pH of the solution.

Mittal et al. (2010) investigated the adsorption performance of materials bottom ash (BA), a power plant waste, and de-oiled soya (DOS), a soya bean industry waste for removal of crystal violet dye through batch and column experiments. In order to describe the impacts of pH, dye concentration, amount of adsorbent, contact time and temperature on dye removal, batch studies were performed. Thermodynamic parameters ($\Delta G°$, $\Delta H°$ and $\Delta S°$) were evaluated for the dye-adsorbent systems, which revealed adsorption process to be endothermic in nature. Pseudo-first and second-order kinetic models have been applied to the experimental data and pseudo-second-order kinetics was found to describe the adsorption of dye (crystal violet) on adsorbents. For bulk removal of dyes, column operations were made and dyes were recovered from eluting HCl solution through the columns and almost 95 % and 78 % of dye was recovered from BA and DOS columns, respectively.

2.1.1.2. Activated Carbon Method

This is the most commonly and effective method among which adsorption treatment process are best available technologies used for the removal of cationic, mordant and acid dyes. It is also known to remove dispersed, vat, direct, pigment and reactive dyes to slightly lesser amount (Raghavacharya, 1997; Rao et al., 1994). Different materials like peat, wood chips, silica

gel, fly ash, corn cobs and rice husks etc. are widely used for the production of commercial activated carbons for the removal of dye from wastewaters because of their widespread availability and low cost (Bansode et al., 2003; Robinson et al., 2001). Moreover, an adsorption process removes the synthetic dye by concentrating it on the surface and retaining its structure unchanged. When the support is to be regenerated the concentrated dye sludge causes a problem for its subsequent disposal into the environment, thus giving major drawback of this method (Forgacs et al., 2004).

2.1.1.3. Membrane Filtration Method

The increasing cost of water and its profligate consumption necessitate a treatment process that is integrated within plant water circuits rather than a subsequent treatment (Machenbach, 1998). Thus, membrane filtration emerged as a potential feasible technology and as an alternative to conventional treatment processes for the removal of CV dyes from wastewater. It has been proved to be a cost effective and less water consumption technology (Koyuncu, 2002). Processes using membranes have provided very interesting possibilities for the separation of hydrolysed dye-stuffs and dyeing auxiliaries that simultaneously reduce the coloration and BOD/COD of wastewaters; usually used to treat the reactive dye bath effluent, because it could potentially reduce the waste volume and simultaneously recover the salt (Sen and Demirer, 2003). The method has the ability to clarify, concentrate and most importantly to separate CV continuously from effluent (Mishra and Tripathy, 1993; Xu and Lebrun, 1991). The method has some special features compared to other methods like resistance to temperature and adverse chemical effects. The advantages of membrane filtration techniques are that it is a quick method with low spatial requirement and the saturate can be reused, but has high capital cost, the possibility of clogging, and membrane replacement affects the applicability of this method.

Usually this technique is applied as a tertiary or final treatment process after biological treatment (Bizuneh, 2012).

2.1.1.4. Ion Exchange Method

Since ion exchangers are inefficient in holding wide range of dyes, thus this method is not widely used for the treatment of dye containing wastewaters. In this technique, the wastewater is passed over the ion exchanger resin until all the available exchange sites are saturated (Bizuneh, 2012). This method is efficiently capable of removing CV but its high cost of organic solvents used for regenerating ion-exchangers limits its use (Mishra and Tripathy, 1993; Robinson et al., 2001; Slokar and Le Marechal, 1998).

2.1.2. Chemical treatment methods

2.1.2.1. Coagulation and Flocculation Method

During past years, Coagulation and flocculation methods are generally used for removing organic materials from wastewaters by partially removing TDS, BOD, COD and color (Aguilar et al., 2005). This process mainly works on the principle of addition of a coagulant followed by a general rapid association between the coagulants and pollutants forming coagulate or flock and subsequently precipitate. The precipitate is then removed by flotation, settling, filtration or other physical techniques by generating sludge, which is further treated to reduce its toxicity (Golob and Ojstrsek, 2005; Mishra and Bajpai, 2005). Although these processes effectively remove the insoluble dyes (Gaehr et al., 1994), its value is doubtful because of the cost of treating the sludge and increasing number of restrictions regarding the disposal of sludge into the environment (Bizuneh, 2012).

2.1.2.2. Photochemical Method

Photocatalytic or photochemical degradation processes results in complete mineralization of dye molecules and thus are gaining importance in the area of wastewater treatment technologies. The photo-activated chemical reactions are characterized by free radical mechanisms initiated by the interaction of photons of proper energy levels with the chemical molecules present in solution/wastewater in presence/absence of catalysts (Gogate and Pandit, 2004). This method causes the degradation of CV molecules into CO_2 and H_2O by UV treatment in presence of H_2O_2 (Peralto-Zamora et al., 1999; Yang et al., 1998). The degradation of CV is caused by the production of high concentrations of hydroxyl radicals, which attack on unsaturated dye molecules resulting in the destruction of chromophore group with no sludge formation and great reduction in foul odours is the additional benefits of this method. However, the rate of dye removal is greatly influenced by the intensity of UV radiation, pH, dye structure as well as the composition of dye bath solution (Slokar and Le Marechal, 1998; Forgacs et al., 2004).

Leticia et al., (2012) reported the synthesis of Sm_2InTaO_7 and its photocatalytic activity on the degradation of CV dye in aqueous solution. They prepared Sm_2InTaO_7 by sol-gel method at 1,200 ℃ and by solid-state reaction at 1,400 °C. They found that Sm_2InTaO_7 prepared by sol-gel method showed better photocatalytic activity than Sm_2InTaO_7 prepared by solid-state reaction for the mineralization of crystal violet dye under UV-light irradiation.

2.1.2.3. Sodium Hypochloride (NaOCl) Method

In this method, NaOCl attacks at amino group of dye molecules by Cl^+ and accelerates the subsequent azo bond cleavage. NaOCl concentration and pH largely affects the rate of decolorization (Slokar and Le Marechal, 1998; Robinson et al., 2001). Apart from this, this method fails to decolorize disperse dyes as it takes longer time to decolorize reactive and metal-

complex dyes. The rate of decolorization increases with an increase in concentration of chloride ions. But its use in higher concentration is not favourable since it's highly reactive in nature, which leads to the production of highly toxic organo-chlorinated compounds (Slokar and Le Marechal, 1997; Banat et al., 1999).

2.1.2.4. Ozonation Method

Ozone is an efficient oxidising agent with high reactivity through which effective degradation of chlorinated hydrocarbons, phenols, pesticides and aromatic hydrocarbons can be achieved (Lin and Lin, 1993; Xu and Lebrun, 1991). The ozone dosage applied to dye effluent largely depends on color intensity and total residual COD to be removed. Ozone decomposition requires high pH value (>10). In alkaline solutions, ozone reacts almost indiscriminately with all compounds present in medium converting them into smaller and biodegradable molecules (Aksu, 2005; Chu and Ma, 2000; Park et al., 1999). The application of ozone in its gaseous state neither increases the volume of wastewater nor sludge generation giving a major advantage to this method (Bizuneh, 2012). However, very short half-life of ozone gives the major drawback to this method as it decomposes in 20 min, thus requiring continuous ozone supply making it very expensive (Gogate and Pandit, 2004; Gosavi and Sharma, 2014; Robinson et al., 2001).

2.1.2.5. Electrochemical Destruction Method

This method was developed in mid 1990's in which destruction of dyes occurs through oxidation and chlorine evolution from NaCl taking place at anode while hydrogen evolution and OH^- ion formation occurs at cathode during electrolysis process. This method demands very little or low consumption of chemicals, leading no/very less sludge generation. The breakdown metabolites are generally not hazardous leaving it safe for treated wastewaters to be released into the water ecosystem. However, the high cost of electricity is the major limitation for the

application of this method at industrial scale (Ogutveren and Kaparal, 1994; Pelegrini et al., 1999).

2.1.2.6. Fenton Reagent Method

The oxidation system based on the Fenton's reagent has been widely used for the treatment of both organic and inorganic pollutants (Beekeepers, 2000). The Fenton's reagent can be used for the effective removal of color and absorbable organic halides from the refining wastewater (Mauskan, 2007). Besides heavy metals, which are caused by metal-complex dyes can be precipitated on the neutralization step with iron oxide. Fenton oxidation process can also be used to decolorize a wide range of dyes because as compared to ozonation, it is relatively cheap and result significant reduction in COD values (Bizuneh, 2012; Park et al., 1999). Refining with fenton reagent is more advantageous than the other methods such as flocculation, precipitation, air flotation, filtration, etc. in which H_2O_2 is used (Sewekow, 1993). Fenton oxidation is limited only to the fact that textile wastewaters usually have high pH, whereas Fenton process requires low pH because at higher pH, large volumes of waste sludge is generated due to the precipitation of ferric iron salts and the process loses its effectiveness (Table 2.2).

Table 2.2: The advantages and disadvantages of the Fenton reagent uses

Advantages	Disadvantages
The first investment cost is low	Additional chemical cost
Decrease in poison for biological refining	Removing mud cost
It can be used in different processes	The potential of polymerization reactions
Getting ineffective of toxic and resistant compounds	Continuing of normal chemical reactions
Sudden beginning time	Potential corrosion problems
Low hydraulic waiting period (1-2 h)	Controlling foam
Chemical mud production	Special safety thoughts

Adopted from Beekeepers, (2000)

Wu et al., (2010) investigated the degradation of CV dye by Fenton oxidation process and found that can be degraded effectively through UV-Vis spectrogram. They elucidated degradation kinetics of CV on the experimental data. The degradation of CV obeys the first-order reaction kinetics. The kinetic model can be described as k=1.5 exp(-(7.5)/(RT))[H_2O_2](0)(0.8718)[Fe^{2+}](0)(0.5062). According to the IR spectrogram, it is concluded that benzene ring of CV has been destroyed by Fenton oxidation. The result will be useful in treating dyeing wastewater containing CV by Fenton oxidation process.

In this way, the various physico-chemical treatment methods are thus found to be effective, but their application is limited due to the excess usage of chemicals, sludge generation, subsequent disposal problems, high installation as well as operating costs (Bizuneh, 2012; Vandevivere et al., 1998). Therefore, as a viable alternative, biological treatment method have gained increasing interest due to their low cost, generation of less amount of sludge, and most importantly is their environment friendly nature (Banat et al., 1996).

2.1.3. Biological Treatment Methods

Bioremediation is a process where removal of pollutants and xenobiotics is achieved by using biological systems. Over the past decades, many microorganisms (bacteria, fungi, yeast, actinomycetes and algae) have been reported for their ability of degrading CV dye. The effectiveness of biological treatment methods largely depends on the adaptation and activity of selected microorganisms as well as on pH, temperature, aeration, media composition etc. (Bumpus and Brock, 1988; Kwasniewska, 1985; Yatome et al., 1991). As CV is a stable and long lasting colorant, it is usually not easily degraded. Nevertheless, many researchers have also reported either the partial or complete biodegradation of CV either by pure or mixed cultures of bacteria, fungi and algae (Table 2.3).

Table 2.3: Microorganisms used by various workers for the decolorization of CV dye wastewater

Microorganisms	**Decolorization (%)**	**References**
Agrobacterium radiobacter	100	Parshetti et al., (2011)
Agrobacterium radiobacter, *Bacillus* spp., *Sphingomonas paucimobilis*, *Aeromonas hydrophila*	91	Cheriaa et al., (2012)
B. subtilis ETL-2211	90	Shah et al., (2013)
Mucor mucedo	78	Moturi and Singara, (2009)
Trametes versicolor	72	Moturi and Singara, (2009)
Polyporus elegans	73	Moturi and Singara, (2009)
Lenzites betulina	75	Moturi and Singara, (2009)
Nostoc linckia	72	Sharma et al., (2011)
Pleurotus ostreatus	92	Kunjadia et al., (2012)
Bacillus spp.	99	Azmi and Banerjee, (2001)
N. coralline	98.3	Azmi et al., (1998)

2.1.3.1. Degradation Mechanism by Bacteria

The ability of bacteria to metabolize CV dye has been investigated by several researchers (Ahmad and Mondal, 2012a, b; Parshetti et al., 2011; Ahmad et al., 2010; Mondal et al., 2010;

Chen et al., 2007; Yatome et al., 1991). Earlier, it was predicted that CV is relatively resistant to biodegradation in the environment because the most important environmental factor affecting CV biodegradation was the pH (Michaels and Lewis, 1986).

Hamid et al., (2015) analyzed the ability of bacterial strains isolated from textile mill site for decolorization of dye effluent. The isolated strains were finally identified by 16S rDNA sequence analysis as *Bacillus sp.* and were used in bioremediation. The physicochemical characterization of effluent generated before and after treatment was also analyzed. Samples were analyzed in UV spectrophotometer and showed the absorption maxima at wavelength of 668 nm. The bacterial strain was found to have sorptive capacity, immobilized on nutrient agar medium. The effluent was treated in a flask containing minimum salt medium, 20 % dye effluent and 5 % (w/v) of bacterial pellet. The flask was placed in an incubator shaker at 37 °C and 200 rpm. After inoculation the sample was analyzed in visible spectrophotometer after every six hours and it was found that 90 % decolorization and 70 % reduction in COD was achieved after 24 hours. Heavy metals were also biosorpted. It was concluded that the isolated bacteria represented a promising application in bioremediation process of textile industrial effluent and possible reusability of the cells for its commercial application can be achieved.

Sriram and Reetha, (2015) evaluated different physicochemical parameters such as pH, temperature, Electrical Conductivity (EC), Total Solids (TS), Total Dissolved Solids (TDS), Total Suspended Solids (TSS), Dissolved Oxygen (DO), Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), total alkalinity, total hardness and chloride content of textile dye effluent. The experiment was carried out to degrade the dye effluents by using five screened bacterial isolates i.e. *Pseudomonas aeruginosa, Bacillus cereus, Bacillus subtilis, Pseudomonas fluorescens and Escherichia coli* from textile dye effluents.

Shah et al., (2013) isolated bacterial strain *Bacillus subtilis* ETL-2211 from textile effluent samples collected from textile industries of Ankleshwar, Gujarat, India for the removal of Crystal violet dye. They selected this bacterial strain on the basis of rapid crystal violet dye (100 mg/L) decolorization and later based on phylogenetic and phenotypic characterization. They compared the decolorization efficiency of *B. subtilis* ETL-2211 across a wide range of pH 5-9. They recorded maximum decolorization at pH 8.

Al-Garni et al., (2013) reported that Crystal violet can be efficiently decolorized by *P. fluorescens* and *Corynebacterium* sp. *P. fluorescens* alone was sufficient to decolorize CV up to 150 µg/mL after 92 hr of incubation and *Corynebacterium* sp alone was sufficient to decolorize concentrations of CV up to 50 µg/mL after 72 hr of incubation. But the mixed culture of both *P. fluorescens* and *Corynebacterium sp.* was found to decolorize CV more than they did single. They found that the decolorization period of mixed culture was reduced more than 37 % and the decolorization rate (µg/hr) increased upto 59 %.

Chen et al., (2007) have elucidated the biodegradation pathway of CV by *Pseudomonas putida* by using High Performance Liquid Chromatography fitted with Atlantis dC18 column (Fig. 3). They identified nine metabolic products resulting from the CV degradation process and concluded that the dye is broken down by the means of demethylation yielding mono-, di-, tri-, tetra- penta- and hexademethylated end products. This biodegradation pathway differs from that of *Nocardia coralline* and *Bacillus subtilis*. However, the maximum CV biodegradation efficiency of *P. putida* achieved in the study was 78.5 % at the concentration of 60 µM. Many bacteria, which were used to degrade CV, were found to be toxic to many other microorganisms (Michaels and Lewis, 1986). However, Chen et al., (2007) have suggested that CV is non-toxic to *P. putida* and it has the potential to remove CV from the environment without producing toxic

by-products and they proposed the demethylation pathway of CV by *P. putida* depicted in Fig. 2.1. They also investigated the effect of pH on the biodegradation of CV and found that the optimum CV degradation takes place at pH 7.5, but at pH higher or lower than 7.5 and 6.0, the CV degradation capability of *P. putida* was significantly reduced.

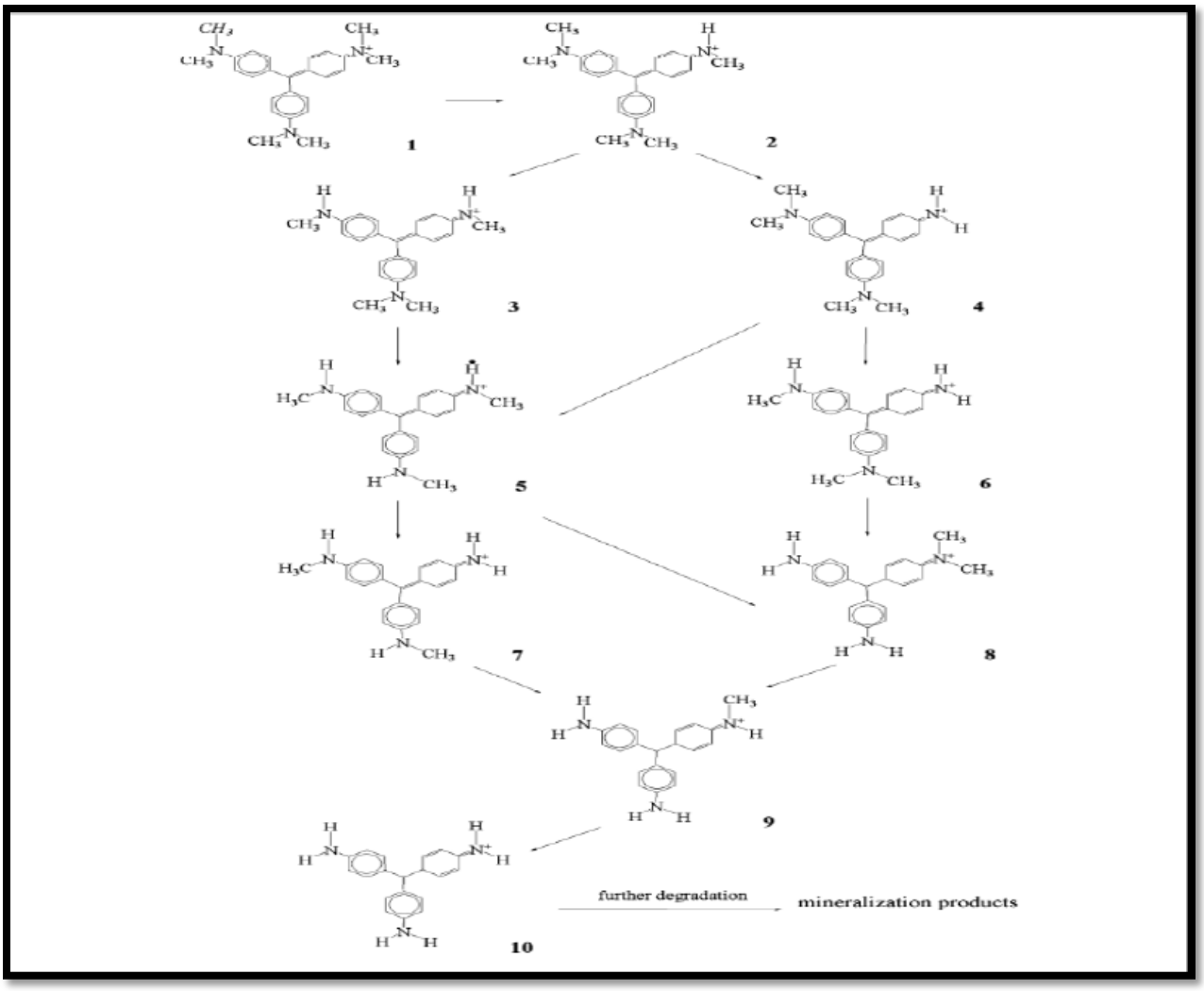


Figure 2.1: Proposed pathway of Crystal violet demethylation by *P. putida* (Chen et al., 2007) 1: N, N, N^1, N^1, N^{11}, N^{11}-hexamethyl pararosaniline; 2: -N, N-dimethyl-N^1, N^1-dimethyl-N-methylpararosaniline; 3: -N, N-dimethyl-N^1-methyl-N^{11}-methyl pararosaniline; 4: -N, N-dimethyl-N, N^1-dimethyl pararosaniline; 5: -N-methyl-N-methyl pararosaniline; 6: -N, N-dimethyl N^1-methylpararosaniline; 7: -N-methyl-N^{11}-methyl-N^{11}-methyl pararosaniline; 8: -N, N-dimethyl pararosaniline; 9: -N-methyl pararosaniline; 10: -Pararosaniline

Yatome et al., (1991) have studied the degradation of CV by *Bacillus subtilis* IFO 13719 and found that CV was remarkably decolorized within the 8 h of incubation period with low cell growth, but it was completely decolorized in 24 h when the cell growth was higher. They also tried some other bacteria like *E. coli*, but bacterium was not able to decolorize CV, even the cells were growing remarkably. Similar results were also observed with two other cultures namely *Pseudomonas cepacia* and *Pseudomonas cruciviae*. Roth et al., (1992) have isolated 21 hydrophobic oleophilic bacterial strains having decolorization activity, but *Mycobacterium* was found to be most active strain for CV decolorization.

2.1.3.2. Degradation Mechanism by Fungi

The role of fungi, their enzymes and their potential use in degradation and detoxification of CV has been well reported and recognized (Assadi et al., 2003; Claus et al., 2002; Mielgo et al., 2001; Ferreira et al., 2000). Concerning the dye degradation, the most widely used fungi are ligninolytic fungi among which wood degrading white rot fungi are found to be very effective in treatment of colored textile effluents because of the production of enzymes such as lignin peroxidase, manganese peroxidase and laccase that can degrade organic pollutants (Toh et al., 2003). Many authors have found these white rot fungi capable to oxidize an array of phenolic, non-phenolic, soluble and in soluble dyes (Libra et al., 2004).

Rani et al., (2014) studied the biodegradation and detoxification of dyes, Malachite green, Nigrosin and Basic fuchsin by using two fungal isolates *Aspergillus niger* and *Phanerochaete chrysosporium*, isolated from dye effluent contaminated soil. Three methods were selected for biodegradation, viz. agar overlay and liquid media methods; stationary and shaking conditions at 25 °C. *Aspergillus niger* recorded maximum decolorization of dye Basic fuchsin (81.85 %) followed by Nigrosin (77.47 %), Malachite green (72.77 %) and dye mixture (33.08 %) under

shaking condition whereas, *P. chrysosporium* recorded maximum decolorization of Nigrosin (90.15 %) followed by Basic fuchsin (89.8 %), Malachite green (83.25 %) and mixture (78.4 %). The selected fungal strains performed better under shaking conditions compared to stationary method. Moreover, the inoculation of fungus also brought pH of dye solutions to neutral from acidic. Seed germination bioassay study exhibited that when inoculated dye solutions were used, seed showed germination while uninoculated dyes inhibited germination even after four days of observation. Similarly, microbial growth was also inhibited by uninoculated dyes. The excellent performance of *A. niger* and *P. chrysosporium* in biodegradation of textile dyes of different chemical structures suggests and reinforces the potential of these fungi for environmental decontamination.

Chakraborty et al., (2013) isolated a novel white rot fungus *Alternaria alternata* CMERI F6, which decolorized 99.99 % of 600 mg/L Congo red within 48 h in yeast extract-glucose medium at 25 °C, pH 5 and 150 rpm. Physicochemical parameters like carbon and nitrogen sources, temperature, pH and aeration were optimized to develop faster decolorization process. Dye decolorization rate was maximal (20.21 mg/L h) at 25 °C, pH 5, 150 rpm and 800 mg/L dye, giving 78 % final decolorization efficiency. Scanning electron microscopy and X-ray Diffraction analysis revealed that the fungus become amorphous after dye adsorption. HPLC and FTIR analysis of extracted metabolites suggested that decolorization occurred through biosorption and biodegradation. Thermogravimetric analysis (TGA), differential thermal analysis (DTA) and acid-alkali and 70 % ethanol treatment revealed the efficient dye retention capability of the fungus. The foregoing results justified the applicability of the strain in removal of Congo red from textile wastewaters and their safe disposal.

Bergsten-Torralba et al., (2009) investigated the capacity of *Penicillium simplicissimum* INCQS 40211 in decolorization and detoxification of textile dyes Reactive Red 198 (RR198), Reactive Blue 214 (RB214), Reactive Blue 21 (RB21) and the mixture of three dyes (MXD). The dye RB21, a phthalocyanine, was totally decolorized in 2 days, and others, monoazo RR198, diazo RB214 and MXD were decolorized after 7 days by *P. simplicissimum*. Initially, the dye decolorization involved dye adsorption by biomass followed by degradation. The acute toxicity after fungal treatment was monitored with the microcrustacean *Daphnia pulex* and measured through Effective Concentration 50 % (EC). *P. simplicissimum* reduced efficiently the toxicity of RB21 from moderately acutely toxic to minor acutely toxic and it also reduced the toxicity of RB214 and MXD, which remained minor acutely toxic. Nevertheless, the fungus increased the toxicity of RR198 despite of the reduction of MXD toxicity, which included this dye. Thus, *P. simplicissimum* INCQS 40211 was efficient to decolorize different textile dyes and the mixture of these with a significant reduction of their toxicity. In addition this investigation also demonstrated the need of toxicological assays associated to decolorization experiments.

Vasdev et al., (1995) have reported the decolorization of three triphenylmethane dyes including CV by three birds nest fungi namely *Cyathus bulleri*, *C. stercoreus* and *C. striatus*. They observed that *C. bulleri* have both laccase and ligninase enzyme activity, but the laccase activity was higher than ligninase activity during the dye decolorization. They also reported the decolorization of CV by using ultra-filtered and dialyzed extracellular culture filtrate of *C. bulleri*, which could be due to the presence of active laccase enzyme in ultra-filtered and dialyzed extracellular fluid. However, *C. bulleri* was found capable to decolorize CV dye up to 90 μM, whereas *P. chrysosporium* has shown to decolorize the dyes to a much lesser extent i.e. 12.3 μM (Bumpus and Brock, 1988).

Yesilada, (1995) has studied CV decolorization by three different white-rot fungi such as *Coriolus versicolor*, *Funalia trogii*, and *Phanerochaete chrysosporium* and one brown-rot fungus, *Laetiporus sulphureus* and found that CV undergoes oxidation process only in presence of H_2O_2 suggesting the involvement of an H_2O_2-dependent enzyme produced by tested fungal strains.

Das et al., (1995) have studied the CV decolorization in a column bioreactor using *P. chrysosporium.* The decolorization process was performed in a glass column bioreactor (31 cm × 5 cm) with an eight tier stainless steel inoculum holder, through which the dye containing medium was recirculated by a peristaltic pump. During the process, CV was passed through column at a concentration of 0.002 % with a recycling rate of 20 mL min^{-1} at 30 °C and results have revealed that almost 92 % CV was decolorized in 82.4 h in recycled medium as compared to 64 % CV decolorization in shake flasks condition in 17 days of incubation period.

In 1988, Bumpus and Brock have studied the CV biodegradation by using white rot fungus *Phanerochaete chrysosporium* and found that in medium, the CV disappeared jointly with the appearance of three metabolic products: N, N, N^1, N^{11}-penta-, N, N, N^1, N^{11}-tetra- and N, N^1, N^{11}-trimethylpararosaniline. The purified ligninase enzymes were found to catalyze the N-methylation of CV, which proved that the lignin-degrading enzyme system is mainly responsible for the CV degradation.

2.1.3.3. Degradation Mechanism by Yeasts

Only few reports are available on the degradation of CV by yeast. Kwasniewska, (1985) has studied the biodegradation of CV by some oxidative red yeast and found that these oxidative yeasts such as *Rhodotorula sp.* and *Rhodotorula rubra* were capable of CV degradation in liquid broth, which was measured in terms of decrease in absorbance indicating the continuous

decrease in CV concentration and after 4 days of incubation period, the absorbance of culture medium at 600 nm became non-measurable indicating the complete degradation of CV by both the oxidative red yeasts. It was also observed that the fermentative yeast *S. cerevisiae* did not degrade CV in liquid medium even after a prolonged incubation period of 30 days.

Jafari et al., (2013) isolated the yeast (strain JKS4), which had high ability to decolorize different azo dyes. Under aerobic condition, the yeast strain showed 85.7 % decolorization at 200 mg/L Reactive Black 5 (as a model azo dye), 1 % (w/v) glucose concentration and 35 °C after 24 h. All the examined dyes were extensively decolorized (53.35-97.9 %) after 24 h. With elongated incubation period, complete decolorization was observed in presence of all dyes.

Aracagok and Cihangir, (2013) used *Yarrowia lipolytica* NBRC 1658 yeast strain, which could decolorize Reactive Black 5 effectively through biodegradation. The optimum conditions such as initial pH, glucose concentration, nitrogen concentration and initial dye concentration were determined. Correlations between decolorization and laccase and manganese peroxidase activities were investigated, but neither laccase nor manganese peroxidase (MnP) activities were determined in culture mediums. *Y. lipolytica* could decolorize 97 % of 50 mg/L RB5 dye within 24 hours and could tolerate upto 300 mg/L of dye. It was found that decolorization occurred during the exponential growth phase. Aerobic batch conditions with 5 g/L glucose and 1 g/L ammonium sulphate at pH 7 were considered to be the optimum decolorizing conditions.

Qu et al., (2012) isolated a yeast strain from sea mud with strong abilities to decolorize various azo dyes aerobically. The strain designated as TCL was identified as *Pichia sp.* on the basis of 18S rDNA analysis. More than 90% of Acid Red B (100 mg/L) was decolorized within 10 h in Martin Broth at 30 °C and strain TCL could tolerate up to 1000 mg/L of the dye. Meantime, the effects of different physicochemical parameters (media, concentrations of

glucose, NH_4Cl, initial dye and NaCl) were investigated to improve the removal efficiency. The significant biodegradation process of Acid Red B rather than inactive surface adsorption was confirmed by UV-Vis, HPLC analysis and colorless microbial cells. In addition, the metabolic products and partial degradation pathway were proposed with the help of HPLC-MS analysis.

To the best of our knowledge, it was the first time that a yeast strain of *Pichia sp.* has been reported with the excellent decolorizing ability against azo dyes under shaking conditions. This work conferred the utilization possibility of strain TCL in the biological treatment of dyeing wastewater.

2.1.3.4. Degradation Mechanism by Actinomycetes

Yatome et al., (1991) presented the first report on CV biodegradation by actinomycetes. They studied the CV decolorization by two actinomycetes namely *Nocardia coralline* and *N. globerula* and found that the decolorization activity of both the actinomycetes was intracellular and the dye was completely decolorized within 24 h. Further, in 1993, they published a report on CV degradation by *Nocardia coralline,* in which they identified Michler's ketone as the main degradation product by Gas chromatography-mass spectrometry (Fig. 2.2). They also observed that the decolorization rate was dependent upon the initial concentration of *N. coralline* in medium. Besides CV decolorization, *N. coralline* was also found capable to decolorize methyl violet, ethyl violet, basic fuchsin and Victoria blue to a great extent (Table 2.4).

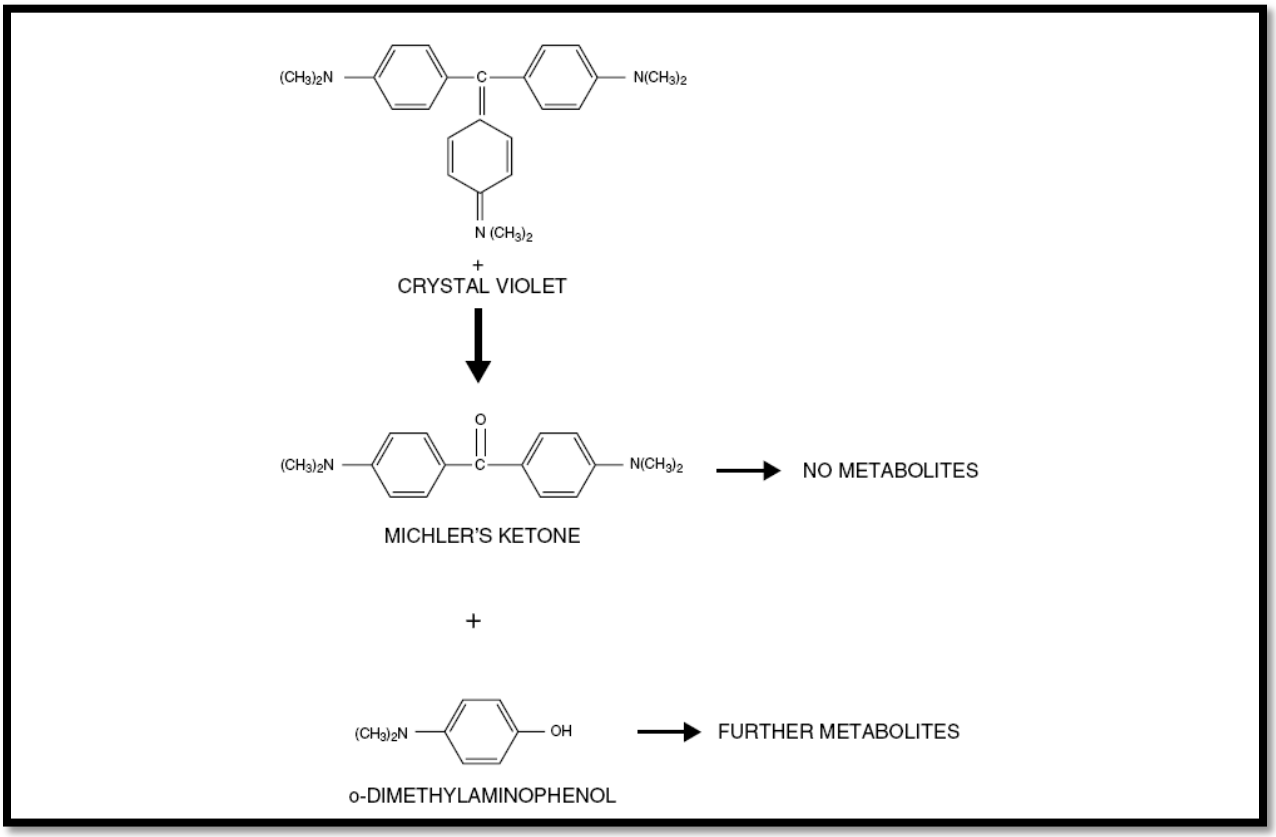


Figure 2.2: Degradation pathway of CV by *Nocardia coralline* (Yatome et al., 1993)

Table 2.4: Decolorization of different triphenylmethane dyes by *N. coralline*

Dyes	**λ_{max}**	**Half life of decolorization (min)**	**Maximum decolorization (%)**
Crystal violet	590	50	98.3
Methyl violet	590	60	71.8
Ethyl violet	600	480	59.8
Basic fuchsin	555	30	70.0
Victoria violet	620	20	33.0

(Adopted from Yatome et al., 1991)

2.2. Effects of nutritional and environmental factors on the microbial degradation and decolorization of CV

The activity of enzymes are greatly influenced by environmental factors such as pH, temperature, aeration and nutrients concentration as these play a vital role in the microbial degradation of industrial wastes. Several studies have been made by various workers to understand the role of various environmental factors in microbial degradation and detoxification of CV for environmental safety (Moturi and Singara, 2009).

Shah et al., (2013) have studied the effect of different carbon and nitrogen sources on degradation and detoxification of CV by varying 1 % each in medium and found dextrose as best carbon source resulting 92 % CV decolorization followed by starch and mannose with 80 % and 65 % decolorization, respectively at the end of 24 h incubation period. However, the decolorization efficiency decreased dramatically with dulcitol, mannitol, lactose, d-xylose and sucrose resulting only 52 %, 45 %, 35 %, 30 % and 25 %, decolorization respectively. Further, the maximum decolorization 90 % was achieved when peptone was used as nitrogen source whereas malt extract has resulted only 15 % decolorization of CV.

In addition, Parshetti et al., (2011) have observed 100 % decolorization of CV by using yeast extract and NH_4Cl within 5 hr of incubation period at a concentration of 1 % and 0.1 %, respectively. However, in presence of urea, peptone and malt extract, the culture exhibited 87.5 %, 81.25 % and 38.23 % CV decolorization, respectively whereas the presence of sucrose, lactose and glucose at 1 % concentration of each, the culture showed 90 %, 39.23 % and 36.17 % decolorization of CV, respectively.

Shah et al., (2013) while studying the decolorization efficiency of *B. subtilis* ETL-2211 across a wide range of pH have found that CV decolorization was maximum (90 %) at pH 8 and

minimum at acidic pH. They also observed that at neutral pH, the strain decolorized 80 % CV, whereas at pH 6 and 9, it was only 45 % and 40 %, respectively. The percentage decolorization of CV decreased markedly at pH 5 (10 %) due to acidic conditions indicating that the maximum growth and decolorization of CV occur at pH 8. Shah et al., (2013) have also studied the effects of temperature on CV decolorization and observed that the dye decolorization activity of strain decreased with increase in temperature. However, the optimum decolorization of CV (95 %) was achieved at 35 °C and least (25 %) at room temperature (RT) and further increase in temperature has resulted continuous decrease in percent decolorization of CV as at 37, 40, 45 and 50 °C to 85, 70, 55 and 25 %, respectively at the end of 24 h of incubation period. It was also depicted that the percent removal of dye decreases with increase in temperature, which may be due to the weakening of bonds between dye molecules and the binding sites of the adsorbent (Chowdhury and Saha, 2010).

Chen et al., (2007) while studying the biodegradation of CV have also observed that environmental factors such as pH and temperature play an important role in the microbial degradation and decolorization of CV. They also examined the effect of pH only in slightly acidic and slightly alkaline conditions in order to avoid possible chemical degradation of dye molecules and found that the optimal pH for CV biodegradation by *P. putida* was 7.5 and resulted 78.5 % CV decolorization. The degradation capacity of *P. putida* decreased significantly at pH higher and lower than 7.5 and 6.0, respectively. They also observed that the optimum temperature for CV degradation was 37 °C by *P. putida*.

Sharma et al., (2011) also studied the effect of pH on dye removal at different pH (4.0-9.0) at 100 mg/L initial dye concentration and found that the maximum dye removal takes place at pH 8.0 and temperature 25 °C.

Moturi and Singara, (2009) studied the effect of pH on decolorization of CV by using four fungal species such as *Polyporus elegans*, *Trametes versicolor*, *Lenzites betulina* and *Mucor mucedo* and found that these fungal strains were capable to decolorize CV upto 73, 72, 75 and 78 % at pH 6.0, 4.5, 4.0 and 2.5, respectively. Thus, all the above studies showed that the maximum decolorization (95-99 %) of CV can be achieved at pH 7.5, temperature 35 °C and shaking condition (140 rpm) by using 1 % glucose and 1 % peptone as a carbon and nitrogen source, respectively.

2.3. Enzymes involved in the degradation and detoxification of Crystal violet dye

In recent years, the use of microbes and plants for the degradation and detoxification of recalcitrant organic pollutants have gained significant recognition as a viable alternative to the existing physical and chemical treatment methods (Movahedin et al., 2006; Franciscon et al., 2009; Kagalkar et al., 2009). Moreover, biological treatment methods are found capable to degrade an array of recalcitrant organic pollutants due to the involvement of different kinds of enzymes. Hence, both intracellular and extracellular enzymes are being explored as biochemical means of wastewater treatment (Nelson and Cox, 2004). Thus, the enzymatic treatment may be an innovative approach for the effective treatment of industrial wastewaters containing different types of recalcitrant organic pollutants by removing them either through precipitation or transforming them into other products (Mugdha and Usha, 2012). Enzymes offered several advantages such as greater specificity, better standardization, easy handling and storing and no dependence on bacterial growth rates.

The structural complexity of CV and very little knowledge about the enzymatic system involved in its degradation has created many challenges to elucidate the mechanism involved in

the enzymatic degradation of CV. However, various workers have studied the enzymes from various microorganisms involved in CV degradation.

Most of the enzymes causing degradation of CV belong to the class of oxidoreductases, which catalyses the electron transfer reactions (Mugdha et al., 2011). Sometimes, a substrate required may not be oxidized directly by enzyme, if the redox potential of substrate is higher than that of enzyme. This phenomenon has been observed in the case of laccase enzymes, which require mediators like 2, 2^1-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) to act as an intermediate substrate for enzyme (Kunamneni et al., 2007, 2008). Many processes have been developed based on the laccases due to their potential in degradation of dyes of diverse chemical structures (Rodriguez et al., 2006; Wesenberg et al., 2003). Laccases are copper-containing enzymes that catalyze the oxidation of electron rich substrates. Laccase alone has a limited effect on bioremediation process due to its specificity for phenolic subunits in lignin. Phenolic compounds were shown to be efficient laccase mediators (Camarero et al., 2005). Laccase utilizes molecular oxygen for degradation purposes. Whereas peroxidase enzymes such as lignin peroxidase and manganese peroxidase acts as electron acceptor, which require hydrogen peroxide or alkyl, peroxide that are not specific towards the electron donor in redox reactions that they catalyze (Moturi and Singara, 2009; Kersten et al., 1990).

The role of fungi and their enzymes in degradation and detoxification of organic pollutants has been well reported globally because of their wide versatility and broad range of substrates (Moturi and Singara, 2009; Assadi et al., 2003; Claus et al., 2002; Mielgo et al., 2001; Ferreira et al., 2000). Several studies have demonstrated the ability of fungal biomass and purified enzymes to degrade and decolorize a category of different types of organic pollutants (Wesenberg et al., 2003). White rot fungi were able to degrade CV using lignin peroxidase (LiP)

and manganese dependent peroxidase enzymes (MnP) (Muragesan et al., 2007). Other enzymes used for this purpose include H_2O_2 producing enzymes such as glucose-2-oxidase along with laccase and phenol oxidase enzymes (Husain, 2009). The production of enzymes such as lignin peroxidase, manganese peroxidase and laccase by various fungal strains during the decolorization of CV is well reported (Moturi and Singara, 2009; Parshetti et al., 2010; Telke et al., 2008).

Moturi and Singara, (2009) studied the production of ligninolytic enzymes (lignin peroxidase, manganese peroxidase and laccase) by fungal strains *Polyporus elegans*, *Trametes versicolor*, *Lenzites betulina* and *Mucor mucedo* during decolorization of CV. They observed high production of lignin peroxidase during initial ten days which reduced in 15 days of incubation period. Enzyme production was highest in *Polyporus elegans* while lowest was recorded in *Mucor mucedo* whereas *Trametes versicolor* and *Lenzites betulina* showed maximum production of this enzyme in 5 and 10 days, respectively (Moturi and Singara, 2009). Additionally, maximum quantity of manganese peroxidase enzyme was produced by *Trametes versicolor* in 5 and 10 days of incubation period whereas *Mucor mucedo* showed the minimum production of manganese peroxidase in 5 days of incubation and remained fail to secrete the enzyme in 10 and 15 days. *Polyporus elegans* and *Lenzites betulina* also showed the maximum production of manganese peroxidase enzyme.

Moturi and Singara, (2009) found *Mucor mucedo* totally unable to secrete laccase enzyme during incubation time. However, the maximum laccase enzyme production was shown by *T. Versicolor*, moderate quantity of enzyme by *P. elegans* and lower quantity by *L. betulina* (Moturi and Singara, 2009). The major drawbacks of the enzymatic treatment are the inactivation

of enzymes by the conditions normally found in the textile wastewater as well as the high cost of enzyme production has limited its application at industrial scale.

Parshetti et al. (2011) have projected a theoretical metabolic trail of enzymatic biodegradation of CV by *A. radiobacter*. The degradation of CV was analyzed by Gas Chromatography and Mass Spectroscopy (GC-MS) and enzyme activities. Five intermediate compounds such as, *N*, *N*, N^1, N^{11}-tetramethylpararosaniline, [*N*,*N*-dimethylaminophenyl] [*N*-methylaminophenyl] benzophenone, 4-methyl amino phenol, *N*, *N*-dimethylaminobenzaldehyde and phenol were detected during the CV degradation. During the CV degradation, the *N*, N^1, N^{11}-tetramethyl pararosaniline was first wrecked into [*N*, *N*-dimethylaminophenyl] [*N*-methylaminophenyl] benzophenone and 4-methyl amino phenol and then in the next step [*N*,*N*-dimethylaminophenyl] [*N*-methylaminophenyl] benzophenone was further degraded into *N*, *N* dimethylaminobenzaldehyde and 4-methyl amino phenol and finally phenol was obtained as the final degradation product of CV (Fig. 2.3).

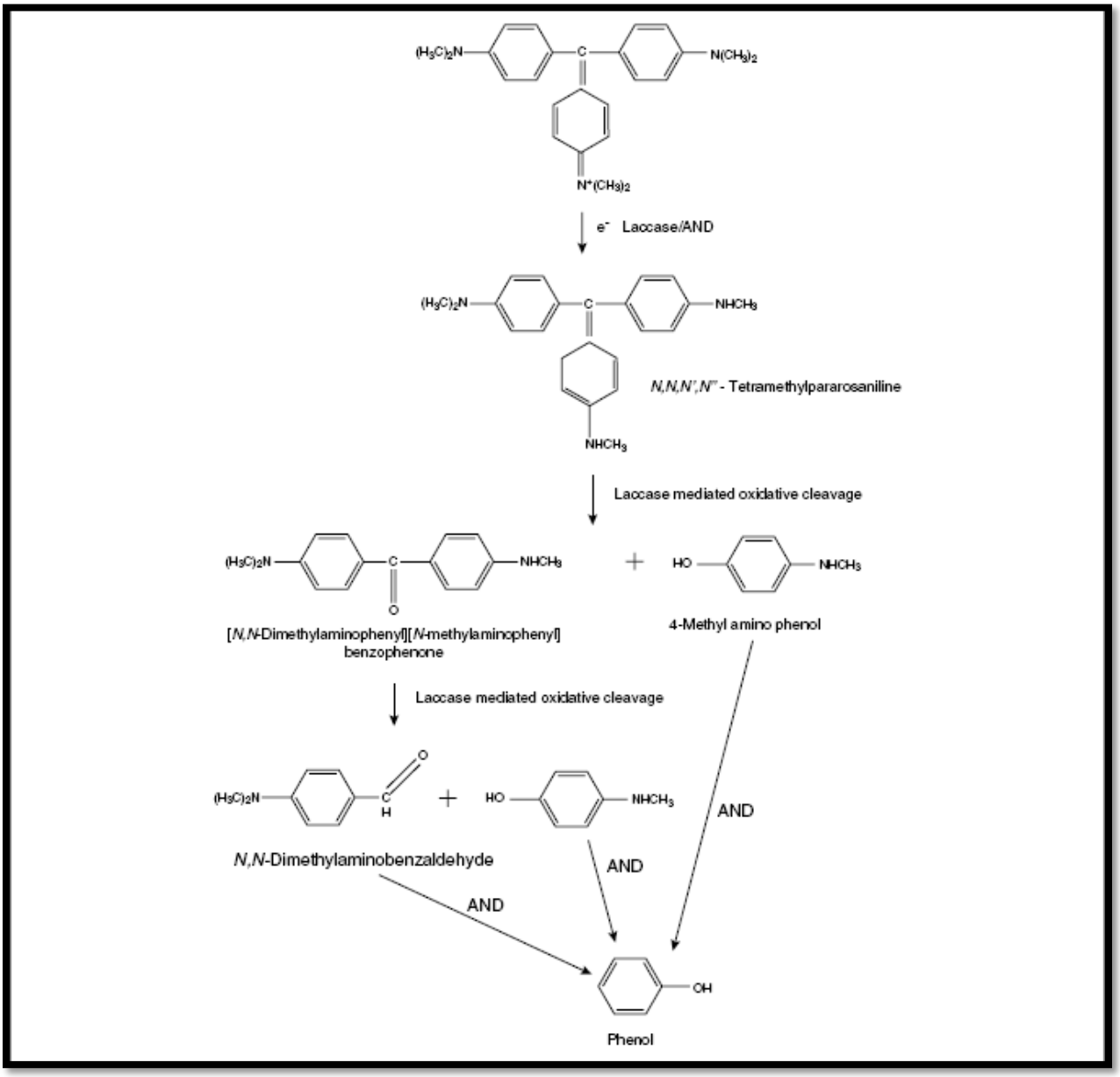


Figure 2.3: Mechanism of enzymatic degradation of CV by *Agrobacterium radiobacter* (Parshetti et al., 2011)

On account of previous reports, Crystal violet dye has been revealed to be one of the most argued and divisive compounds due to its unfavorable effects on environment as well as severe health hazards on living organisms. CV also acts as a mitotic fatal, carcinogenic mediator and a persuasive clastogen promoting tumor growth in some species of fishes Thus, it is

concluded that CV is an intractable impurity, which possesses noxious effects on aquatic as well as on terrestrial ecosystems. Hence, a recurrent and effectual treatment method should be employed for the degradation and detoxification of wastewater containing CV. In this regard, physico-chemical treatment methods are very lavish and also spawn large quantity of different types of derivative pollutants. Therefore, this thesis is based on the study of development of effective bacterial consortium, which is potent in decolorization as well as degradation of Crystal violet dye.

Chapter 3

ISOLATION, SCREENING AND CHARACTERIZATION OF BACTERIA CAPABLE OF THE DECOLORIZATION OF CV

Isolation, screening and characterization of bacteria capable of the decolorization of CV

3.1. Introduction

Over the preceding two decades, various treatment technologies such as physical and chemical methods have been engaged for the exclusion of color from textile dye effluents. But these methods have certain limited applications, since these produce large amount of sludge, solid wastes and are very expensive. As compared, the biological methods based on the microorganisms are accomplished of decolorizing as well as degrading the recalcitrant compounds of the textile wastewaters and are considered as lucrative and ecofriendly for the treatment of these effluents. These biological methods are environment friendly and most probably lead to complete mineralization of xenobiotic compounds. Many scientists have reported few organisms, which are proficient of decolorizing dye at lab scale, but few reports indicating their exploitation in the treatment processes are also available. The most extensively studied microorganism is white-rot fungus *Phanerochaete chrysosporium* (Reddy, 1995). In the 1970's, the efforts of isolating bacterial cultures competent of degrading azo dyes started with the reports on isolation of *Bacillus subtilis* (Horitsu et al., 1977).

Further, the bacterial cultures isolated from soil and slush samples had shown high dye decolorizing knack and belonged to *Bacillus sp., Alcaligenes sp.* and *Aeromonas sp.* (Sharma et al., 2004). Parikh and Madamwar, (2005) showed 80 % decolorization of Acid red 97 and FF sky blue dye by isolated Cyanobacterial strains like *Gloeocapsa pleurocapsoides* and *Phormidium ceylanicum*, after 26 days of incubation period whereas Waffa and Moawad, (2003) studied decolorization of direct yellow and erio red dyes by bacterial and actinomycetes strains. Other strains such as *Pseudomonas sp., Escherichia coli* and sulphate reducing bacteria are also reported for efficient dye decolorizing ability (Kothari, 2002 and Yoo, 2000). The efficacy of

these biological treatment methods mainly depend on the survivability and adaptability of microorganisms used during the treatment processes. This part of study was commenced to separate bacterial cultures proficient of decolorizing and degrading Crystal violet dye used in textile industries in Kanpur region. The use of isolated bacterial strains either individually or as consortium was predicted to develop natal processes for the treatment of industrial effluent containing different dyes.

3.2. Materials and methods

3.2.1. Collection of dye wastewater sample and its physicochemical analysis

Kanpur is one of the most industrialized areas in India. It is known as chemical hub and therefore, was chosen for effluent sample collection. Kanpur is one of India's earliest industrial towns with varied industrial activities ranging from textile engineering and leather works. The effluent sample was collected from a handloom textile company from the Panki Industrial area of Kanpur city (site 3), India. Kanpur is located between 26.4670° North, 80.3500° East and covers area of 3,029 km^2 (1,170 m^2) (Mani and Bharagava, 2017).

The wastewater was collected in a pre-sterilized container (5 liter), brought to laboratory and maintained at 4 °C for further analysis. The parameters such as pH, temperature, color and odour of collected wastewater sample were recorded on the spot. The collected wastewater was also analyzed for the presence of crystal violet dye by spectrophotometer (Evolution 201, Australia) at 590 nm. The other physico-chemical factors such as BOD, COD, TDS, TSS, EC, phosphate, nitrite, residual chlorine, carbonates, and sulphates were analyzed in laboratory as per the standard methods (APHA, 2012).

3.2.2. Media Composition

The Crystal violet dye used in experimental study was purchased from spectrochem, India and its standard stock solution was prepared and used throughout the study. The medium used throughout the study was modified Minimal-salt-medium (MSM) amended with CV dye, which contained (in gL^{-1}) K_2HPO_4, 5.22; KH_2PO_4, 4.08; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.55; NH_4Cl, 0.4; agar, 15.

3.2.3. Isolation and purification of CV degrading bacterial strains

The bacterial colony was isolated from textile dye effluent sample through serial dilution procedure and purified by frequent streaking routine on the same culture medium. The morphologically distinct bacterial colonies showing clear zones of inhibition around their colonies due to dye decolorization were selected for further studies (Mani and Bharagava, 2017).

3.2.3.1. Screening of CV degrading bacterial strains

The isolated bacterial strains having capability to decolorize CV dye were screened through primary and secondary screening test on solid MSM-agar-dye amended media method (Cheriaa and Bakhrouf, 2009).

3.2.3.1.1. Primary screening test

The isolated bacterial strains were primarily screened by nutrient enrichment technique on solid MSM amended with agar and different concentrations of CV dye. The plates were streaked with isolated bacterial strains and incubated at 35 °C for 48 hours. The bacterial strains that were capable to grow and endure highest concentration of CV dye were selected for secondary screening test and the pure culture stocks were preserved on glycerol stock solutions at -20 °C.

3.2.3.1.2. Secondary screening test

The secondary screening test of primarily screened bacterial strains was carried out at 35 °C by shaking culture method. Six primarily screened bacterial strains were grown in 250 mL Erlenmeyer flasks containing 100 mL autoclaved modified MSM amended with the escalating concentration of CV dye at 35 °C under aerobic conditions. The bacterial strains were screened on the basis of their growth performance and decolorization potential for CV dye assessed spectrophotometrically (Evolution 201, United States) at 600 nm and 590 nm, respectively. After screening, the compatibility tests were also performed.

3.2.4. Decolorization study

A 100 mL of autoclaved MSM broth was amended with 100 ppm CV dye and a loop full of overnight grown culture and was incubated at 35 °C with 100 rpm. The experiment was performed in triplicates and flasks were monitored at regular time intervals. A separate set of uninoculated flask was maintained in equivalent as control.

The decolorization of CV was monitored in terms of bacterial growth and decrease in color intensity by measuring the absorbance at 600 nm for bacterial growth and at 590 nm for CV decolorization at regular time intervals of 50 minutes. The decolorization of CV was expressed in terms of decrease in absorbance at 590 nm against the initial absorbance at the same wavelength (λmax) using spectrophotometer (Evolution 201, United States). The percent decolorization was calculated by using the formula.

$$\% \text{ Decolorization} = \frac{\text{Initial OD-Final OD} \times 100}{\text{Initial OD}}$$

Where, OD = Optical Density at 590 nm

3.2.4. Characterization of isolated CV decolorizing bacterial strains

3.2.4.1. Morphological and biochemical characterization

The bacterial species isolated from textile wastewater were characterized according to the Bergey's Manual of Determinative bacteriology (Whitman et al., 2012) and Cwan and Steel's manual of medical microbiology (Barrow and Feltham, 2009). The morphological characters examined include gram staining, shape, color, surface texture, margin and elevation. Biochemical tests were performed by inoculating broth culture of the isolates into a string of media. These tests include catalyze, oxidase, methyl red test, casein hydrolysis test, hydrogen sulfide test, citrate utilization test and triple sugar iron sugar fermentation (glucose, sucrose, and lactose).

1) Primary biochemical test

A. Gram's staining

Gram's staining method was discovered over 100 years ago by Dr. Hans Christian Gram, a Danish physician, in 1884. This method is most commonly used for direct microscopic examination of specimens and subculture. It is very useful stain for identifying and classifying bacteria into two major groups: the gram-positive and gram-negative.

Principle:

The cell wall of gram-negative bacteria is generally thinner than those of Gram-positive. Experimental evidences suggested that during de-staining of Gram-negative bacteria with alcohol extract the lipid, which results increased porosity or permeability of cell walls. Thus, the crystal violet complex can be extracted and Gram-negative organism is decolorized by alcohol. All these evidence point to the cell wall retention of the primary stain.

Procedures:

1. Thin smear of material was made for study and allow to air dry.

2. Fix the material to slide by passing the slides 3 or 4 times through the flame of a bunsen burner so that the material does not wash off during the staining procedure.
3. Flood the crystal violet for one minute.
4. Pour off excess dye and wash gently in tap water and drain the slide against a paper towel.
5. Expose the smears to Gram's iodine for one minute by washing with iodine, then adding more iodine and leaving it on the smear until the minute is over.
6. Wash with tap water and drain carefully (Do not blot.)
7. Wash with 95% alcohol for 30 seconds.
8. Wash with tap water at the end of 30 seconds to stop the decolorization.
9. Counter stain with 0.25% safranin for 30 seconds.
10. Wash, drain, blot, and examine under oil immersion.

Interpretation:

Gram-positive bacteria appear purple and Gram-negative bacteria appear pink or red.

2) Secondary biochemical tests

A. Starch hydrolysis (Amylase) test:

Starch is a high molecular-weight, branching polymer composed of glucose molecules linked together by glycosidic bonds. The degradation of this macromolecule first requires the presence of an extracellular enzyme amylase for its hydrolysis into shorter polysaccharides, namely dextrins and ultimately into disaccharide, which is catalyzed by maltase, yields low-molecular weight, soluble glucose molecules that can be transported into the cell and used for energy production through the process of glycolysis. Starch is used to demonstrate the hydrolytic activities of these exoenzymes. The medium is composed with nutrient agar supplemented with starch, which serves as the polysaccharide substrate. The detection of hydrolytic activity

following growth period is made by performing the starch test to determine the presence or absence of starch in medium.

Procedures:

1. Flood the starch agar plate cultures with Gram's iodine solution, allow the iodine to remain in contact with medium for 30 seconds, and pour off the excess.
2. Examine the cultures for the presence or absence of a blue-black color surrounding the growth of each test organism.
3. Based on the observation, determined and record the organism capable of hydrolyzing the starch.

Interpretation:

If the starch has been hydrolysed, a clear zone of hydrolysis will surround the growth of organism. This is a positive result else negative result.

B) Casein hydrolysis test:

Casein, the major milk protein, is a macromolecule composed of amine acid subunits linked together by peptide bonds (CO-NH). Before their assimilation into the cell, proteins must undergo step by step degradation into peptones, polypeptides and ultimately into their building blocks, amino acids. This process is called peptonisation, or proteolysis, and it is mediated by extracellular enzymes called proteases. The function of this protease is to cleave the peptide bond CO-NH by introducing water into the molecule. The reaction liberates amino acids. The low-molecular-weight soluble amino acid can now be transported through cell membrane into the intracellular amino acid pool for use in the synthesis of structural and functional cellular proteins. Milk agar is used to demonstrate the hydrolytic activity of these exoenzymes. The medium contain nutrient agar supplemented with milk that contains the protein substrate casein.

Similar to other proteins, milk protein is a colloidal suspension that gives the medium its color and opacity because it deflects light rays rather than transmitting them.

Procedures:

1. Examine the milk agar plate cultures for the presence or absence of a clear area, or zone of proteolysis, surrounding the growth of each of bacterial test organisms.
2. Based on the observation, determine and record the organism capable of hydrolyzing milk protein casein.

Interpretation:

The medium surrounding the growth of organism showing loss of opacity shows positive result whereas medium surrounding the growth of organism remaining opaque shows negative result.

C) Triple sugar-iron agar test:

This test is designed to differentiate among the different groups or genera of Enterobacteriaceae, which all are gram-negative bacilli capable of fermenting glucose with the production of acid, and to distinguish the Enterobacteriaceae from other gram-negative intestinal bacilli. This differentiation is made on the basis of differences in carbohydrate fermentation patterns and hydrogen sulphide production by various groups of intestinal organisms. The TSI agar slants contain lactose and sucrose in 1% concentration and glucose (dextrose) in a concentration of 0.1%, which permits the detection of utilization of this substrate only. The acid-base indicator phenol red is also incorporated to detect by a change in color of medium from orange-red to yellow in presence of acids.

Procedure:

1. Prepare the triple sugar-iron agar slants.

2. Using aseptic techniques inoculate each experimental organism into its appropriately labeled tube by means of a stab and streak inoculation. The last tube will serve as control.
3. Incubate for 18 to 24 hours at 37 °C.
4. Examine the color of both butt and slant of all agar slant cultures. Based on the observation determine the type of reaction that has taken place (acid, alkaline, or none) and carbohydrate that has been fermented (dextrose, lactose, and/or sucrose, all or none) in each culture.
5. Examine all cultures for the presence or absence of blackening within the medium. Based on the observation, determine whether or not each organism was capable of H_2S production.

Interpretation:

Alkaline slant (red) and acid butt (yellow) with or without gas production (breaks in agar butt); only glucose fermentation has occurred. Acid slant (yellow) and acid butt (yellow) with or without gas production. Lactose and /or sucrose fermentation has occurred. Alkaline slant (red) and alkaline butt (red) or no change (orange-red) in butt indicates absence of carbohydrate fermentation.

D) Catalase test:

During aerobic respiration, microorganisms produce hydrogen peroxide and in some cases, an extreme toxic superoxide. Accumulation of these substances will result in death of organisms unless they can be enzymatically degraded. These substances are produced when aerobes, facultative anaerobes, and microaerophiles use the aerobic respiratory pathway in which oxygen is the final electron acceptor, during the degradation of carbohydrates for energy production. Organisms capable of producing catalase rapidly degrade hydrogen peroxide. Aerobic organism that lack catalase can degrade especially toxic superoxides using the enzyme

superoxide dismutase. The end product of a superoxide dismutase is H_2O_2, but this is less toxic to bacterial cells than superoxides.

Procedures:

1. Using aseptic technique, inoculate each experimental organism into its appropriately labeled tube by means of a streak inoculation. The last tube will be served as a control.
2. Inoculate all cultures for 24 to 48 hours at 37 °C.
3. Allow three or four drops of the 3 % hydrogen peroxide to flow over the entire surface of each slant culture.
4. Examine each culture for the presence or absence of bubbling or foaming.
5. Based on the observation, determine and record whether or not each organism was capable of catalase activity.

Interpretation:

If catalase is present, the chemical reaction is indicated by bubbles of free oxygen gas (O_2).

E) Hydrogen sulphide test:

Gaseous H_2S may also be produced by the reduction of inorganic sulphur compounds such as thiosulfates ($S_2O_3^{2-}$) sulfates (SO_4^{2-}), or sulfites (SO_3^{2-}). The medium contains sodium thiosulfate, which certain microorganisms capable of reducing to sulfite with liberation of hydrogen sulfide. The sulphur atoms act as hydrogen acceptors during oxidation of inorganic compound. In this experiment, the SIM medium contains peptone and sodium thiosulfate as the sulphur substrates; ferrous sulfate ($FeSO_4$), which behaves as H_2S indicator; and sufficient agar to make medium semisolid and thus, enhance anaerobic respiration. Ferrous ammonium sulfate in medium serves as an indicator by combining with gas, forming an insoluble black ferrous

sulfide precipitation and is indicative of H_2S production. SIM agar may also be used to detect motile organisms.

Procedure:

1. Prepare SIM agar deep tubes.
2. Aseptically inoculate each experimental organism into its appropriately labeled tube by means of stab inoculation. The last tube will serve as a control.
3. Incubate all cultures for 24 to 48 hours at 37 °C.
4. Examine all SIM cultures for the presence or absence of black coloration along the line of the stab inoculation.
5. Based on the observation, determine and record whether or not each organism was capable of producing hydrogen sulfide.
6. Observe all cultures for the presence (+) or absence (-) of motility.

Interpretation:

Presence of precipitation is evidence of a positive reaction whereas the absence of precipitation is evidence of a negative reaction. Motility is recognized when culture growth (turbidity) of flagellated organisms is not restricted to the line of inoculation. Growth of non-motile organisms is confined to the line of inoculation.

F) Citrate utilization test:

This test is used to differentiate among the enteric bacteria on the basis of their ability to utilize/ ferment citrate as sole carbon source. The utilization of citrate depends on the presence of an enzyme citrate produced by organism that breaks down the citrate to oxaloacetic acid and acetic acid. These products are later converted to pyruvic acid and carbon dioxide enzymatically. The citrate test is performed by inoculating the microorganisms into an organic synthetic

medium, Simmon's citrate agar, where sodium citrate is only source of carbon and energy. Bromothymol blue is used as an indicator. When the citric acid is metabolize, the CO_2 generated combines with sodium and water to form sodium carbonate an alkaline product, which changes the color of the indicator from green to blue.

Procedure:

1. Prepare Simmon's citrate agar (pH 6.9) slants.
2. Pour the medium into culture tubes and sterilize by autoclaving at 15 lb pressure for 15 minutes and prepare the slants.
3. Inoculate Simmon's citrate agar slants with experimental strains by means of stab and streak inoculation. The last tube is kept as uninoculated comparative control.
4. Incubate the slants at 37 °C for 48 hours.

Interpretation:

The changes in color of indicator from green to blue constitute a positive test whereas no change in color shows negative test.

G) Indole production test:

Tryptophan, an essential amino acid, is oxidized by some bacteria by enzyme tryptophanase resulting in the formation of indole, pyruvic acid and ammonia. The indole test is performed by inoculating a bacterium into tryptone broth. The indole produced during the reaction is detected by adding Kovac's reagent (dimethylaminobenzaldehyde), which produces a cherry-red reagent layer.

Procedure:

1. Prepare (1 %) tryptone broth: dissolve 10g of peptone in one litre of distilled water. Sterilize at 15 psi (121 °C) for 15 minutes.

2. Inoculate tryptone broth with the experimental strains. The last tube is to be kept as control.

3. Incubate inoculated and uninoculated tubes at 35 °C for 48 hours.

4. After 48 hours of incubation, add 1 mL of Kovac's reagent to each tube including control.

5. Shake the tubes gently after intervals for 10-15 minutes.

6. Allow the tubes to stand to permit the reagent to come to the top.

7. Examine the tubes as to the color in the reagent "layer".

Interpretation:

Development of cherry red color in the top layer of tube indicates positive test for indole production. Absence of red color is indole negative.

H) Oxidase test:

Oxidase enzymes play a vital role in the operation of electron transport system during aerobic respiration. Cytochrome oxidase catalyze the oxidation (O_2), resulting in the formation of H_2O_2 or H_2O. Aerobic bacteria, as well as some facultative anaerobes and microaerophiles exhibit oxidase activity. The oxidase test aids in differentiation among members of the genera *Neisseria* and *Pseudomonas*, which are oxidase-positive and Enterobacteriaceae, which are oxidase-negative. The ability of bacteria to produce cytochrome oxidase can be determined by the addition of test reagent *p*-aminodimethylaniline oxalate to colonies grown on a plate medium. The light pink reagent serves as an artificial substrate, donating electrons and thereby becoming oxidized to a blackish compound in the presence of the oxidase and free oxygen.

Procedures:

1. Add two or three drops of *p*-aminodimethylaniline oxalate to surface of growth of each test organism.

2. Observe growth for the presence or absence of a color change from pink, to maroon and finally to purple. Positive (+), color change in 10-30 seconds; negative test (-), no colour change, or light pink color.
3. Based on observation, determine and record whether or not each organism was capable of producing cytochrome oxidase.

Interpretation:

If color changes in 10-30 seconds then result is positive (+), but if no colour change or light pink color appears then result is negative (-).

I) Methyl red test:

Methyl Red (MR) test determines whether the microbe performs mixed acids fermentation when supplied glucose. Mixed acid fermentation is one of two broad patterns, 2-3-butanediol fermentation being another. In mixed acid fermentation, three acids (acetic, lactic and succinic) are formed in significant amounts. The mixed acid pathway gives 4 mol of acidic products (mainly lactic and acetic acid), 1 mol of neutral fermentation product (ethanol), 1 mol of CO_2, and 1 mol of H_2 per mol of glucose fermented. This large amount of acid results significant decrease in pH of medium below 4.4. This is visualized by using pH indicator, methyl red (p-dimethylaminoaeobenzene-O-carboxylic acid), which is yellow above pH 5.1 and red at pH 4.4.

Procedures:

1. Inoculate two tubes containing MR-VP Broth with a pure culture of microorganisms under investigation.
2. Incubate at 35 °C for up to 4 days.
3. Add about 5 drops of methyl red indicator solution to tube.

4. Examine the tubes for color change.

Interpretation:

When the culture medium turns red after addition of methyl red, then the test is positive whereas if the culture medium remains yellow, then the test is assumed to be negative.

J) Voges Proskauer test:

The Voges-Proskauer test determines the capability of some organisms to produce non acidic or neutral end products, such as acetyl methyl carbinol, from organic acids that result from glucose metabolism. The reagent used in this test is Barritt's reagent, consists of a mixture of alcoholic α a-naphthol and 40 % potassium hydroxide solution. Detection of acetyl methyl carbinol requires this end product to be oxidized to a diacety compound. This reaction will occur in the presence of α a-naphthol catalyst and a guanidine group that is present in peptone of MR-VP medium.

Procedures:

1. Inoculate a tube of MR/VP broth with a pure culture of test organism.
2. Incubate for 24 hours at 35 °C.
3. At the end of incubation time, add 10 drops of Barritt's A reagent and 10 drops of Barritt's B reagent to each broth tube.
4. Shake gently for several minutes.
5. Observe test tubes for color change.

Interpretation:

Formation of red color within 15 to 20 minutes is a positive result. No red color formation after 15 to 20 minutes is a negative result.

3.2.4.1. Genomic DNA preparation, PCR amplification and 16S rRNA gene sequencing analysis

DNA extraction

1. The overnight grown bacterial cells were obtained by centrifugation and transferred in 5 mL of 50 mM Tris Buffer (pH 8.0) and 50 mM EDTA.
2. The obtained cell suspension was freezed at -20 °C.
3. To the frozen suspension, 0.5 mL of 250 mM Tris Buffer (pH 8.0) and 10 mg/mL lysozyme was added, and thawed at room temperature. When thawed, were placed on ice for 45 min.
4. 1 mL of 0.5 % SDS, 50 mM Tris (pH 7.5), 0.4 M EDTA and 1 mg/mL proteinase K was added to the cell suspension and placed in 50 °C water bath for 60 min.
5. After this, the bacterial cells were extracted with 6 mL of Tris-equilibrated phenol and were centrifuged at 10,000 rpm for 15 min. Obtained top layer was transferred to new tube.
6. Add 0.1 volume of 3 M Na-acetate (mix gently), then add 2 volume of 95 % ethanol (mix by inverting).
7. Rolled out DNA was transferred to 5 mL of 50 mM Tris (pH 7.5), 1 mM EDTA, 200 g/mL RNAse and dissolved overnight by rocking at 4 °C.
8. DNA was extracted by mixing with equal volume of chloroform by inverting and centrifuged at 10,000 rpm for 5 min. The top layer was transferred to a new tube.
9. Add 0.1 volume of 3 M Na-acetate to tube, mix gently, then add 2 volume of 95 % ethanol and mixed by inverting.

10. DNA was spool out and dissolved in 2 mL of 50 mM Tris (pH 7.5) and 1 mM EDTA and finally, the purity of DNA was checked by electrophoresis and spectrophotometric analysis.

PCR amplification and 16S rRNA gene sequencing analysis

Biomass was scrapped from nutrient agar plates and the total genomic DNA was prepared from overnight grown bacterial cultures by alkaline lysis method as described by Kapley et al. (2001). The 16S rRNA gene from isolated bacterium was amplified using about 5 µl DNA as template and universal primers (27F) 5´-AGAGTTTGATCMTGGCTCAG-3´ and (1492R) 5´-CGGTTACCTTGTTACGACTT-3´ and 1180, 1256 and 1264 bp product was amplified for the strains SJ4, SJ5 & SJ10, respectively. The reaction mixture contained 1 µL template DNA, 0.25 µL forward primer, 0.25 µL reverse primer, 25 µL master mix and rest nuclease free water in a final volume makeup of 50 µL. The thermocycling reactions were carried out by using Veriti® 96-Well Thermal Cycler (Applied Biosystems, USA) as initial denaturation at 95 °C for 2 minutes followed by 35 cycles of denaturation for 30 seconds at 95 °C, annealing for 30 seconds at 52 °C, extension for 2 minutes at 72 °C and final extension for 15 minutes at 72 °C. The PCR product was gel purified using 1 % agarose gel at 100V for 60 min and gel was visualized under UV light.

Phylogenetic analysis

The evolutionary history was contingent using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches (Felsenstein, 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum

Composite Likelihood method and are in the units of the number of base substitutions per site (Tamura et al., 2004). The analysis involved 11 nucleotide sequences and all positions with more than 95 % site coverage were included, fewer than 5 % alignment gaps, missing data, and ambiguous bases were allowed at positions. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

Nucleotide Sequence accession numbers

The partial sequences obtained were subjected to BLAST analysis using the online option available at www.ncbi.nlm.nih.gov/BLAST (Altschul et al., 1997), suggesting the identity of the isolates. The final sequences obtained were submitted to NCBI for obtaining accession no.

3.2.5. Statistical analysis

All the experiments were performed in triplicates. The results obtained from each set of data have been expressed in terms of mean and standard deviation.

3.3. Results and Discussion

3.3.1. Physico-chemical characteristics of dye wastewater

The wastewater collected from textile handloom, Kanpur, India was dark purple in color with sharp pungent and fishy smell, which causes nuisance and reject the esthetic worth of environment and receiving water resources (Fig 3.1). The pH of collected textile wastewater was noticeably below neutral level than that of NEQ standards. High pH reduces fish production as well as slows down the growth of aquatic macrophytes and vice-versa (Argo, 2003). The temperature of wastewater was also above the standard limits, which is harmful for aquatic life affecting the dissolved oxygen concentration and also can persuade the activity of bacteria in water resources. The collected sample also showed higher EC, TDS and TSS than that of NEQS. High values of TDS and EC can cause osmotic stress at plants root zone making it difficult to

absorb water for its growth and thus causing lower crop production when used in irrigation (Mojiri, 2011).

The chemical oxygen demand (COD) and biological oxygen demand (BOD) values were also higher than permissible limits (Table 3.1), which is very harmful to the aquatic life. Higher BOD causes bad taste to drinking water (Singh et al., 1998). Phosphate range was 0.45 mg/L in sample. Higher value of phosphate leads to eutrophication and oxygen depletion in water bodies and leads to osteoporosis and kidney damage in humans. Nitrite and sulphates content in collected textile wastewater was found to be lower than standard values. The value of carbonates and residual chlorine of sample was also higher indicating that textile wastewater to be highly polluted and harmful.

Table 3.1: Physico-chemical characteristics of Textile wastewater

S.No.	Parameters	Test value	Standard value
1.	Color	Dark	Colorless
2.	Odour	Pungent	No odour
3.	pH	8.5	5.5-9.0
4.	Temperature	22	40
5.	TDS	6360	3500
6.	TSS	1320	100
7.	BOD	333.4850	30
8.	COD	456	250
9.	EC	6.45	-
10.	Phosphate	0.45	-
11.	Nitrite	0.27	-
12.	Residual chlorine	3.34	1
13.	Carbonates	4.3	-
14.	Sulphates	0.32	2

Figure 3.1: Sample collection sites in Kanpur

3.3.2. Isolation, screening and characterization of bacteria capable of the decolorization of CV

3.3.2.1. Isolation and purification of CV degrading bacterial strains

The textile wastewater sample was screened for CV dye decolorizing bacterial strains by inoculating 10 mL of wastewater into flask containing 100 mL of MSM broth. The flasks were incubated at 35 °C under shaking conditions (110 rpm). After 48 hr of incubation period, 1mL of culture broth was appropriately diluted through serial dilutions and plated on MSM-CV dye amended agar plates (Fig 3.2). The linguistically dissimilar bacterial isolates viewing lucid zones around their colonies due to decolorization of CV dye were selected and further screened on the basis of their aptitude to decolorize CV in MSM-CV dye amended agar plates at different concentrations of CV dye (Fig 3.3). The bacterial cultures tolerating higher concentration of CV dye was isolated through streak plate method.

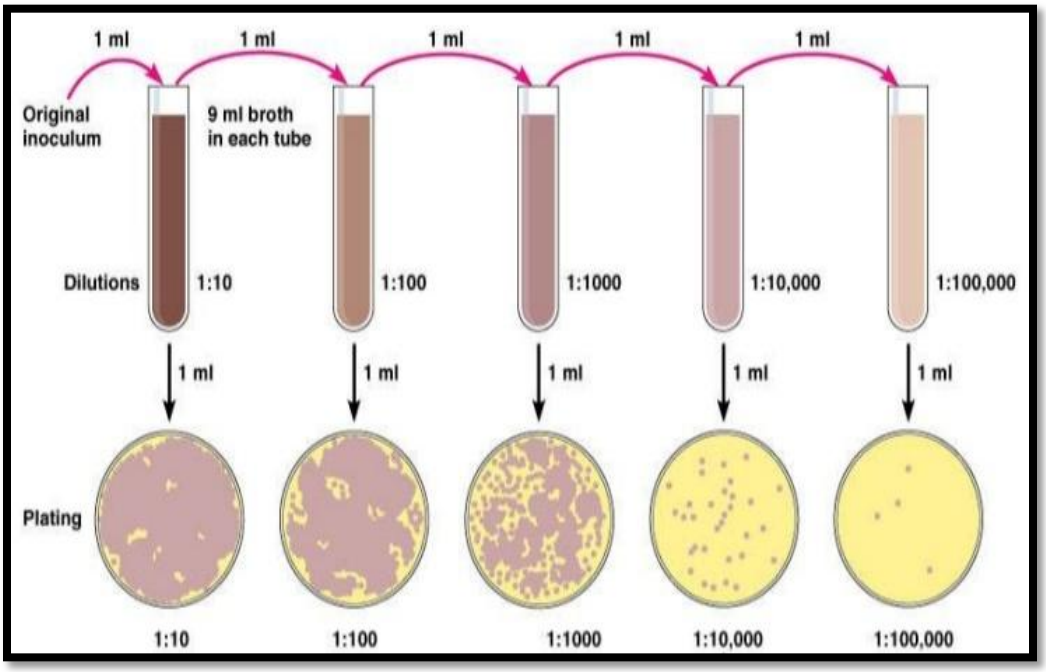


Figure 3.2: Serial dilution procedure for isolation of bacterial colonies

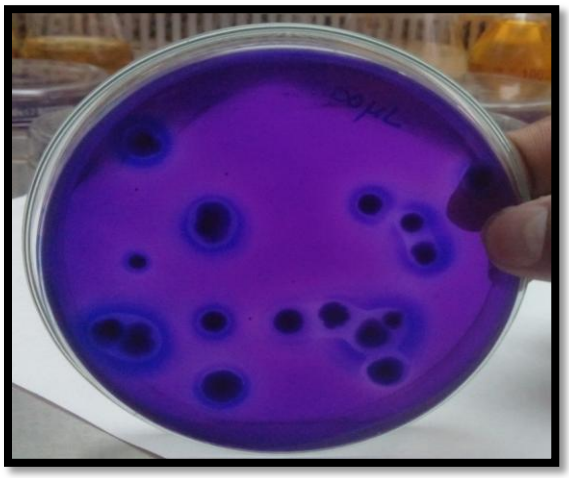


Figure 3.3: Isolation of bacteria on the basis of Zone formation on MSM-Dye-Agar media

Ten (10) bacterial strains were isolated from textile effluent disposal site where the chances to get the potent CV degrading bacterial strains were maximum. The isolated bacterial strains were streaked successively for several times to get distinct and pure colonies. The pure bacterial strains were maintained at 4 °C and used for screening test.

3.3.2.2. Screening of CV degrading bacterial strains:

In primary screening test of ten (10) isolated bacterial strains performed on solid modified MSM-agar medium amended with different concentrations of CV dye in plates, only six (6) potent bacterial strains tolerating the maximum concentrations of CV dye were selected for secondary test performed in MSM-CV-dye broth amended with 1500 ppm stock solution at 35 °C under aerobic conditions for 6 consecutive days and finally three bacterial strains SJ4, SJ5 and SJ10 were selected as the most potential CV degrading bacterial strains for further study.

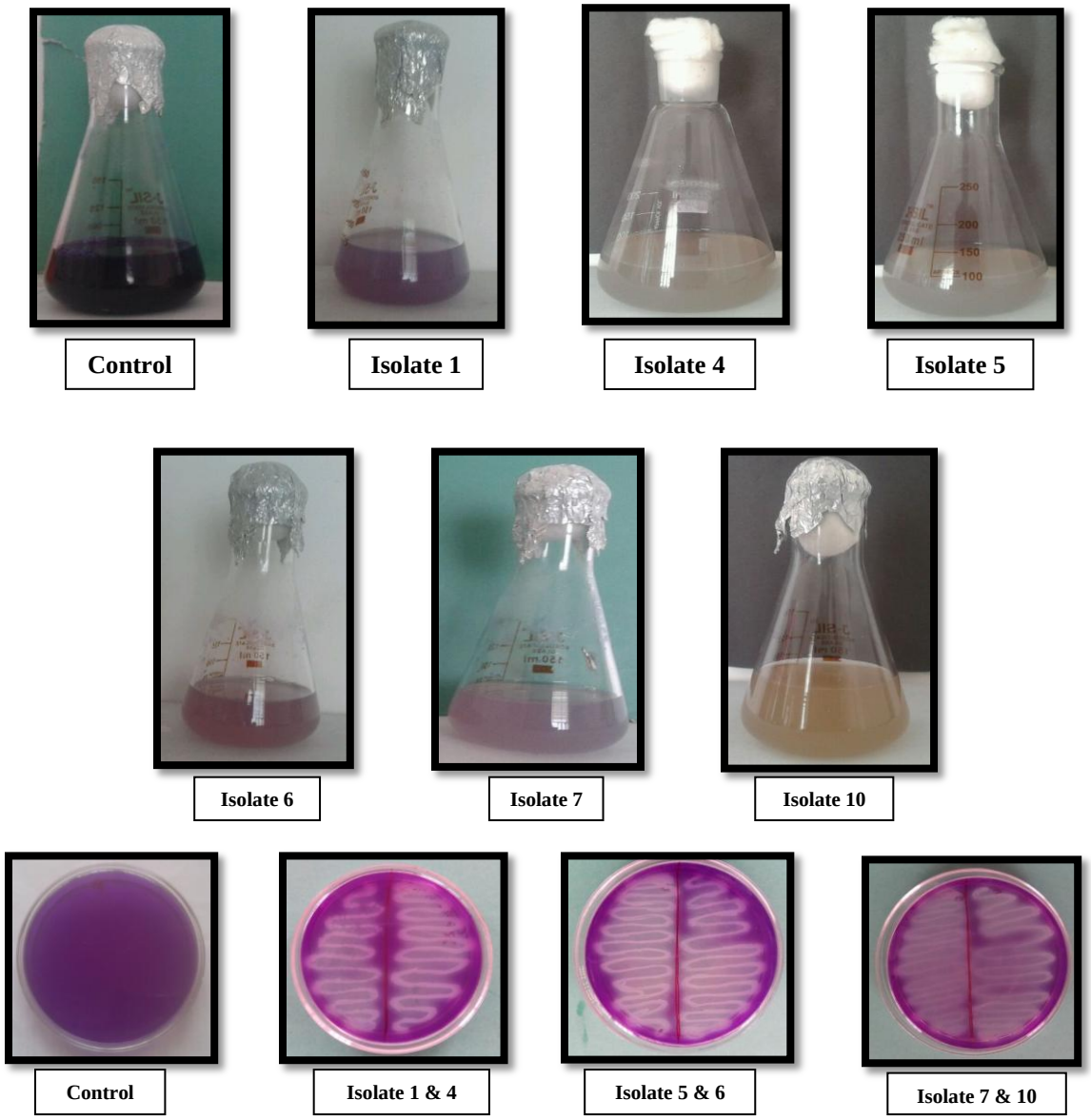
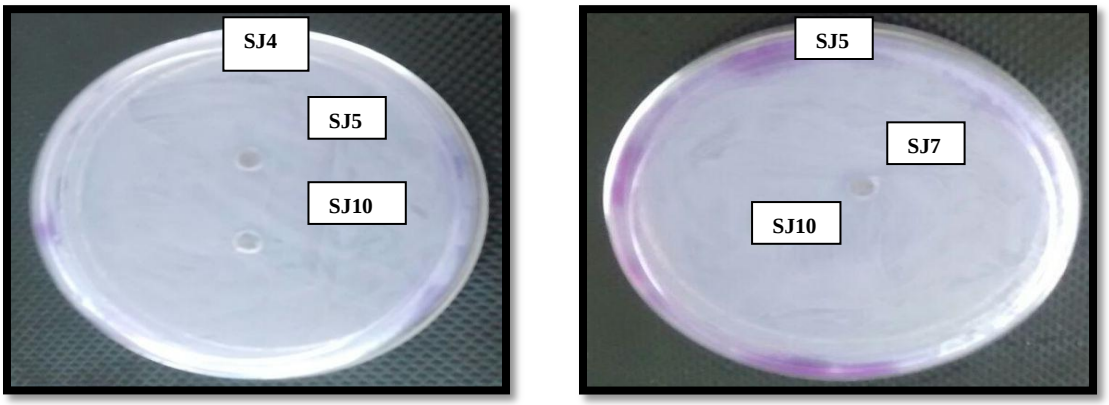


Figure 3.4: Decolorization of CV dye by the individual isolates

Out of six potent bacterial strains, only four strains have shown much decolorization percentage than others (Fig 3.4). The CV degrading potential of all the four strains i.e. strain SJ4 degraded about 90 %, strain SJ5 degraded about 93 %, strain SJ7 degraded about 85 % and strain SJ10 degraded about 95 %. The Crystal violet degrading potential of each strain was expressed as decrease in absorbance at 590 nm against the initial absorbance at the same wavelength using a spectrophotometer (Evolution, 201) and the percentage decolorization was calculated by the following formula discussed before.

3.3.2.3. Compatibility test:

For revealing the capability of each strain to exist with other strains without any inconsistency, a compatibility experiment was performed before developing the bacterial consortium. In this test, one strain was spreaded on the nutrient rich agar plate and other two strains were inoculated on the wells made on the plates after spreading. Likewise all strains were spreaded one by one and other two were inoculated on the wells as shown in figure (3.5). All strains showed no signs of inheritance towards each other showing compatibility to each other, but since strains SJ4, SJ5 and SJ10 has shown much decolorization percentage than SJ7, thus all three strains were further used in the development of bacterial consortium.



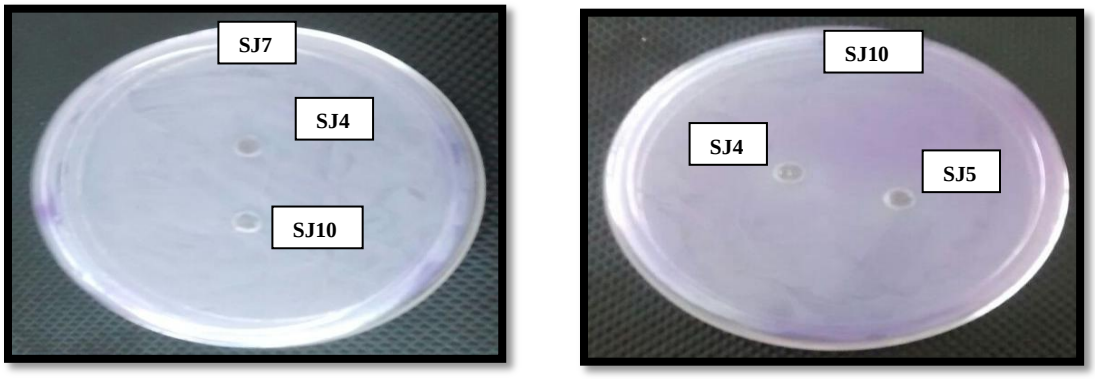


Figure 3.5: Compatibility test of selected strains

3.3.3. Characterization of isolated Crystal violet degrading bacterial strains

3.3.3.1. Biochemical characterization of bacterial strains

Three morphologically dissimilar bacterial strains SJ4 [Gram negative], SJ5 [Gram negative] and SJ10 [Gram negative] were lastly screened for the degradation of Crystal violet dye (Figure 3.6). The various morphological and biochemical tests given by all the three strains are given in Table 3.2 & 3.3 and Figure 3.7.

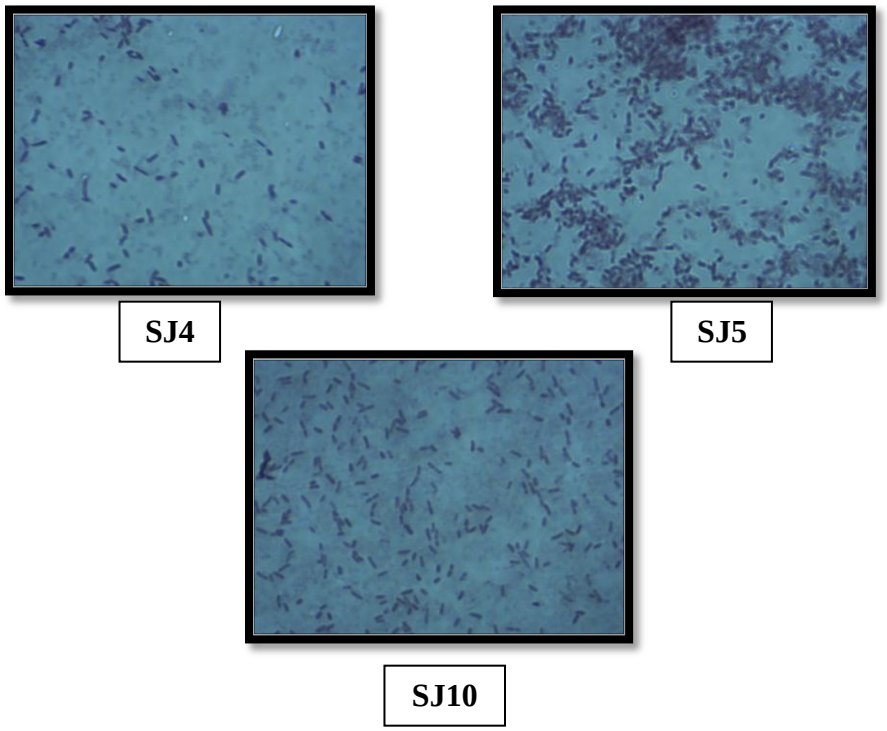


Figure 3.6: Microscopic view of all three bacterial strains

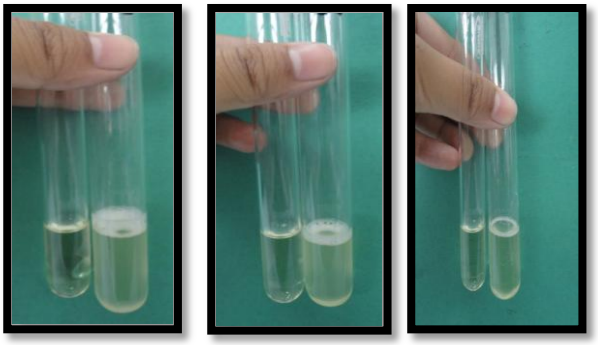
Table 3.2: Morphological characteristics of isolated bacterial strains

S.No.	Morphological Characteristics	Bacterial strains		
		SJ4	**SJ5**	**SJ10**
1	Gram staining	Gram negative	Gram negative	Gram negative
2	Shape	Short rods with rounded ends	Rod with rounded ends	Rod shaped with rounded ends
3	Color	Beige (slightly yellowish)	Transparent	Off-white
4	Surface Texture	Normal	Smooth	Normal
5	Margin	Even	Even	Even
6	Elevation	Flat	Convex	Convex
7	Motility	Motile	Motile	Motile

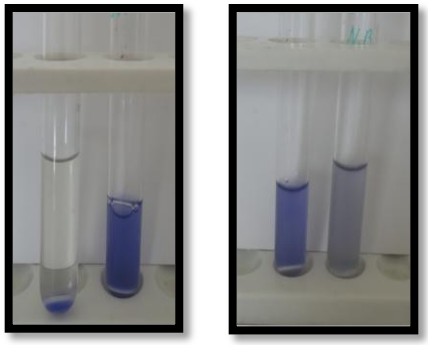
Table 3.3: Biochemical characteristics of isolated bacterial isolates

S.No.	Biochemical Characteristics	SJ4	SJ5	SJ10
1.	Catalase test	+ve	+ve	+ve
2.	Oxidase test	+ve	+ve	-ve
3.	Indole test	+ve	+ve	-ve
4.	Methyl red test	-ve	+ve	+ve
5.	Voges Proskauer	-ve	-ve	-ve
6.	Citrate utilization test	-ve	-ve	-ve
7.	Casein hydrolysis	+ve	-ve	+ve
8.	Glucose fermentation	+ve	-ve	-ve
9.	Sucrose fermentation	-ve	+ve	+ve
10.	Lactose fermentation	+ve	+ve	+ve
11.	Hydrogen sulfide test	-ve	-ve	+ve

12. Starch hydrolysis +ve +ve +ve



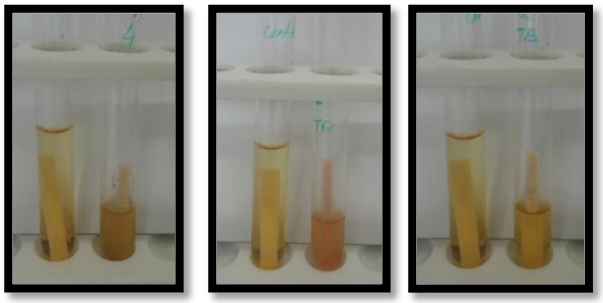
SJ4 SJ5 SJ10



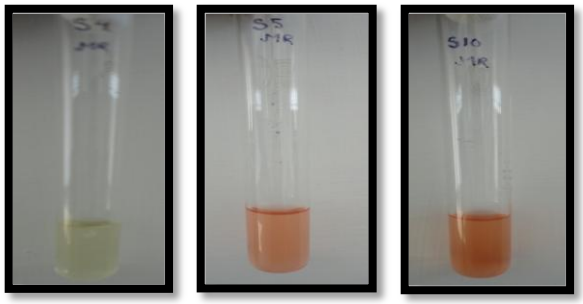
SJ4 SJ5 & SJ10

Catalase Test

Oxidase Test



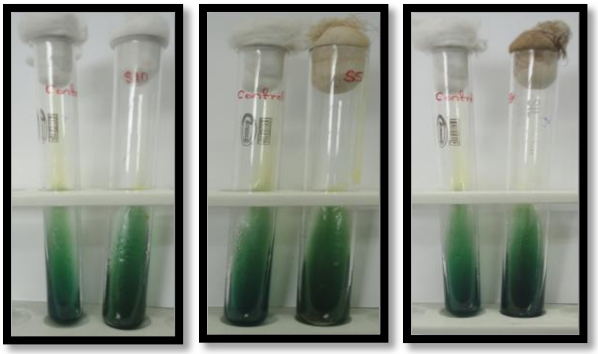
SJ4 SJ5 SJ10



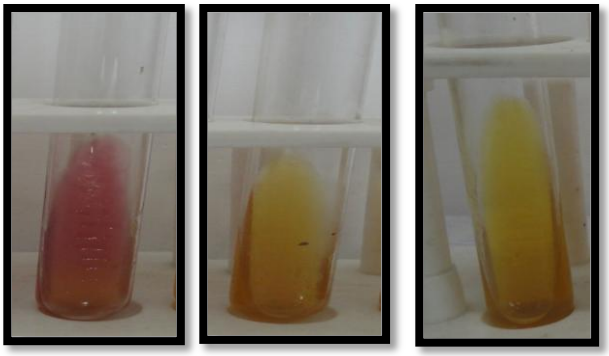
SJ4 SJ5 SJ10

Indole test

Methyl red test



SJ4 SJ5 SJ10



SJ4 SJ5 SJ10

Citrate Utilization test

Triple sugar agar test

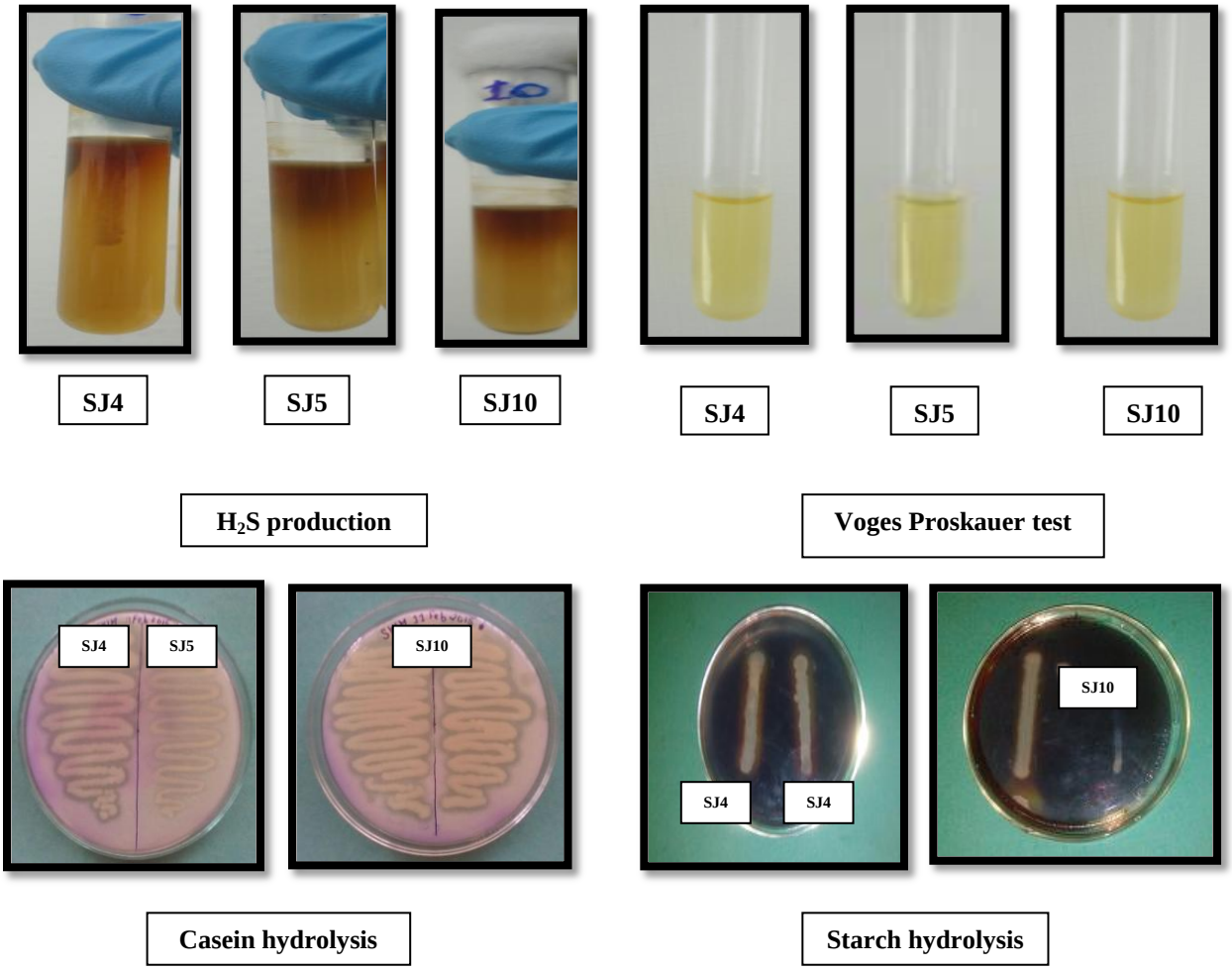


Figure 3.7: Pictorial representation of biochemical tests performed

3.3.3.2. Molecular characterization of isolated bacterial strains:

Hagstrom et al., (2000) reported that a 16S rRNA sequence similarity of ≥97 % is a reasonable level for grouping the bacteria into species. The BLAST analysis of sequences obtained bacterial strains SJ4 showed 99 % similarity with *Aeromonas hydrophila* strain ATCC 7966, SJ5 showed 100 % similarity with *Aeromonas caviae* strain CECT 4221 and SJ10 showed 99 % similarity with *Aeromonas media* strain RM. This similarity values clearly indicates that the isolated bacterial strains were of genus *Aeromonas* and identified as *Aeromonas hydrophila, Aeromonas Caviae* and *Aeromonas media,* respectively as shown in the figure 3.11, 3.12 and

3.13. The partial 16S rRNA gene sequences of 1180 bp, 1256 bp and 1264 bp were submitted to GenBank and an accession number KU720586, KU720587 and KU847363 were assigned to SJ4, SJ5 and SJ10 strains, respectively. The PCR amplification of 16S rRNA gene of isolated bacteria has been shown in figure 3.8a and the phylogenetic tree of SJ4, SJ5 and SJ10 has been shown below in fig 3.8b, 3.8c, and 3.8d.

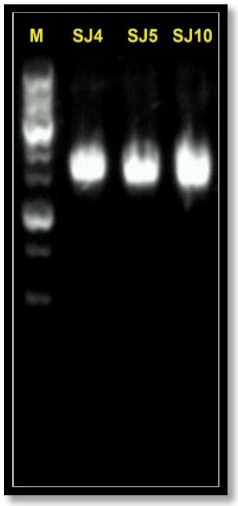


Figure 3.8a: PCR amplification of 16S rRNA gene of isolated bacteria; Lane M 1500 bp Ladder; Lane 1 SJ4; Lane 2 SJ5; Lane 3 SJ10

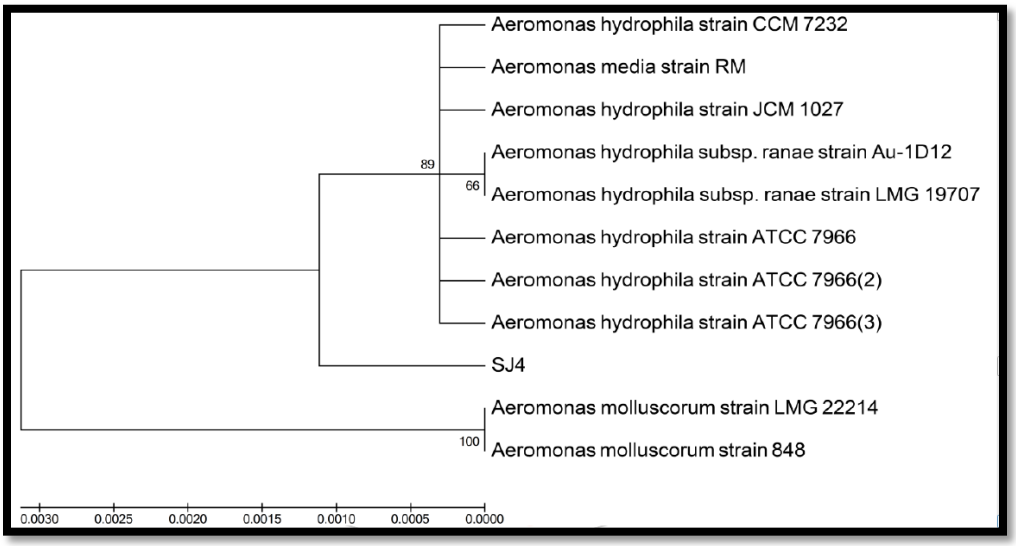


Figure 3.8b: Phylogenetic tree showing the relationship of isolated bacterium SJ4 with its neighboring species

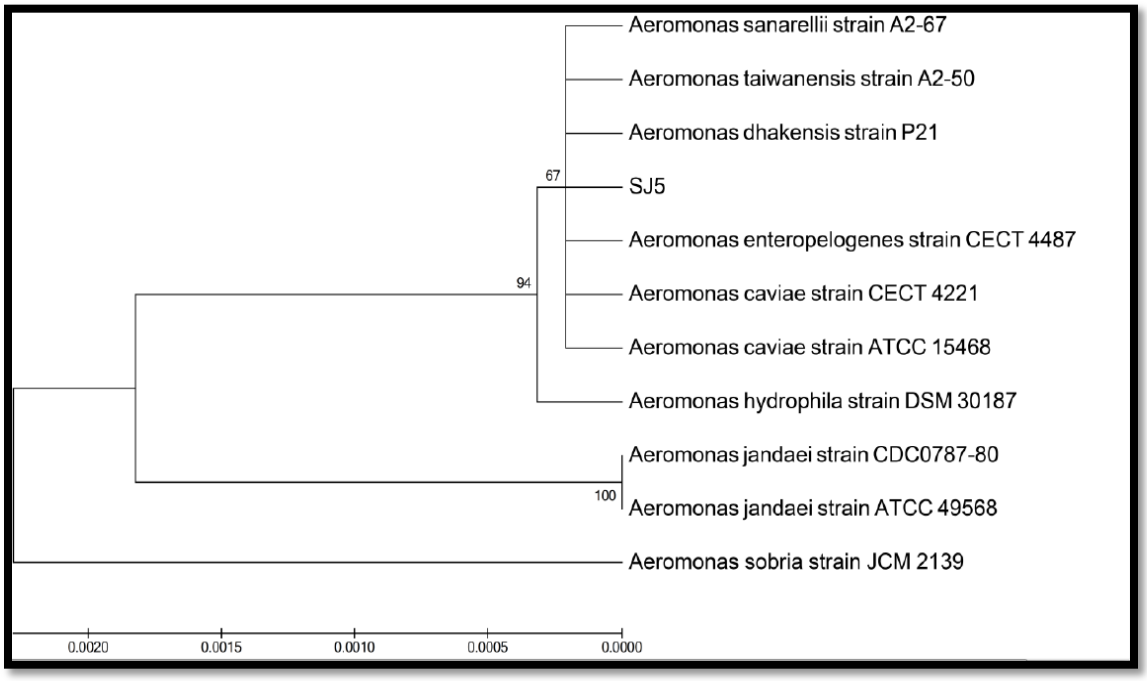


Figure 3.8c: Phylogenetic tree showing the relationship of isolated bacterium SJ5 with its neighboring species

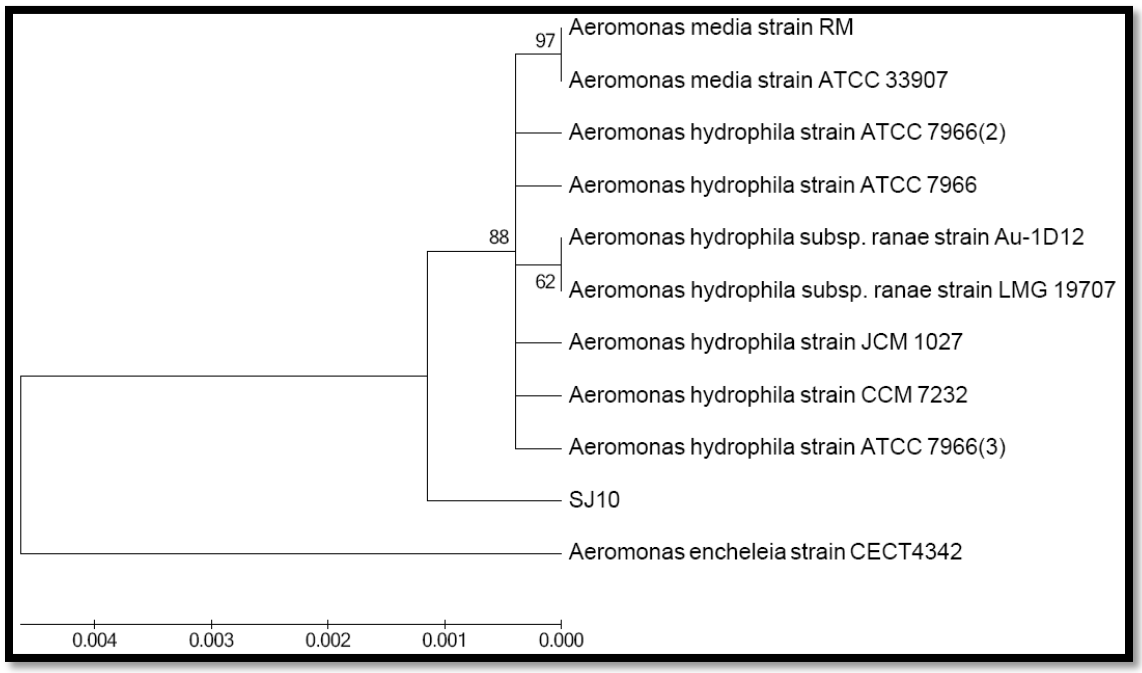


Figure 3.8d: Phylogenetic tree showing the relationship of isolated bacterium SJ10 with its neighboring species

Chapter 4

DEVELOPMENT OF A BACTERIAL CONSORTIUM FOR THE OPTIMUM DEGRADATION AND DECOLORIZATION OF CV

Development of a bacterial consortium for the optimum degradation and decolorization of CV

4.1. Introduction

The textile industrial effluents consist of various dyes with complex compositions, which vary tremendously even the same place therefore, the decolorization of these effluents necessitate a suitable option, conceivably a bacterial consortium in combination with running conditions. Together with dyes, the textile dye effluent also consists of salts, chelating agents, by-products, precursors, high ionic strength & extreme pH values and surfactants, which make its decolorization quite niggling despite of availability of some high decolorizing potential strains (Faraco and Pezzella, 2009). Biological remediation approaches used are ecofriendly and lucrative as compared to abiotic treatments. But the presence of azo, nitro and sulfo groups make dyes more challenging to microbial degradation and alternately their residues accumulate in nature (Joe et al., 2008). But, there have been copious attempts to develop biological processes for the treatment of textile effluents using bacteria, fungi and enzymes secreted by them (Banta et al., 1996; McMullan et al., 2001; Wesenberg et al., 2003). White rot fungi that produce lignolytic enzymes, were able to degrade dyes and various other organic compounds using lignin peroxidase (LiP), manganese dependent peroxidase enzymes (MnP) and Laccase (Fu and Viraraghvan, 2001; Muragesan et al., 2007).

Decolorization of CV dye generally instigates with the declined cleavage of bonds anaerobically resulting in colorless aromatic amines, followed by complete degradation of aromatic amines strictly under aerobic conditions (Kothari, 2006). Thus, anaerobic/aerobic processes using consortia are frequently used for the complete mineralization of dyes. The endurance of wide variety of organisms in miscellaneous culture capable of decolorizing a wide

range of dyes has been recently uncovered in the researches. The microbial consortium is complex in nature which enables them to act successfully on a variety of pollutants. For environmental remediation, microbial consortium is frequently used devoid of analyzing the component microbial populations (Mohorcic et al., 2004). In naturally occurring microbial consortia, bacteria plays a central role which has the degrading capacity of the pollutants (Liu and Suffita, 1993; O'Neill et al., 2000). Several bacteria capable of dye decolorization either individually or in consortia, has been reported (Patil et al., 2008). In this respect, present study deals with the studies on the decolorization of Crystal violet dye by individual bacterial strains as well as by the developed bacterial consortium from the previously isolated bacterial strains showing maximum ability of decolorizing Crystal violet dye.

4.2. Materials and methods

4.2.1. Development of bacterial consortium

Selected organisms namely; *Aeromonas hydrophila* (SJ4), *Aeromonas caviae* (SJ5) and *Aeromonas media* (SJ10) were selected to develop consortia for decolorization. For developing a consortium, individual bacterial isolates were grown overnight and further, added in equal proportion in order to get 3 % inoculum in reaction mixture. Bacterial isolates were mixed and their ability to decolorize Crystal violet dye was studied.

4.2.2. Dye decolorization experiments

Decolorization of Crystal violet dye was studied under shaking culture conditions at 35 °C in 250 mL Erlenmeyer flasks containing 100 mL of MSM broth medium. Crystal violet dye was added in the reaction mixture with final concentration of 100 mg/L. Aliquots (3 mL) from reaction flasks were withdrawn at regular intervals and centrifuged at 10,000 rpm for 20 min. For residual dye content (decolorization), the cell free supernatant was analyzed.

4.2.3. Decolorization of industrial effluent

Industrial effluent containing a mixture of various textile dyes was obtained from a drainage line emerging out from a textile handloom in Kanpur Industrial area as previously shown in pictures. For removing insoluble materials from the textile effluent, it was centrifuged at 12,000 rpm for 10 min (Joe et al., 2008). This supernatant was finally inoculated with equal proportions of overnight grown culture of *Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas media* and incubated at 30 °C under shaking condition.

4.2.4. Analysis of decolorization

The CV dye decolorizing activity of isolated bacteria SJ4, SJ5 and SJ10 individually as well as of developed bacterial consortium was determined in terms of decrease in absorbance at 590 nm against the initial absorbance at the same wavelength (λmax) using spectrophotometer (Evolution 201, Australia) and was calculated as the mean amount of decolorization for three replicates and was expressed in terms of percentage of decolorization (Deepak et al., 2004). The flasks containing sterilized modified MSM broth was inoculated with pre-cultured strain and filter sterilized (0.22 µm) CV dye and incubated on rotary shaker (110 rpm) at 35 °C. At regular intervals, 4 mL sample was withdrawn from flasks and centrifuged at 10,000 rpm for 20 min. The supernatant obtained was used for determining the percentage decolorization of CV.

At the end of experiment, the media containing individual bacterial cultures were autoclaved in order to kill the bacteria and deactivate its enzyme activity. Further, the bacterial culture was reaped by centrifugation at 4 °C and 10,000 rpm followed by twice washing with sodium phosphate buffer (pH 6.5-7). The supernatant was discarded and 5 % pellets was inoculated in different media composition with the previously provided conditions and was used as control. The color of pellet was visually scrutinized to establish whether the dye was adsorbed

to the cells or being degraded by the bacterial cells. The uninoculated MSM-CV amended broth was used as blank. The decolorizing activity of the industrial wastewater by developed bacterial consortium was also analyzed in terms of decrease in absorbance from 200-750 nm. The percent decolorization was calculated by using the formula:

$$\% \text{ Decolorization} = \frac{\text{Initial OD-Final OD}}{\text{Initial OD}} \times 100$$

Where, OD = Optical Density at 590 nm

4.2.5. Statistical analysis

All the experiments were performed in triplicates. The results obtained from each set of data have been expressed in terms of mean and standard deviation.

4.3 Results and Discussion

4.3.1. Decolorization of CV dye by individual strains as well as bacterial consortium

Bacterial isolates used to develop consortium consisted of all three isolated strains *Aeromonas hydrophila* (SJ4), *Aeromonas caviae* (SJ5) and *Aeromonas media* (SJ10). Individually, SJ4 has decolorized CV dye upto 89 %, SJ5 has decolorized upto 90 % and SJ10 has decolorized upto 93 % but in consortium CV dye was decolorized upto 95 % without any extra provided conditions. The bacterial growth and decolorization pattern of individual bacterial culture has been shown in figure 4.1 and of bacterial consortium has been shown in figure 4.2. Decolorization of broth containing CV dye by bacterial consortium of isolates SJ4, SJ5 and SJ10 with control has also shown in figure 4.3. Cheriaa et al., (2012) developed consortium of four bacterial isolates (*Agrobacterium radiobacter; Bacillus spp.; Sphingomonas paucimobilis, and Aeromonas hydrophila*) (CM-4) for degradation and decolorization of triphenylmethane dyes and obtained 91 % and 99 % of decolorization of Crystal violet and malachite green dye, respectively.

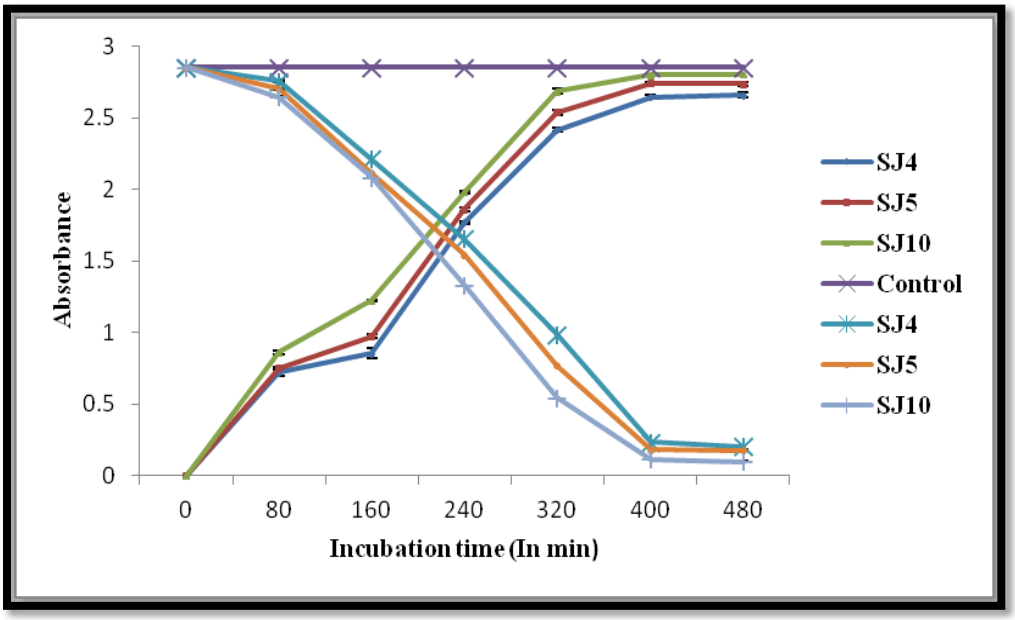


Figure 4.1: Graphical representation of bacterial growth and CV decolorization of strains SJ4, SJ5 & SJ10

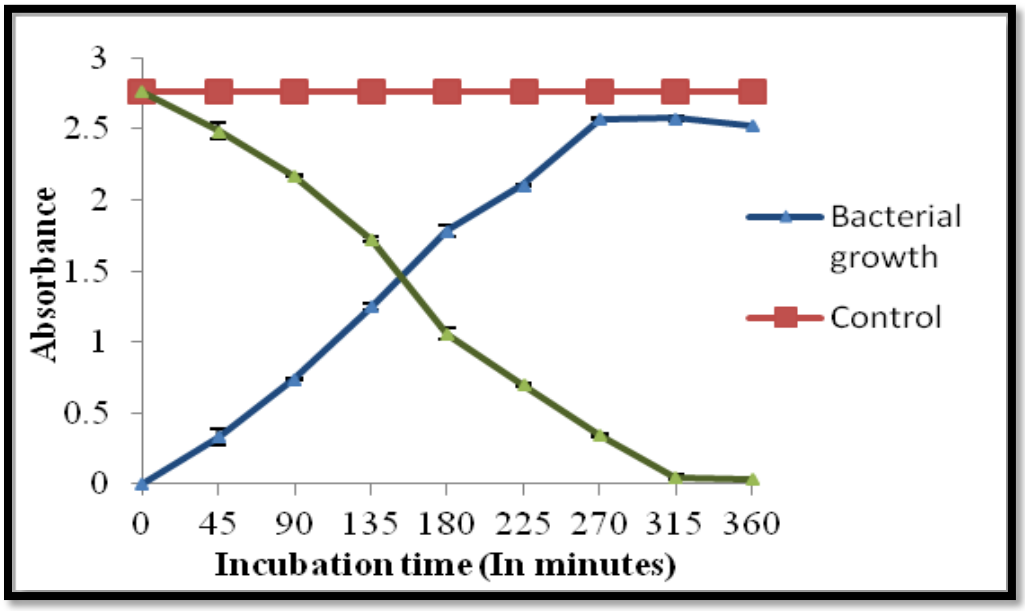


Figure 4.2: Graphical representation of bacterial growth and decolorization of CV by developed bacterial consortium

Figure 4.3: Decolorization of broth containing CV dye by consortium of Isolates SJ4, SJ5 and SJ10 with control

4.3.2. Decolorization of industrial effluent by developed bacterial consortium

Decolorization of textile industry effluents containing colored dye is a more difficult task due to the complex character of dyes as well as the presence of various organic compounds (Fu and Viraraghavan, 2001; Wesenberg et al., 2003). The decolorization potential of developed bacterial consortium from previously isolated strains was further studied on the effective decolorization as well as degradation of industrial effluent from which the bacterial strains were isolated. After incubation period of 6 days, the effluent was visually observed to be decolorized from dark purple color upto 91 % as shown in figure 4.4. The decolorization did not proceed beyond the periods indicated. In order to observe whether the bacterial consortium has only decolorized the effluent or further has degraded the organic constituents present in the effluent, GC-MS/MS analysis was performed which will be discussed in later chapter.

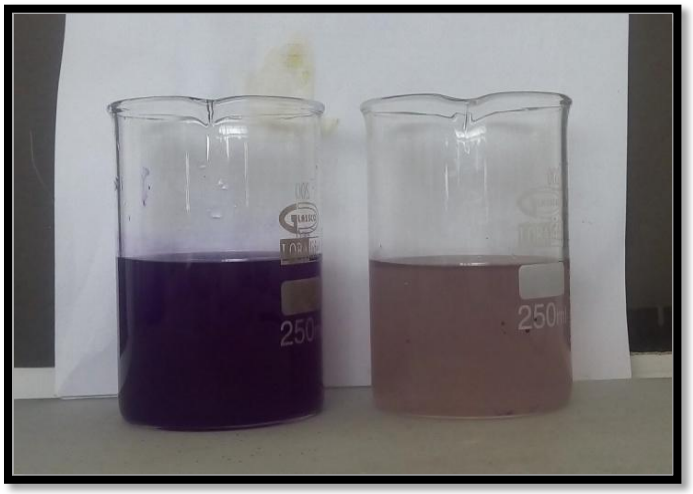


Figure 4.4: Decolorization of Wastewater by the developed bacterial Consortium

Despite many articles reporting the ability of bacteria to decolorize various dye structures, limited information subsist on their capability to decolorize textile dye effluents rather the ability of fungi, which has been well reported on the decolorization of textile effluents (Nilsson et al., 2006; Asgher et al., 2009). Schliephake et al. (1993) showed complete decolorization by *Pycnoporus cinnabarinus* within 3 days of incubation period of pigment plant effluent whereas Nilsson et al. (2006) has shown 60-70 % of decolorization of unstipulated untreated textile effluent within 10 days by *Pleurotus flabellatus.* Cammarota and Sant Anna, (1992) shown 70 % decolorization of Kraft bleach plant E1 stage effluent by *P. chrysosporium* within 5.8 days, Vanhulle et al. (2008) shown 3.7 fold decrease in color of crude effluent from dye manufacture within 14 days by *Pleurotus sanguineus* whereas Aggelis et al. (2002) initiated 50 % decolorization of effluents within 31 days by *P. ostreatus* from green olive debittering process.

Chapter 5

OPTIMIZATION OF NUTRITIONAL AND ENVIRONMENTAL PARAMETERS FOR THE OPTIMUM DEGRADATION AND DECOLORIZATION OF CV

5. Optimization of nutritional and environmental parameters for the optimum degradation and decolorization of CV

5.1. Introduction

Amongst the most alarmed environmental pollutions that threatening our biodiversity, water pollution are a key one where effluents from dye-based industries supply as primary resource. The total textile dyes used in dyeing procedure remains unreacted and approximately 15 % of dyes are directly lost in effluents (Zainith et al., 2016; Jadhav and Govindwar, 2006; O' Neill et al., 1999). Dye-containing wastewater are generated from the industries includes pigment manufacture, textile, printing, dyeing, leather, food and cosmetic industries (Sujata and Bharagava, 2016; Ahmad, 2009; Amini et al., 2008). The effluents from these industries contain extensive variety of complex structured dyes and other products such as dispersants, acids, bases, salts, detergents, humectants, oxidants etc. The existence of dyes even in very small amount in water is highly visible and thus affects the transparency and gas solubility of water building it polluted body (Banat et al., 1996).

Oodles of information is available on the degradation and decolorization of Crystal violet dye using axenic cultures of bacteria, fungi, algae and yeast including *Agrobacterium radiobacter*, *Sphingomonas paucimobilis*, *Cyathus bulleri*, *Rhodotorula rubra*, *Nocardia coralline* and *Phanerochaete chrysosporium* (Swamy and Ramsay, 1999; Aksu and Tezer, 2000; Aksu and Donmez, 2005). The pH play a very effective role in the decolorization of dyes and for color removal, optimal pH has been reported to be amid 6-10 for most of the dyes. On the other hand, under extremely alkaline pH (> pH 12) and acidic pH (< pH 6), the decolorization rate tends to decrease. Kumar et al., (2009) concluded that percentage removal of dye decreased with an increase in dye concentration while studying the effect of different initial dye concentrations

from 25 to 300 ppm. The major drawbacks for the application of cultures in biological process is to maintain purity in large scale as well as inability of cultures to degrade all different dyes present in the actual effluent (Chakraborty et al., 2003).

In present study, effort has been made for the degradation of a Crystal violet dye (CV) by using a latent bacterial consortium developed from isolated strains from effluent contaminated site. Effect of various parameters, such as static & shaking culture condition, pH, temperature, carbon & nitrogen sources, different inoculum concentrations, initial dye concentration and repeated addition of CV dye aliquots have been studied.

5.2. Materials and methods

5.2.1. Dyes and Chemicals

Crystal violet dye, a triphenylmethane dye was procured from spectrochem, India (C.I. 42555). All chemical used were of highest purity or analytical grade, obtained from recognized chemical suppliers. The other chemical for preparing various media were purchased from Hi-media Pvt. Ltd. Mumbai, India. Complete Medium Broth used for decolorization study consisting of (g/L) K_2HPO_4, 5.22; KH_2PO_4, 4.08; $MgSO_4$.$7H_2O$, 0.2; $CaCl_2$, 0.55; NH_4Cl, 0.4; agar, 15. Carbon sources such as starch, glucose, fructose, maltose and sucrose; Nitrogen sources such as peptone, yeast extract, urea, sodium nitrate and ammonium sulphate. The pH of the medium was adjusted to 7.0. A standard stock solution was prepared and used throughout the study.

5.2.2. Effect of static and shaking condition

100 mL medium containing flask was inoculated with loopful of fresh culture and supplemented with 100 ppm CV dye and incubated at static and shaking conditions (110 rpm) at

35 °C for 8 hrs with parallel abiotic control and decolorization percentage was measured as mentioned earlier (Gahlout et al., 2013).

5.2.3. Effect of physicochemical parameters

The effect of physicochemical parameters like temperature (25-45 °C) and pH (5-9) on decolorization of CV were monitored in flasks containing 100 mL of autoclaved modified MSM-CV amended medium, inoculated with fresh culture was incubated at 35 °C under shaking flask conditions. Abiotic control (without culture) was also kept as a blank and the decolorization efficiency was monitored.

5.2.4. Effect of carbon and nitrogen sources

During this nutritional parameter study, the culture containing broth was supplemented with extra carbon and nitrogen sources in order to boost the decolorization performance of CV. The medium was supplemented with different carbon sources like glucose, sucrose, starch, maltose and fructose, individually at a concentration of 0.1 % each and various organic & inorganic nitrogen sources such as yeast extract, peptone, urea, sodium nitrate and ammonium sulphate in another set were added at a concentration of 0.5 % each to medium for studying the effect on decolorization process.

5.2.5. Effect of Different inoculum concentrations on CV decolorization

The effect of different inoculum concentrations such as 1mL, 2mL, 3mL, 4mL and 5mL was also optimized on the decolorization activity of CV by the developed bacterial consortium. Abiotic control was also run parallerly.

5.2.6. Effect of initial dye (CV) concentrations

The effect of initial dye concentration (200-1000 ppm CV) on decolorization in flasks containing medium inoculated with culture was examined under shaking condition was

incubated at 35 °C and decolorization percentage was determined at every 2 hrs of incubation period. Abiotic control was also run parallerly.

5.2.7. Effect of repeated addition of (CV) dye aliquots

Repetitive decolorizing ability of developed bacterial consortium was studied by repeated spiking of dye (100 ppm) at each cycle. After each cycle, the samples were withdrawn for percent decolorization.

5.2.8. Statistical analysis

All the experiments were performed in triplicates. The results obtained from each set of data have been expressed in terms of mean and standard deviation.

5.3. Results and Discussion

5.3.1. Effect of static and shaking condition on decolorization process

The decolorizing efficiency of bacterial consortium was evaluated by incubating the flasks under shaking (110 rpm) and static conditions at 35 °C. Shaking/agitation condition favored the decolorization of CV with about 95 % decolorization after 8 hr of incubation period whereas, 45 % decolorization was obtained under static condition within the same incubation time period, thus suggesting shaking condition to be favorable for decolorization (Fig. 5.1).

The higher percentage decolorization obtained under shaking condition may be owing to the increase in biomass and oxygen transfer between the cells and medium. Parshetti et al., (2011) showed complete decolorization of CV dye (10 ppm) under static conditions and Kurade et al., (2011) have found 98 % decolorization of single dye Scarlet RR within 18 hr under static conditions whereas Bouraie and El Din, (2016) have shown 76 % decolorization of Reactive Black 5 dye under static condition and 56 % decolorization under shaking condition.

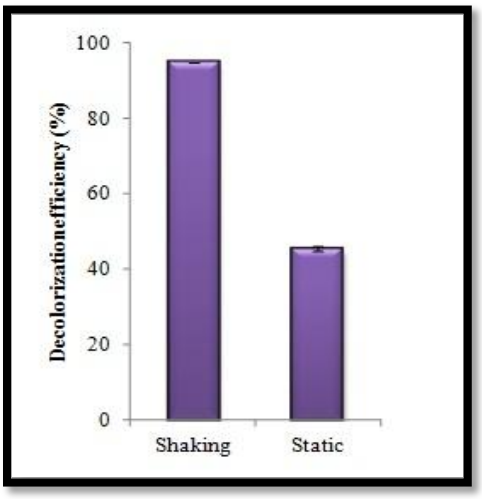


Figure 5.1: Effect of shaking and static conditions on CV dye decolorization

5.3.2. Effect of pH and temperature on dye decolorization

The pH of culture medium plays a very demanding role in the growth, metabolic activity, ligninolytic enzyme production and xenobiotic pollutant degrading potential of bacteria. Therefore, to examine the effect of pH on CV dye decolorization ability by bacterial consortium, the initial pH of medium was adjusted in a wide range of 5-9. The results obtained specify that bacterial consortium has the capability of decolorizing CV dye efficiently in wide ranges of pH 6-9. Maximum decolorization (97 %) was obtained at pH 7, thus suggesting that neutral condition favors bacterial activity in CV dye decolorization. At pH 6, 8 & 9, the decolorization values was found to be 85 %, 95 % and 90 %, respectively, whereas the decolorization percentage (9 %) was markedly found decreased at pH 5 due to acidic conditions. Similar results were also showed by Mali et al., (1999) and Chang et al., (2000) on decolorization of dye. Bouraie and El Din, (2016) and Shah et al., (2013b) has shown maximum decolorization competence at pH 7of Reactive Black dye.

An essential parameter which varies from one microorganism to other microorganisms is temperature and a slight variation in it may affect the growth and enzyme activities of

microorganisms. The effect of varying incubation temperatures on decolorization of CV by bacterial consortium was analyzed and it was constructed that dye decolorization activity of strain decreased with the increase in incubation temperature and vice-versa. The effect of pH and temperature on decolorization of CV dye is shown in fig 5.2a and 5.2b. The highest decolorization was at 35 °C (97 %) and least percentage decolorization was at 25 °C (75 %). 85 % of decolorization was recorded at 40 °C, followed by 80 % and 83 % at 45 °C and 30 °C, respectively at the end of incubation period. Shah et al., (2013a) recorded 90 % of decolorization at pH 8 and 95 % decolorization at 35 °C. Similar results were also reported by Wang et al., (2009).

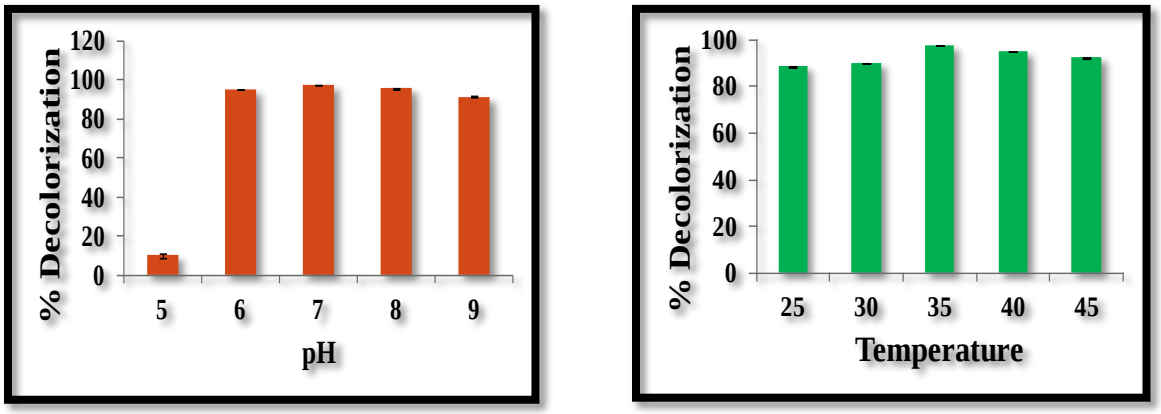


Figure 5.2a: Graphical representation of effect of pH and temperature on decolorization of CV dye by developed bacterial consortium

Control **pH 5-9 & Temperature 25 °C**

pH 5-9 & Temperature 30 °C

pH 5-9 & Temperature 35 °C

pH 5-9 & Temperature 40 °C

pH 5-9 & Temperature 45 °C

Figure 5.2b: Decolorization of broth containing CV dye by consortium at different temperatures from pH 5-9 with control

5.3.3. Effect of carbon and nitrogen sources on dye decolorization

The modified MSM-CV amended medium was supplemented with different carbon sources such as starch, glucose, fructose, sucrose, and maltose at a concentration of 0.1 % each for increasing the decolorization efficiency. Maximum decolorization obtained was 97.06 % in the flask receiving glucose as an additional carbon source; followed by sucrose (95 %) and lactose (93 %) showing higher dye decolorization efficiency as compared with other carbon sources (maltose and starch) used as shown in figure 5.3a and 5.3b. The decolorization capability of bacterial consortium decreased in the absence of the carbon source suggesting the efficacy of external co-substrate in promoting the decolorization of CV. Similar results were found by Parshetti et al., (2011).

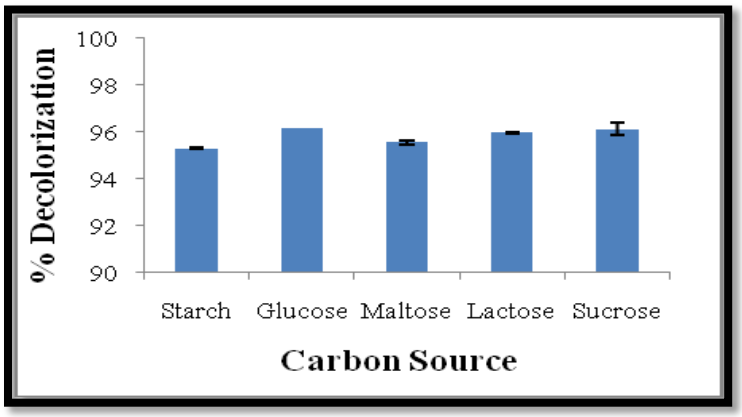


Figure 5.3a: Graphical representation of effect of different carbon sources on decolorization of CV dye by developed bacterial consortium

Figure 5.3b: Decolorization of broth containing CV dye and different carbon sources by consortium with control

In case of nitrogen sources, yeast extract has shown a maximum of 95.08 % decolorization on 8 hr of incubation period followed by ammonium sulphate and sodium nitrate which exhibited good decolorization with 93.09 % and 91.2 %, respectively. Peptone has shown 90.17 % decolorization whereas urea has shown least decolorization i.e. 58.34 % at the end of incubation period. The effect of different nitrogen sources on decolorization of CV has been shown in fig 5.4a and 5.4b.

The presence of various nutritional sources in medium might be principal to stimulatory or inhibitory effect on decolorization of CV dye resulting in variation of percentage decolorization. Shah et al., (2013a) showed 90 % of decolorization by using peptone as a

nitrogen source and Gahlout et al., (2013) has shown 96 % dye decolorization by using yeast extract as nitrogen source. Mahmood et al., (2011) showed 75-100 % decolorization of Remazol Black-B azo dye by using 4 % yeast extract and Guo et al., (2008) showed similar results of K-2BP dye decolorization by using either yeast extract or peptone as a nitrogen source.

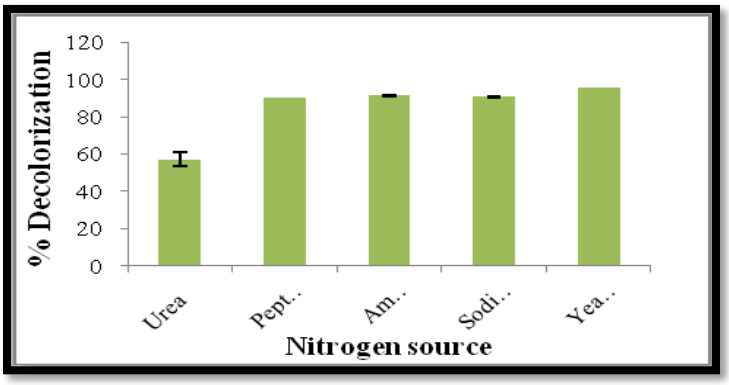


Figure 5.4a: Graphical representation of effect of different nitrogen sources on decolorization of CV dye by developed bacterial consortium

Figure 5.4b: Decolorization of broth containing CV dye and different nitrogen sources by consortium with control

5.3.4. Effect of different dye (CV) concentrations and repeated dye on decolorization at optimized conditions

According to Bhatt et al., (2005), the authentic concentration of dyes in textile effluents/wastewater has been reported to be in a series from 60-250 mgL^{-1}. Therefore, in order to investigated removal efficiency of dye under different optimized conditions, different

concentrations of inoculum as well as CV dye increasing from 200-1000 ppm were studied and established that the dye was well tolerated and decolorized by bacterial consortium in all the tested concentrations of dye and as we increased the inoculum concentrations, the dye decolorization efficiency was also increased with the decrease in incubation period. The dye decolorization was maximum at 5mL (97 %) inoculum and decreased with the decrease in inoculum concentration such as 4mL (94 %), 3mL (92 %), 2mL (88 %) and 1mL (69 %) as shown in figure 5.5a and 5.5b.

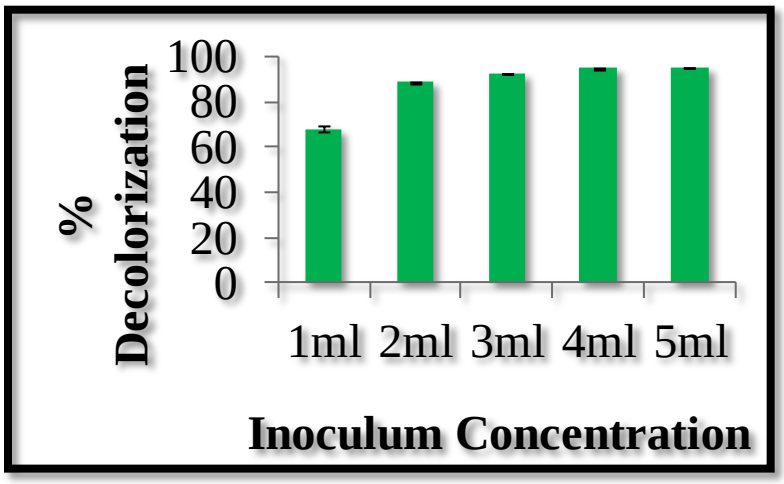


Figure 5.5a: Effect of different inoculum concentrations on decolorization of CV

Figure 5.5b: Decolorization of broth containing CV dye and different concentrations of inoculum with control

At lower concentration (200 ppm), the decolorization was observed to be highest i.e. 98 % but on further increasing dye concentration, the decolorization of CV was compact to 35 % after 24 hr of incubation time. The result showed in Fig. 5.5c has revealed that the decolorization rate was maximum at 200 ppm. At high concentration (1000 ppm), decolorization ability of bacterial consortium was greatly suppressed by CV, which might be due to the toxicity of dye to bacterial cells or due to the dissemination of the bacterial cells with dye products or might be because of the obstruction of dynamic sites of azoreductase enzymes by dye molecules (Sumathi and Manju, 2000; Sponza and Isik, 2004; Vijaykumar et al., 2007). Kashif et al., (2011) has reported 78 % decolorization of 0.5 g/L of solar golden yellow R dye by *Pleurotous ostreatus* and Vaithanomast et al., (2010) has reported more than 90 % decolorization of 1.0 g/L of reactive dye RBBR and RB5 by *Datronia species*.

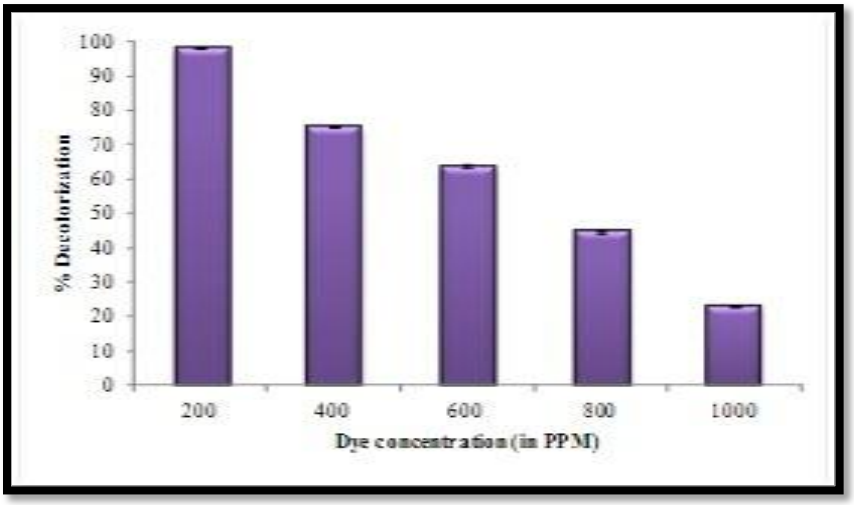


Figure 5.5c: Effect of different dye concentrations on decolorization of CV The target of this The target of this study was to estimate the effectiveness of bacterial consortium to decolorize CV dye by its repeated addition of 100 ppm dye solution under the optimized conditions. Fig. 5.5d shows that bacterial consortium was able to decolorize CV dye consecutively up to 10

cycles with reduction in decolorization efficiency ranging from 99 to 35 % for 1-10 cycles, respectively. In first cycle, 99 % decolorization was obtained within 8 hr. The successive addition of dye resulted in faster rate of decolorization process till 6th cycle and decolorization efficiency of organism decreased and required more time for decolorization of CV dye from 7th cycle to last cycle which might be by reason of nutrient diminution in flasks that may have lead to the unstable enzyme production and uncontrolled growth of bacteria. Vijaya and Sandhya, (2003) showed decolorization of methyl red dye only upto three cycles.

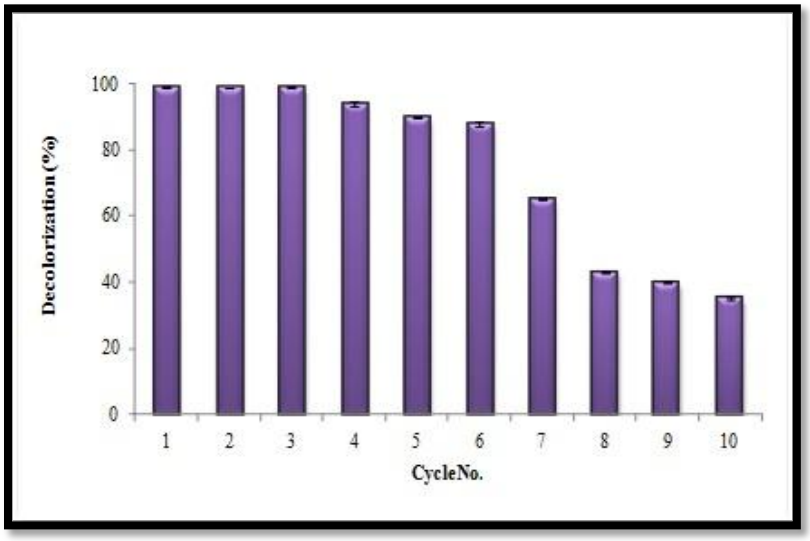


Figure 5.5d: Effect of repeated dye addition on CV dye decolorization

Chapter 6

DEGRADATION AND DECOLORIZATION OF CV BY DEVELOPED BACTERIAL CONSORTIUM AT OPTIMIZED NUTRITIONAL AND ENVIRONMENTAL CONDITIONS

Degradation and decolorization of CV by developed bacterial consortium at optimized nutritional and environmental conditions

6.1. Introduction

The activity of enzymes during microbial degradation process of industrial effluents is widely affected by environmental factors such as pH, temperature, aeration and nutrients concentration, etc. Many researchers have studied to understand the various environmental factors in microbial degradation and detoxification of CV for environmental safety (Moturi and Singara, 2009).

In 2003, Dong et al. reported that decolorization of Reactive brilliant blue by *Rhodocyclus gelatinosus* XL-1 was enhanced with amplification in peptone concentration as a carbon source and yeast extract as a nitrogen source in reaction medium (Chen et al., 2003). In some studies, glucose has been reported to enhance the decolorization of various dyes (Haug et al., 1991; Kapdan et al., 2002; Shah et al., 2013). Shah et al., (2013) found maximum decolorization of CV upto 90 % by *Bacillus subtilis* ETL-2211 when peptone was used as nitrogen source. Parshetti et al., (2011) have observed 100 % decolorization of CV by using 1 % concentration of yeast extract and 0.1 % concentration of NH_4Cl within 5 h of incubation period.

In this present study, effect of various environmental and nutritional parameters have been studied for achieving maximum degradation and decolorization of Crystal violet dye by the developed bacterial consortium (*Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas media*).

6.2. Material and methods

6.2.1 Dyes and Chemicals

Crystal violet dye, a triphenylmethane dye was procured from spectrochem, India (C.I. 42555). All chemical used were of highest purity or analytical grade, obtained from recognized

chemical suppliers. Medium Broth used for decolorization study consisted of (g/L) K_2HPO_4, 5.22; KH_2PO_4, 4.08; $MgSO_4$.$7H_2O$, 0.2; $CaCl_2$, 0.55; NH_4Cl, 0.4; agar, 15. Sucrose and Yeast extract has been used as nutritional sources.

6.2.2. Effect of optimized environmental and nutritional conditions on degradation and decolorization of CV dye

In previous chapter, we have explored the effect of pH from 5-9 range on CV dye decolorization by developed bacterial consortium and scrutinized that the decolorization of CV was maximum at pH 7 and also, we have investigated the effect of temperature from 25-45 °C range out of which 35 °C temperature was found to be the most appropriate condition. In order to amplify the decolorization competence, the medium was also supplemented with different carbon sources such as glucose, sucrose, starch, maltose and fructose and different nitrogen sources such as yeast extract, ammonium sulphate, sodium nitrate, urea and peptone at a concentration of 0.1 % and 0.5 %, respectively. So, here we have studied the effect of optimized pH i.e. 7 and temperature i.e. 35 °C with optimized nutrient sources i.e. sucrose and yeast extract on the decolorization and degradation of CV dye.

6.2.3. Statistical analysis

All the experiments were performed in triplicates. The results obtained from each set of data have been expressed in terms of mean and standard deviation.

6.3. Results and discussion

6.3.1. Effect of optimized environmental and nutritional conditions on degradation and decolorization of CV dye

The degradation and decolorization of CV dye by using the developed bacterial consortium (*Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas media*) at optimized

environmental conditions i.e. 35 °C at pH 7and nutritional conditions i.e. 0.1 % glucose and 0.5 % yeast extract was found to be suitable conditions (Shah et al., 2013; Chen et al., 2007). The CV dye decolorization was found to be maximum at the optimized environmental and nutritional parameter i.e. 99 % as shown in fig 6.1. Mahmood et al. (2011) showed complete decolorization of Remazol Black-B dye at pH 7 and 35 °C with yeast extract as carbon source whereas Shah et al. (2013) showed 90 % of decolorization of CV dye at pH 8 and 35 °C temperature with starch and peptone as carbon and nitrogen sources, respectively.

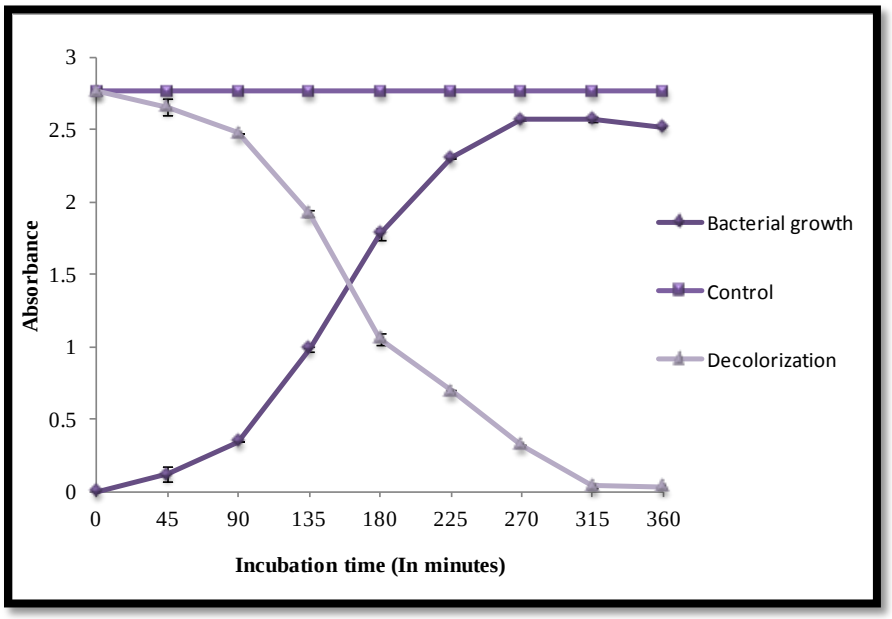


Figure 6.1: Graphical representation of bacterial growth and decolorization of CV by developed bacterial consortium at optimized conditions

Chapter 7

DETECTION AND CHARACTERIZATION OF ENZYMES RESPONSIBLE FOR DEGRADATION AND DECOLORIZATION OF CV

Detection and characterization of enzymes responsible for degradation and decolorization of CV

7.1. Introduction

Among the most alarmed environmental pollutions, water pollution is a major one where effluents from dye-based industries supply as a principle source (Mani and Bharagava, 2016). Textile industry uses vast amount of water for processing operations like desizing, scouring, bleaching, mercerizing, dyeing, printing and finishing stages, thereby releasing large amount of effluents. Wastewater can be distinct as exploited water loaded with a variety of dissolved and suspended squanders from residential, commercial, agricultural and industrial firms and every so often holds plentiful pathogenic organisms from human origin also. These abundant organic materials with nutritive principles can also stimulate the growth of some undesired aquatic organisms. Therefore, effective and economical wastewater treatment skill is compulsory to overcome health and environmental hazardous problems (Kothari et al., 2006; Banat et al., 1996; Brown and Vito, 1993; Brown and Laboureur, 1983).

In recent years, microbes and plants used for degradation and detoxification of recalcitrant organic pollutants have gained noteworthy appreciation as a feasible substitute to the existing physical and chemical treatment methods (Movahedin et al., 2006; Franciscon et al., 2009; Kagalkar et al., 2009). Microorganisms involved treatment methods capable of decolorizing or degrading the recalcitrant compounds are considered to be environmental friendly as well as can lead to mineralization of intended compounds (Sharma and Saini, 2004). Under aerobic conditions, decolorization of dyes through bacteria usually results in adsorption of dyestuffs on bacteria, rather than oxidation (Pagga and Brown, 1986). With the involvement of different kinds of enzymes, biological treatment methods are found capable of degrading an array of recalcitrant organic pollutants.

In previous few years, numerous organisms have been reported to decolorize and degrade dyes up to mineralization level. In this chapter, we have discussed about the detection and characterization of ligninolytic enzymes released throughout the degradation and decolorization of Crystal violet dye.

7.2. Material and methods

7.2.1. Preparation of cell free extract

The bacterial cells were grown in 250 mL Erlenmeyer flasks containing 100 mL of medium, pH 7 and incubated at 35 °C for 24 hr. After the incubation period, a sample of 2 mL was withdrawn from the flask and harvested by centrifugation at 10,000 rpm at 4 °C for 15-20 min. The clear culture supernatant obtained was directly used as extracellular enzymes in order to determine the enzymatic status. These harvested cells (75 mg/mL) were perched in a potassium phosphate buffer (50 mmol/L) at pH 7.4 and sonicated (Sonics-Vibracell ultrasonic processor, USA), keeping sonifier output at 40 (amps) and giving 7 strokes each of 30 sec, with 1 min interval at 4 °C. The homogenate was centrifuged at 10,000 rpm for 20 min and supernatant was used as a source of crude enzyme.

7.2.2. Enzyme assays

Laccase Activity: Laccase activity was performed by monitoring absorbance of reaction mixture by adding 10 µL crude enzyme. The reaction mixture contained 100 µL of 0.1 M guaiacol dissolved in 100 mM Na-Acetate buffer at pH 5. Increase in absorbance was recorded for 30 mins at 450 nm, which was proportional to laccase activity.

Lignin peroxidase activity: Lignin peroxidase activity was determined by monitoring the conversion of propanaldehyde at 300 nm in a reaction mixture of 10 µL enzyme extract and 100

µL containing 100 mM n-propanol, 250 mM tartaric acid, and 10 mM H_2O_2 according to Kalyani et al., (2008).

7.2.3. SDS-PAGE Preparation

Denaturing sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on Polyacrylamide Gel Electrophoresis unit (GX-SCZ2, Genetix Biotech Asia Pvt Ltd) by using 10 % polyacrylamide in gel. The samples were loaded in duplicates and concentration of enzyme extract was calculated by comparing with the standard curve at 595 nm absorbance. The composition of separating gel and stacking gel has been discussed below:

Materials

- Casting gel unit for electrophoresis
- Siliconized Pasteur pipettes
- Syringes equipped with blunt, stub nosed, needles
- Vacuum chamber for degassing gels
- Micropipettes (10-300 µL)
- Stock 30 % T: 0.8 % C Acrylamide monomer
- 1.5 M Tris-HCl buffer, pH 8.8
- 10 % (w/v) SDS
- 10 % (w/v) Ammonium persulfate

Separation Gel (mixed just prior to use)

- 10 mL of Acrylamide monomer
- 2.6 mL of Tris-HCl Buffer, pH 8.8
- 0.1 mL of 10 % (w/v) SDS
- 3.8 mL of H_2O

Stacking gel (mixed just prior to use)

- .67 mL of Acrylamide monomer
- 1.25 mL of Tris buffer, pH 8.8
- 0.05 mL of 10 % (w/v) SDS

- 2.975 mL of H_2O

Procedure:

1. Assemble the slab gel unit with the glass sandwich set in the casting mode with 1.5 mm space.
2. Prepare a separating gel in a separate small beaker.
3. Add separating gel to a side arm flask, stopper the flask and attach to a vacuum pump equipped with a cold trap. Turn on the vacuum and degas the solution for ~ 10 min. During this period, gently swirl the solutions in the flask.
4. Exit the vacuum, open the flask and add 100 µL of ammonium persulfate and 10 µL of TEMED to the solution.
5. Add stopper to flask and degas for additional 2 min with gentle swirl to mix the solutions.
6. Transfer appropriate amount of degassed solution to casting chamber without any air bubble formation.
7. Immediately fill in water to the top of separating gel for preventing formation of meniscus.
8. Let it settle for 20-30 min to gelate.
9. Prepare a stacking gel as separating gel preparation method and add 0.05 mL of ammonium persulfate and 0.005 mL of TEMED.
10. Pour the stacking gel onto the separating gel.
11. Insert the well-forming comb without trapping air and wait for 20-30 min to let it gelate.
12. Take out the comb after complete gelation,.
13. The prepared samples were mixed with sample buffer and were heated in the boiling water for 5-10 min.
14. Now load the samples into wells and protein markers into the first lane. Now cover the top and connect the anodes.
15. Set an appropriate volt and run the electrophoresis.
16. After completion of total running time, stop SDS-PAGE running when the downmost sign of protein marker reaches foot line of the glass plate.

On completion of Electrophoresis, the gel was subjected to coomassie brilliant blue R-250 staining and decolorization of gel obtained around the protein bands was interrelated with

enzyme activity and the molecular weight was estimated by comparing with standard protein marker (Molecular Standard Mixture Recombinant, 15-150 kDa; Sigma).

7.2.4. Statistical analysis

All the experiments were performed in triplicates. The results obtained from each set of data have been expressed in terms of mean and standard deviation.

7.3. Results and discussion

7.3.1. Enzyme assay

The biotransformation process of toxic pollutant is only possible through the catalytic species present in microorganisms i.e. oxido-reductive enzymes. Oxidative degradation takes place by the action of enzymes such as peroxidases and laccases (Ayed et al., 2010). The possible roles of oxido-reductive enzymes in biodegradation of toxic dyes have been well documented by Dhanve et al. 2008, Jadhav et al. 2009, Telke et al. 2009, etc. Protein separated by SDS-PAGE gel was treated for enzyme renaturation showing two protein bands corresponding to ~40 kDa and ~60 kDa with clear zones of decolorization, suggesting positive Lignin peroxidase and Laccase activity, respectively of purified proteins by using standard protein marker as shown in figure 7.1.

Lignin Peroxidase was the major enzyme as compared with the laccase at 8 hr of incubation period at the optimized conditions secreted by *A. hydrophila*, *A. caviae* and *A. media* was found to be associated with the degradation and decolorization of CV dye (Table 7.1). *Aeromonas hydrophila* has produced 3.06 U/mL of LiP and 1.065 U/mL of Laccase enzyme, *Aeromonas caviae* has produced 2.50 U/mL of LiP and 0.946 U/mL of Laccase enzyme whereas *Aeromonas media* has produced 1.39 U/mL of LiP and 1.0142 U/mL of Laccase enzyme

showing *A. hydrophila* strain to be more able to produce ligninolytic enzymes than other two strains.

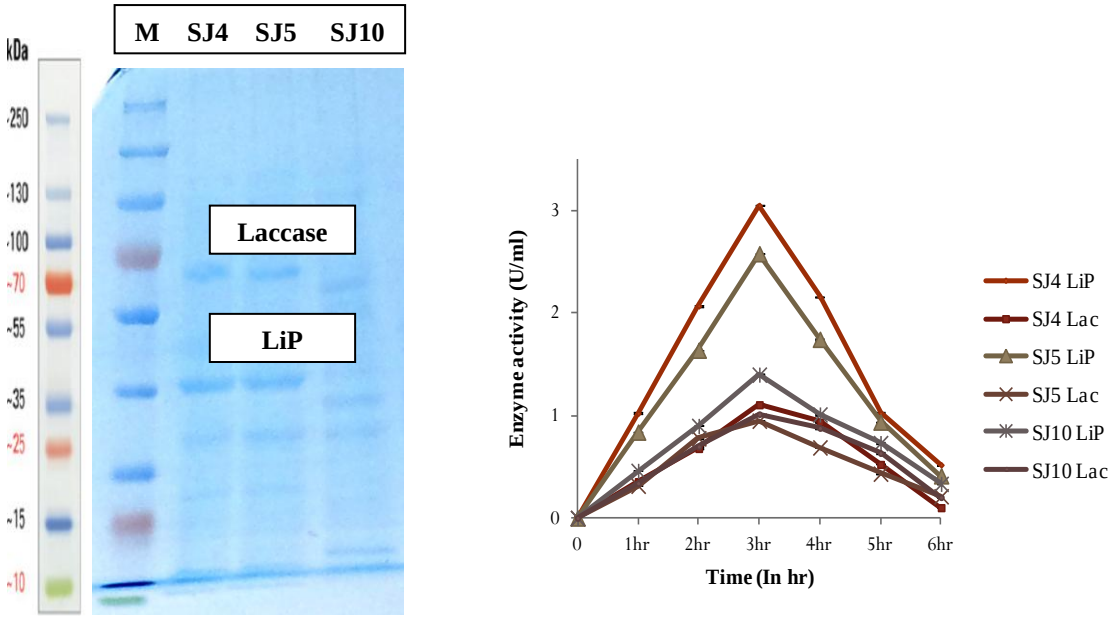


Figure 7.1: (a) SDS-PAGE analysis of crude enzyme and **(b)** Laccase and LiP production by *Aeromonas hydrophila* (SJ4), *Aeromonas caviae* (SJ5) and *Aeromonas media* (SJ10) during the degradation and decolorization of Crystal violet dye

Table 7.1: Total Proteins produced and enzyme activity of all three strains

Samples	Crude enzyme concentrations (µg/mL)	Laccase activity (Unit/mL)	Lignin Peroxidase activity (Unit/mL)
SJ4	552.7767	1.065	3.06
SJ5	537.7767	0.946	2.50
SJ10	547.7767	1.0142	1.39

Chapter 8

CHARACTERIZATION OF METABOLIC PRODUCTS BY HPLC/GC-MS/LC-MS-MS ANALYSIS

8. Characterization of metabolic products by GC-MS-MS analysis

8.1. Introduction

In textile dyeing and printing procedures, a wide variety of dye groups are being more progressively worned. They masquerade toxicity in the form of carcinogenicity, mutagenicity and genotoxicity to aquatic organisms, as well as to humans, animals and plants (Senthilkumaar et al., 2006; Mittal et al., 2010). In humans and animals it causes skin irritation, digestive tract irritation, nausea, vomiting, liver and kidney damage, etc and in plants causes reduced seed germination, root and shoot length. The effluent also hinders the activity of microorganisms contributing the soil fertility. Besides the effectiveness of bacterial treatment, it is found that sometimes after treatment, the dyes get transformed into compounds which are more toxic than of parent compounds. Hence, in order to know the toxic effects of yields formed after biological treatment processes, Parshetti et al. (2011) have performed some microbial toxicity and phytotoxicity by using bacteria contributing soil fertility. The seed germination test was performed on four kinds of plant species for determining the toxicity of degradation products of CV. They found that degradation products of CV were less toxic to bacteria as well as to all four kinds of plant species as compared to undegraded CV.

Bioassays which can alleviate these constrictions are, therefore, recommended for the valuation of natural risks (Conder et al., 2001). In present study, the efficiency of developed bacterial consortium to degraded CV dye as well as the textile effluent was analyzed by GC-MS/MS.

8.2. Materials and methods

8.2.1. Characterization of metabolic products of textile wastewater and degraded textile wastewater by bacterial consortium

8.2.1.1. Liquid-Liquid extraction

A portion of 200 mL of textile effluent sample was centrifuged at 10,000 rpm for 20 min for removing any biotic culture as well as added perched particles from effluent. The supernatant was then extracted thrice with the equal volumes of ethyl acetate. The extracted ethyl acetate sample was then dehydrated over anhydrous Na_2SO_4 and evaporated to dryness in rotary evaporator. The dry residue obtained were dissolved in small volume of HPLC grade n-hexane and used for GC-MS/MS analysis and considered as control.

Further, the textile effluent was again centrifuged at 12,000 rpm for 10 min for removing insoluble materials (Joe et al., 2008). The supernatant attained was finally inoculated with equal proportions of overnight grown culture of *Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas media* and incubated at 35 °C under shaking condition for 6 days. After incubation period, the wastewater was visually found to be decolorized. In order to observe the effect of bacterial consortium on textile wastewater, the GC-MS/MS analysis was done. For this, a portion of 200 mL of degraded textile wastewater sample was centrifuged at 10,000 rpm for 20 min at 4 °C to undo bacterial biomass and other balanced particles from effluent. The supernatant was vaccum evaporated at 40 °C to concentrate the metabolites in charge to reduce the volume upto 50 %, and then 100 mL aliquot of sample was extracted thrice with the equal volumes of ethyl acetate (Parshetti et al., 2011). The extracted ethyl acetate sample was dried over anhydrous Na_2SO_4 and evaporated to dryness in rotary evaporator. The dry residue obtained were dissolved in small volume of HPLC grade n-hexane and used for metabolites analysis (Parshetti et al., 2011).

8.2.1.2. GC-MS analysis

The Gas Chromatography-Mass Spectrometry analysis (GC-MS/MS) of metabolites was carried out by using PerkinElmer (UK) equipped with a PE auto system XL gas chromatograph

and a PE-5MS capillary column (20 m × 0.18 mm internal diameter, 0.18 mm film thickness). Helium was used as a carrier gas (flow rate: 1 mL min^{-1}) using splitless injector (injector temperature was 280 °C). The column temperature was programmed as 50 °C (5 min); 50-300 °C (10 °C min^{-1}, hold time: 5 min). The MS transfer line and ion source temperatures were kept at 200 and 250 °C, respectively. The MS was operated in full SCAN mode. A solvent delay of 3.0 min was selected. In full-scan mode, the electron ionization (EI) mass spectra were recorded in range of 30-550 (m/z units) at 70 eV. The metabolites were identified by using National Institute of Standards and Technology (NIST) library available with instrument and by comparing the retention time (RT) and fragmentation pattern.

8.2.2. Characterization of metabolic products of degraded Crystal violet dye by bacterial consortium

8.2.2.1. Liquid-liquid extraction

The portion of 200 mL of degraded culture medium sections was centrifuged at 10,000 rpm for 20 min at 4 °C to split bacterial biomass and extra perched particles from biotic sample. The clear supernatant obtained was vaccum evaporated at 40 °C to deliberate metabolites in order to condense volume upto 50 %, and then 100 mL aliquot of biotic sample was extracted thrice with the equal volumes of ethyl acetate (Parshetti et al., 2011). The extracted ethyl acetate sample was desiccated over anhydrous Na_2SO_4 and evaporated to parchedness in rotary evaporator. The dry residue obtained was dissolved in small volume of HPLC grade methanol and was used for metabolites analysis (Parshetti et al., 2011).

8.2.2.2. GC-MS analysis

The Gas Chromatography-Mass Spectrometry analysis (GC-MS/MS) of metabolites was carried out by using PerkinElmer (UK) equipped with a PE auto system XL gas chromatograph and a PE-5MS capillary column (20 m × 0.18 mm internal diameter, 0.18 mm film thickness).

Helium was used as a carrier gas (flow rate: 1 mL min^{-1}) using splitless injector (injector temperature was 280 °C). The column temperature was programmed as 50 °C (5 min); 50-300 °C (10 °C min^{-1}, hold time: 5 min). The MS transfer line and ion source temperatures were kept at 200 and 250 °C, respectively. The MS was operated in full SCAN mode. A solvent delay of 3.0 min was selected. In full-scan mode, the electron ionization (EI) mass spectra were recorded in range of 30-550 (m/z units) at 70 eV. The metabolites were identified by using National Institute of Standards and Technology (NIST) library available with instrument and by comparing the retention time (RT) and fragmentation pattern.

8.2.3. Statistical analysis

All the experiments were performed in triplicates. The results obtained from each set of data have been expressed in terms of mean and standard deviation.

8.3. Results and discussion

8.3.1. Degradation analysis of textile wastewater

We scrutinized the skill of developed bacterial consortium (*Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas media*) in decolorizing textile effluent. After 6 days of shaking action of dye containing effluent, we observed noteworthy decolorization of textile effluent, which was or else dark purple in color as previously shown in figure 4.4. In GC-MS/MS analysis of textile effluent, we have seen many peaks for compounds. But in GC-MS/MS analysis of bacterially treated textile effluent, we have observed that some of the peaks have either disappeared or breakdown into other smaller peaks as compared to peaks of control (Table 8.1). The results demonstrated that treatment of textile wastewater with the developed bacterial consortium has somehow efficiently worked in either removing some compounds from wastewater or breaking some larger compounds into lesser compounds as shown in

chromatograms of textile wastewater and bacterially treated textile wastewater (Fig 8.1). The compounds in textile wastewater and treated wastewater have been compiled in table 8.1.

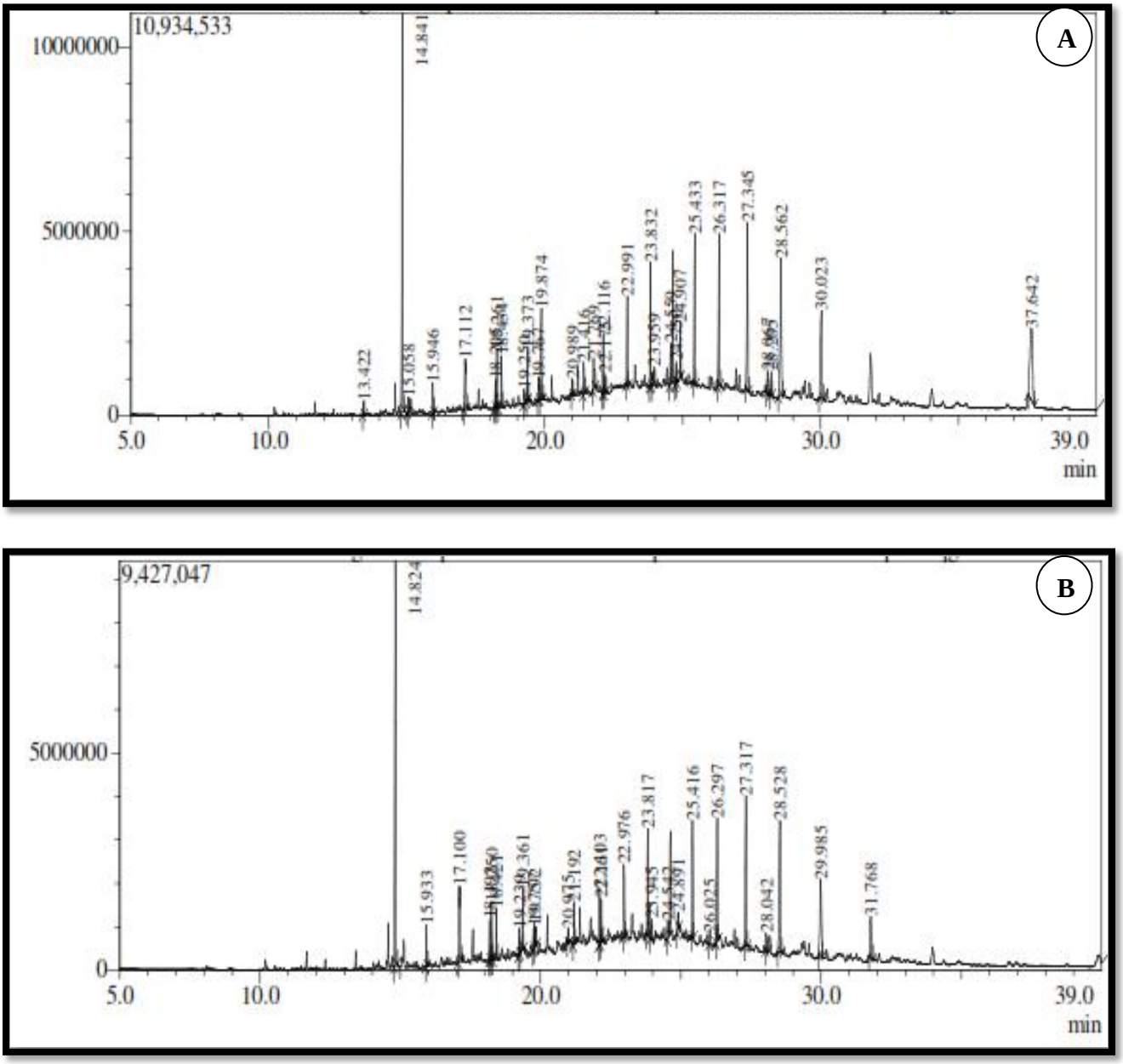


Figure 8.1: A. Chromatogram of Textile Wastewater and **B.** Chromatogram of bacterial consortium treated Textile Wastewater

Table 8.1: Detected and Characterized Organic compounds and their metabolites in textile wastewater and bacterial consortium treated wastewater

S.No.	Compound name	RT	U-T	T
1.	Pentadecane	13.422	+	-
2.	Benzoic acid, 4-ethoxy-, ethyl ester	15.058	+	-
3.	3-Hexadecanol	18.250	-	+
4.	3-Heptadecanol	18.261	+	-
5.	3-Ethyl-3-undecanol	19.757	-	+
6.	4-Heptanol, 4-methyl-	19.767	+	-
7.	Hexadecane, 2,6,10,14-tetramethyl	19.792	-	+
8.	Hexadecanoic acid	19.874	+	-
9.	2-Ethyl-5-methyl-octahydrocyclopenta[b]pyran-3-ol	20.975	-	+
10.	tetrahydro-2,2-dimethyl-5-(1-methylpropyl)-	20.989	+	-
11.	Docosane	21.192	-	+
12.	Dotriacontane	21.416	+	-
13.	Octadecanoic acid	21.769	+	-
14.	Heptacosyl acetate	22.161	-	+
15.	1-Dodecanol, 2-octyl-	22.175	+	-
16.	Tricosane	22.991	+	-
17.	Tetracosane	23.817	-	+
18.	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	24.750	+	-
19.	4,4'-((p-Phenylene)diisopropylidene)diphenol	26.025	-	+
20.	Triacontane	27.317	-	+
21.	Pentacosane	27.345	+	-
22.	Docosane, 2,21-dimethyl-	28.205	+	-
23.	Pentatriacontane	31.768	-	+
24.	Tetrakis(2,3-Ditert-Butylphenyl)-4,4'-Biphenylene Diphosphonate	37.642	+	-

RT- Retention time; U-T- untreated; T- treated; +- positive; - negative

8.3.2. Degradation analysis of degraded Crystal violet dye sample

The identified intermediates such as Phenol, 2,6-bis(1,1-dimethylethyl) (a), 2',6'-Dihydroxyacetophenone (b) and Benzene (c) were formed with the molecular weight 206.3239 g/mol, 152.149 g/mol and 78.11 g/mol with corresponding mass spectrum at GC retention times, 17.97, 10.36 and 7.72 respectively in comparison to control toxic compound i.e. Crystal violet

(407.99 g/mol) (Table 8.2). From the three compounds detected by GC-MS/MS (Fig 8.2) in extracted samples collected during color removal. We cogitated that Crystal Violet may have been first biotransformed into Phenol, 2,6-bis(1,1-dimethylethyl), which might have transformed to 2',6'-Dihydroxyacetophenone and finally might have transformed to Benzene.

Several studies have been done on the degradation of Crystal violet dye showing conversion of it into colorless leuco-derivatives through several microorganisms, but the literature regarding degradation of Crystal violet dye through bacterial consortium (*Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas media*) has been less found. Yatome et al., (1991, 1993) and Chen et al., (2008) has reported Michler's Ketone as key degradation product of CV dye by growing cells of *Bacillus subtilis* IFO 13719, *Nocardia coralline* and *Shewanella decolorationis* NTOU1, respectively. Parshetti et al., (2011) has reported phenol as the ultimate degraded product of Crystal violet, but we have found the derivative of phenol product i.e. Phenol, 2, 6-bis(1,1-dimethylethyl). It is a colorless solid alkylated phenol, which along with its derivatives are used in industries as UV stabilizers and anti-oxidants for hydrocarbon based products, which prevents clogging in aviation fuels and most importantly has very low toxicity (LD_{50} 9200 mg/kg). Further, 2', 6'-Dihydroxyacetophenone was also found along with other products with lowest molecular weight among these. It belongs to organic compounds containing acetophenones family, which is used for analysis of fragile peptides, disulphide bonding and small proteins (Gorman et al., 1996). And the end product Benzene, a colorless and highly flammable liquid is used as a herald in the production of chemicals with more multifarious structure.

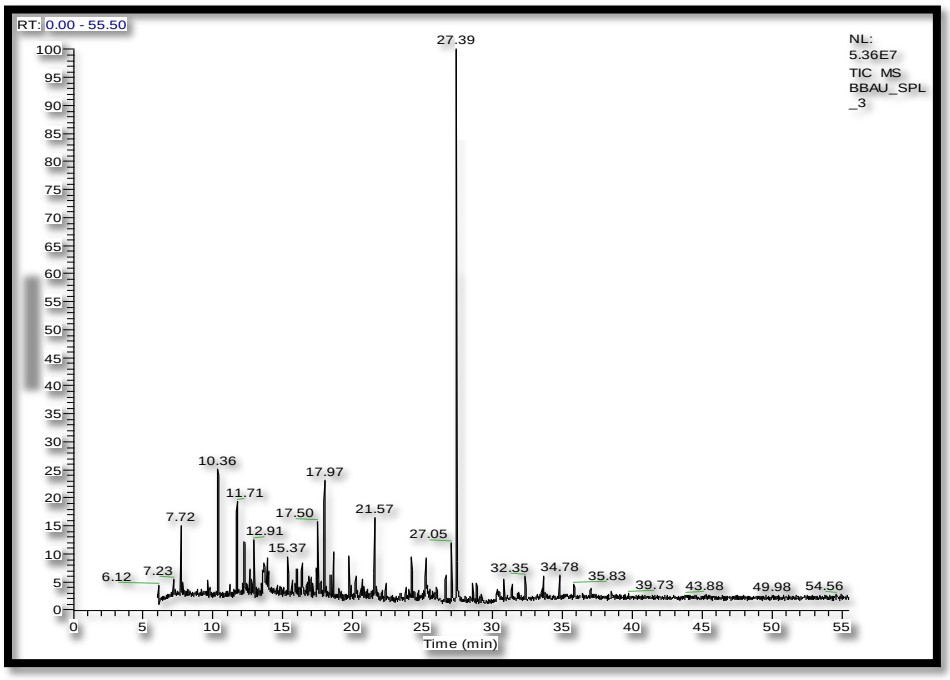


Figure 8.2: Chromatogram of bacterially degraded Crystal violet dye

Table 8.2: Identification of metabolites of Crystal violet through GC-MS/MS with their retention time

Compound Name	**RT**
Phenol, 2,6-bis(1,1-dimethylethyl)	17.97
2’,6’-Dihydroxyacetophenone,	10.36
Benzene	7.72

Chapter 9

TOXICITY ASSESSMENT OF CRYSTAL VIOLET BEFORE AND AFTER BACTERIAL TREATMENT PROCESS

Toxicity assessment of crystal violet dye before and after bacterial treatment process

9.1. Introduction

Plants play important ecological role/functions in providing habitat for natural world protect soil from erosion and also provide enormous immensity of organic matter to soil, which is important for maintaining its fertility (Wang, 1991). The effluents generated from textile industries cause toxic effects on seed germination rates as well as to biomass concentrations of several plant species, which has been previously indicated in many reports. The reason for toxicity of textile effluent may be due to the presence of several dyes or their partially degraded products, which are mutagenic or carcinogenic (Kalyuzhnyi and Sklyar, 2000). Thus, it becomes utmost important for the treatment of textile industry effluents becomes their final discharge in to the environment (Kumar et al., 2009). The present study deals with the toxicity of Crystal violet dye as well as its degraded metabolites carried out by phytotoxicity studies on *Phaseolus mungo* seeds and animal toxicity on *Caenorhabditis elegans*.

9.2. Materials and methods

9.2.1. Dye and chemicals

The Crystal violet dye used in experimental study was purchased from spectrochem, India (C.I. 42555) and its standard stock solution was prepared and used throughout the study. The medium used throughout this study was modified Minimal-salt-medium (MSM) amended with CV dye, which contained (in gL^{-1}) K_2HPO_4, 5.22; KH_2PO_4, 4.08; $MgSO_4$.$7H_2O$, 0.2; $CaCl_2$, 0.55; NH_4Cl, 0.4; agar, 1.5. The seeds of *Phaseolus mungo* were obtained from the local market of Lucknow (U. P.), India. *C. elegans* strains, Bristol N2 (wild type), were procured from *Caenorhabditis* Genetics Centre, (University of Minnesota, MN, USA).

9.2.2. Liquid-liquid extraction

After decolorization, a portion of 200 mL of degraded culture medium samples was centrifuged at 10,000 rpm for 20 min at 4 °C to split the bacterial biomass and other poised particles from biotic sample. The supernatant was then diged out thrice with the equal volumes of ethyl acetate metabolites (Parshetti et al., 2011). The extracted ethyl acetate sample was dried over anhydrous Na_2SO_4 and evaporated to dryness in rotary evaporator. The dry residue obtained were dissolved in water to figure final concentration of 1000 ppm and used for phytotoxicity study (Parshetti et al., 2011).

9.2.3. Phytotoxicity study

The toxic effect of CV dye and its degraded metabolite on seed germination percentage (at regular 24 hr), radical length and phytotoxicity percentage after 3 days was evaluated on *Phaseolus mungo*. Since pulses are an important crop of Indian agriculture, therefore the study was carried out on *Phaseolus mungo* at room temperature (Kalyani et al., 2008; Saratale et al., 2013). For this study, the dried ethyl acetate extracted metabolites of degraded CV dye was dissolved in sterile distilled water to make a concluding concentration of 500 ppm. The dye solutions were also prepared with concentration of 500 ppm for phytotoxicity study. Seedlings were raised in glass petriplates (10 cm diameter) at room temperature (32 ± 2 °C). Surpass to sowing, the seeds were sterilized in 1 % $HgCl_2$ for 1 min and thoroughly washing with distilled water thrice (Somasegaran and Hoben, 1985). Seeds of *Phaseolus mungo* were usually supplied with equal amount of distilled water as a control, CV dye (500 ppm) and their obtained metabolites (500 ppm) for 3 days. The toxicity effect was then measured in terms of above said parameters after 3 days of incubation period.

9.2.4. Animal toxicity study

9.2.4.1. Relative quantification of acetylcholine (Ach)/acetyl cholinesterase (AchE)

Ach levels were determined using Amplex Red Ach/AchE estimation kit as indicated in the manufacturer's procedure. The treatment plates added with age-synchronized embryos were incubated at 22 °C for 48 hr. M9 buffer was used to wash adult worms thrice and sonicated in 1x reaction buffer (supplied in the kit) for 3 min. At 7000 rpm, worm suspension was then centrifuged for 7 min. 100 µL of reaction mixture was prepared by adding 200 µL of 20 mM Amplex Red solution, 100 µL of 200 U/mL HRP solution, 100 µL of 100 U/mL AchE solution, and 100 µL of 20 U/mL choline oxidase solution in 10 mL q.s. of 1x reaction buffer in black well plates in which 100 µL of supernatant was added. The plates were incubated at room temperature for 30 min and fluorescence was read using 96 well plate fluorimeter (BMG Polarstar Galaxy) excitation at 544 nm and emission at 590 nm. The relative fluorescence obtained was normalized with protein content of sample calculated using Bradford method and RFU per µg of protein was calculated to estimate the relative Ach levels (Urra et al., 2016).

9.2.4.2. Synaptic Ach levels as deduced through aldicarb assay

An indirect assay, Aldicarb assay, was used to check the relative effect on neurotransmission and Ach levels within the synapse. Aldicarb is basically a carbamate insecticide, which acts as an AchE inhibitor. Approximately 40 worms were transferred to 0.5 mM aldicarb NGM plates. The worms were keep counted every 30 min for paralysis and were poked using eye lash. As a convention, the worms lacking movement even after poking thrice were considered as paralyzed. Any worms lost or damaged were disregarded from the study. The experiment was done in duplicate sets and percentage of worms paralyzed was calculated (Hosono et al., 1992).

9.2.4.3. Effect on nAchR as deduced through levamisole assay

Levamisole ((S)-6-Phenyl-2,3,5,6-tetrahydroimidazole[2,1-b][1,3]thiazole) a cholinergic agonist is used in Levamisole assay, which is done to check the post synaptic defects in worms. Approximately 20 to 30 worms were transferred to each well of 96 well plates incubated at 22 °C for 48 hr. Equal volume of 50 µM levamisole solution was added to each well and worms were scored for paralysis. The worms that lacked movement were considered as paralyzed. Percentage of worms paralyzed at every time interval was calculated (Fleming et al., 1997).

9.2.4.4. RNA isolation, cDNA synthesis and quantitative real time PCR:

Total RNA from adult age-synchronized animals was extracted by using Trizol reagent (Invitrogen, Life Technologies). 0.1 % DEPC water was used to wash worms from treatment plates. The worms were crushed in 250 µL Trizol reagent using micropestles (Tarson) and for making the final volume up to 1 mL, rest 750 µL of Trizol was added, which was followed by addition of 200 µL of chloroform and was mixed for 2 to 5 min on a vortex mixer. The suspension was then centrifuged at 14000 rpm, 4 °C for 15 min and supernatant was gently pipetted out in fresh vials. RNA was precipitated by addition of 500 µL chilled isopropanol. The vials were kept at room temperature for 10 min and again centrifuged at 14000 rpm, 4 °C for 10 min. The obtained RNA pellets were washed twice with 75 % ethanol (chilled) at 7500 rpm, 4 °C for 5 min. The RNA pellet was finally dissolved in 15 µL of 0.1 % DEPC water and for quantification, RNA absorbance was read using nano drop (QuaWell Q5000).

cDNA synthesis was done from 1 µg of total *C. elegans* RNA in a 96 well thermal cycler (BioRad, C1000) with steps including, incubation at 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min and 4 °C eternally RNA using high capacity cDNA synthesis Kit (Applied Biosystems). Until utilization, quantified cDNA samples by nanodrop were stored at -80 °C. 125 ng of cDNA was used as template for each reaction of qRT-PCR with gpd-1 as housekeeping

control using Light Cycler 480 machine (Roche Diagnostics, Germany). For each primer pair, a melting curve analysis was performed. The thermocycling program in brief was an initial incubation of 50 °C for 2 min hold; 95 °C for 10 min followed by 40 cycles of denaturing at 95 °C for 15 s, annealing at 58 °C for 30 s and final extension at 72 °C for 20 s. Differential expression was calculated by $2^{-\Delta\Delta CT}$ method. Gpd-1 was used as internal control and used to normalize ratios between samples (Shen et al., 2001).

Equation for calculation of differential expression:

$$\Delta\Delta CT = \Delta CT_{\text{refrence}} - \Delta CT_{\text{target}}$$

$$\Delta CT = CT_{\text{Refrence in control}} - CT_{\text{Refrence in treatment}}$$

9.2.4.5. Nile-red staining

The toxicity of Crystal violet dye and its degraded metabolite was evaluated by investigating the effect on lipid content of *Caenorhabditis elegans* by staining it with lipid specific dye, Nile Red (9-diethylamino-5-benzo[α]phenoxazinone) as described by Ashrafi et al., 2003. Briefly, a stock solution of Nile red was prepared by dissolving 0.5 mg Nile red dye in 1 mL of acetone. The stock solution was further diluted to 1:250 and was seeded onto NGM plates. Embryos were transferred onto the Nile red-containing treatment plates and incubated for 48 hrs at 22 °C. The worms were then washed thrice with M9 Buffer and were anesthetized by adding 10 µL of 100 mM sodium azide in 100 µL of worm suspension. The cover slip was sealed using transparent nail paint. The worms were observed in fluorescence microscope for visualization lipid droplets using Rhodamine filter.

9.2.5. Statistical analysis

All data were presented as mean±SD and analyzed by one-way ANOVA followed by Bonferroni's multiple comparison tests for the possible significance identification between the various groups.

9.3. Results and Discussion

9.3.1. Phytotoxicity study

The wastewater discharged in the environment from textile industries causes destructive collision on the nearby flora and fauna and hence, this study is of scrupulous implication. Seed germination and plant growth bioassay is considered as most common technique for evaluating the phytotoxicity (Kapanen and Itavaara, 2001). Thus, the primary aim of this study was to assess the phytotoxicity of dye and its metabolites obtained after degradation of Crystal violet dye by developed bacterial consortium (*Aeromonas hydrophila, Aeromonas caviae* and *Aeromonas media*). The toxicity analysis of CV dye and its metabolites obtained after bacterial degradation has been described in Table 9.1.

Table 9.1: Phytotoxicity of Crystal Violet and it's metabolites formed after its decolorization

Experiment	Germination %			Radicle length (avg. in cm)	Phytotoxicity %
	24 hr	48 hr	72 hr		
Control (tap water)	76.67±5.774	94.44±5.774	94.44±5.774	4.122	-
Crystal violet untreated	40.00±10.00	43.33±5.774	53.33±11.547	0.744	81.941±2.470
Crystal violet treated	56.67±5.774	63.33±5.774	83.33±10.00	2.378	42.317±0.467

The CV dye is toxic to plants regarding its seed germination, length and phytotoxicity percentage, which was confirmed when the study showed 60 %, 56.67 % and 46.67 % inhibition in seed germination after 24 hr, 48 hr and 72 hr of incubation period, respectively as compared to distilled water, but was reduced to 43.33 %, 36.67 % and 16.67 % after 24 hr, 48 hr and 72 hr of incubation period, respectively, when the seeds were treated with metabolites obtained after

bacterial treatment. Besides the inhibition of seed germination, radicle length was also affected in both CV dye and metabolite treated plants, but when compared an improvement was observed in the treated plant (Fig 9.1). Likewise, phytotoxicity percentage was also found to be much reduced in treated plants i.e. 42.317 % as compared to CV dye treatment i.e. 81.941 %. Thus, this study recommended that the treatment with bacterial degraded CV dye metabolites is effective in reducing the toxicity. Similar reduction has also been reported by Kalyani et al., (2008), Parshetti et al., (2011) and Durve et al., (2012). Toxicity study of some textile dyes on germination and early seedling growth of four plant; clover, wheat, lettuce and tomato had been also previously studied by Moawad and Wafaa, (2003). So, phytotoxicity studies revealed the detoxification of CV dye through biodegradation by bacterial consortium.

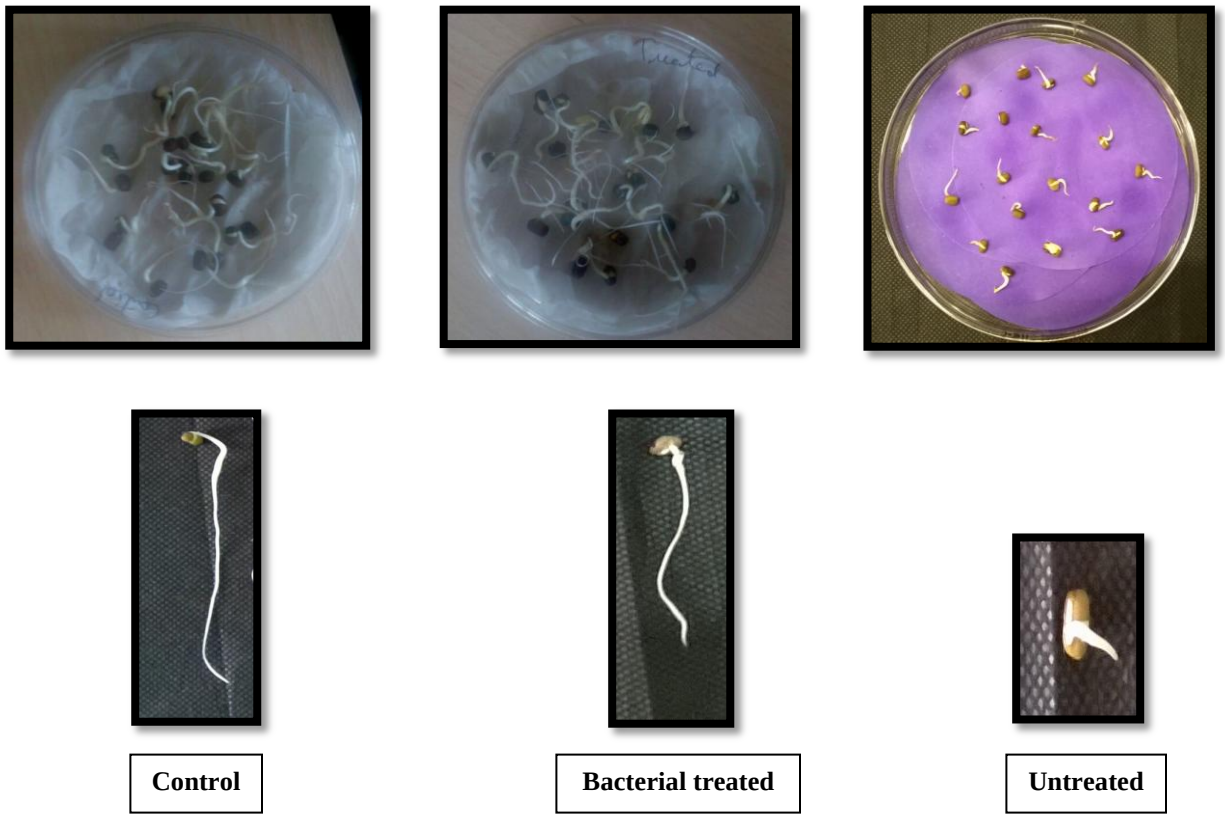


Figure 9.1: Effect of toxicity on *Phaseolus mungo*

9.3.2. Animal toxicity study

A very little variability could be recorded from the total Ach levels and AchE activity of the worms treated with CV dye and dye metabolites. A marginal upsurge in the Ach levels as well as AchE activity was available after the treatment with CV dye, which was slightly subsided by the consortia treated CV dye. The effect of reduced and non-reduced CV dye on the cholinergic transmission using aldicarb and levamisole was also studied. The percentage of worms paralyzed at a particular point of time reflects the synaptic Ach levels. The consortia treated samples reflected increased synaptic Ach levels suggesting increased cholinergic transmission. The cholinergic transmission in worm's musceranic and nicotinic pathways, where the musceranic and nicotinic pathways participate the elicit response of acetylcholine. The nicotinic acetylcholine signaling receptor could be studied using levamisole assay. Both treated and untreated CV samples negatively modulated the nAchR signaling. Overall, the synthetic CV and consortia treated CV samples increased the overall cholinergic transmission with diminishing effect upon nAchR signaling. Contrary to above, the AchE activity was also slightly increased (Fig: 9.2). To find the suitable explanation of this, we performed qPCR for the genes involved in regulation of cholinergic transmission. We observed significant of regulation in the ace2, cho1, cha1 and unc 17. The increased AchE activity could be arbitrated to the up regulation of ace2, cho1, cha1 and is correlated with the findings of aldicarb assay. The above findings are also substantiated by observation of the increased levels of unc17 (gene coding for the Ach transport). The increased unaltered nAchR signaling as recorded after levamisole assay was also substantiated by the genes unc 38 (necessary component of nAchR) and unc50 (participates in nAchR trafficking) (Fig: 9.3A).

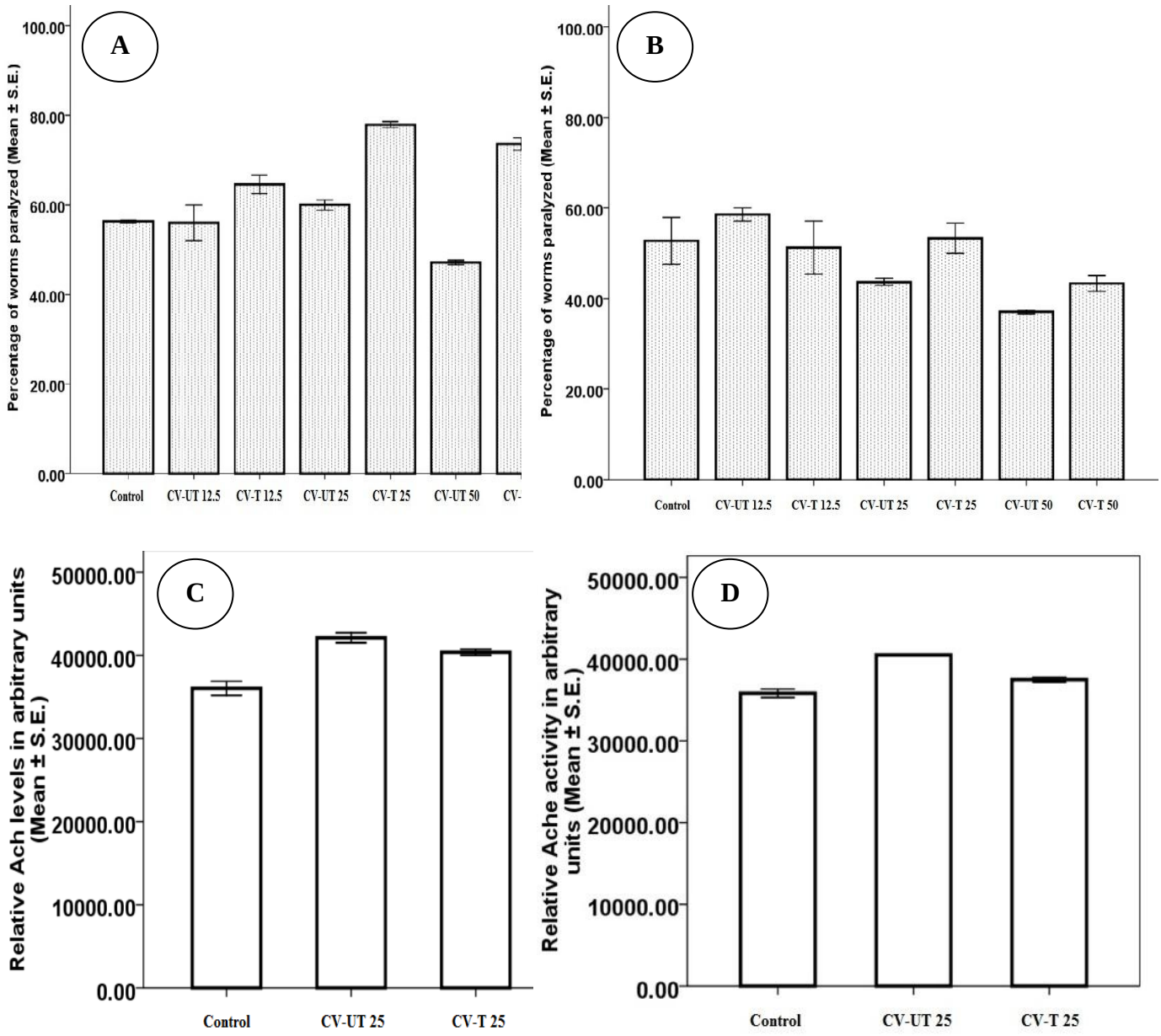


Figure 9.2: Effect of CV-UT (Untreated) and CV-T (Treated) on synaptic Ach (A), nAchR activity (B), gross Ach (C) and AchE levels (D)

9.3.2.1. Nile-red staining

By allosteric mechanisms, lipids influence nicotinic receptor function such as stabilizing varying extents of pre-existing resting, open, desensitized, and uncoupled conformations. Lipids also stabilize different conformational states and at biological synapses, the receptor function is modulated by lipid-nicotinic receptor interactions which are elucidated through the nile-red

staining. Nile red staining, a suitable and quantitative method is used to stain the main fat stores in *C. elegans*. Nile red staining of Crystal violet and bacterial degraded CV dye (metabolites) treated worms was evident for decrease in the total fat content with Crystal violet dye treated having more reflective effect, suggesting diminished nAchR signaling as shown in fig 9.3B.

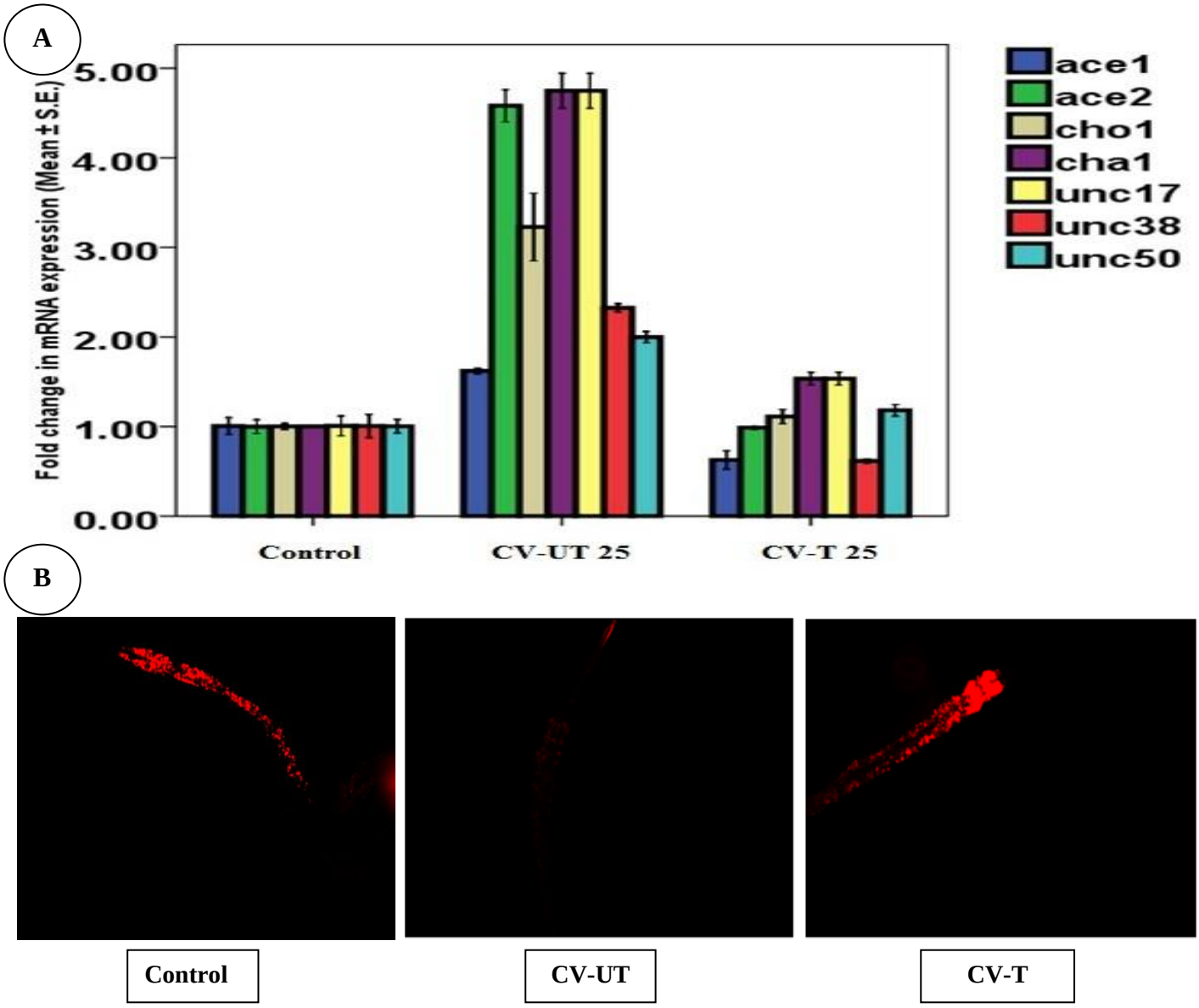


Figure 9.3: Effect of CV-UT (Untreated) and CV-T (Treated) on mRNA expression of genes related to Ach transmission (A) and lipid content (B)

Chapter 10

SUMMARY AND CONCLUSION

Summary and Conclusion

The textile effluents/wastewaters and mire produced by effluent treatment plants is a well-off foundation of bacterial populations with high possibility of decolorizing different groups of dye eluting. The pH of collected textile wastewater was noticeably below neutral level and temperature was above the standard limits, which is harmful for aquatic life affecting the dissolved oxygen concentration and also can persuade the activity of bacteria in water resources. The collected sample also showed higher EC, TDS, TSS, COD, BOD, carbonates and residual chlorine but lower rates of nitrites, sulphates and phosphates.

Total 10 bacterial strains (SJ1-SJ10) were isolated on the basis of their colony characteristics, Gram's reaction, cell morphology and growth pattern. The samples enclosed jointly with both types of bacteria with the dominance of Gram-negative affiliates. Initially, all ten isolates were weathered for their potentiality in decolorizing Crystal violet dye and finally 4 bacterial strains (SJ4, SJ5, SJ7 & SJ10) were selected on the basis of their CV dye decolorizing ability to higher concentration. The all four strains have shown compatibility to each other. Since strains SJ4, SJ5 and SJ10 have shown much decolorization potential than SJ7, therefore these three strains were further selected for the development of bacterial consortium. These three bacterial strains were well thought-out for the characterization, based on the Gram's reaction, cell morphology, colony morphology, growth pattern in MSM-CV amended broth, decolorization and biochemical tests. These species were identified as *Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas media* by 16S rDNA sequencing. The phylogenetic relationship between the identified isolated bacteria and other intimately related bacteria found in the GenBank database was performed.

Various traits of environmental and nutritional parameters were studied for the efficient and enhanced decolorization of Crystal violet dye by the developed bacterial consortium (*Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas media*). The results showed that the bacterial consortium has the high capacity for rapid and efficient decolorization of CV dye. The bacterial consortium showed increased biomass concentration as well as maximum decolorization under shaking condition as compared with static condition which showed sharply reduced decolorization percentage. The most suitable pH for decolorization of CV dye was 7 under shaking condition and optimum temperature was 35 °C. Further, the increase in inoculum concentration has shown continues increase in decolorization percentage with decrease in incubation time. Nutritional parameters were also observed for their consequence on decolorization of CV dye by bacterial consortium. Results revealed that maximum decolorization was obtained with 0.1 % concentration of glucose followed by sucrose and lactose as carbon source. Most applicable nitrogen source was found to be 0.5 % concentration of yeast extract followed by ammonium sulphate and sodium nitrate. Decolorization of CV dye was effected as the concentration of CV dye was increased. The lower concentration of CV has shown maximum decolorization which got reduced with the increase in concentration. The efficiency of bacterial consortium in decolorizing repeated addition of 100 ppm of CV dye solution under the optimized conditions was also evaluated. Results showed that the successive addition of dye resulted in faster rate of decolorization process till 6^{th} cycle and decolorization efficiency of organism decreased and required more time for decolorization of CV dye from 7^{th} cycle to last cycle. The newly developed bacterial consortium (*Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas media*) efficiently decolorized CV dye under various cultural, environmental, and

nutritional parameters, stating the importance of this bacterial consortium in treatment of dye wastewater.

Textile industry uses broad variety of structurally dissimilar but extremely variable compositions of dyes which are eluted in the effluent, therefore, the treatment of these dye containing effluents are difficult. The treatment becomes more difficult with the added heterogeneous hazardous chemicals, wide array of pH and different salt concentrations. The developed bacterial consortium also decolorized and degraded CV dye under optimized cultural, environmental, and nutritional conditions displaying the ability/efficiency of the developed bacterial consortium to decolorize and degrade the various dyes and chemical containing textile effluents as well as synthetic CV dye under optimized conditions. The GC-MS/MS analysis of the textile wastewater and bacterially treated textile wastewater was also performed. In GC-MS/MS analysis of textile effluent, we have seen many peaks of harmful compounds but in GC-MS/MS analysis of bacterially treated textile effluent, we have observed that the peaks have either disappeared or breakdown into other smaller peaks as compared to the peaks of control. In GC-MS/MS analysis of metabolites, shown structural changes after CV dye degradation. Three degraded products were formed namely as Phenol, 2,6-bis(1,1-dimethylethyl) (a), 2',6'-Dihydroxyacetophenone (b) and Benzene (c) after degradation which revealed the significant use of developed bacterial consortium in large scale treatment of textile effluent.

A noteworthy amplification in the production of ligninolytic enzymes (Laccase and Lignin peroxidase) was found during the decolorization and degradation process of CV dye indicating the participation of enzymes during the degradation of CV dye. Lignin peroxidase enzyme was observed to be produced more than laccase enzyme. The metabolites obtained after the degradation of CV dye was found to be abridged in toxicity as compared to the CV dye

which was clearly displayed by the phytotoxicity test performed on seeds of *Phaseolus mungo.* Only few articles have been reported on the bacterial decolorization and degradation of CV dye through bacterial consortium. Previously, many articles have been reported on treatment of industrial dyestuffs with single cultures and on a small scale. The only way for eventually controlling the pollutants releasing from textile and dye stuff industries is considered to be biological treatment methods. However, additional research would be required to develop a feasible unconventional process for the treatment of dye wastewaters/effluents.

The continuous dumping of wide variety of dye containing textile wastewater/effluents full of hazardous chemicals has created environmental pollutions as well as medical and visual problems linked with human health and agriculture. Thus, the bioremediation of dumping and contaminated area of textile effluents should be of primary importance. An effluent has wide diversity of Gram-positive as well as Gram-negative bacterial species due to adaptation and thus, has the capability of decolorizing different types of dyes here in it.

Gram-negative bacteria dominated in the samples serene from textile handloom outlet. For rapid decolorization and degradation of CV dye, the bacterial consortium of three strains (*Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas media*) isolated from collected sample was developed, which was found to be the most valuable treatment of CV dye as well as textile effluent than in anoxic conditions. The best decolorization of CV dye and growth pattern of bacterial consortium was recorded to be pH 7 at 35 °C with 0.1 % concentration of glucose and 0.5 % of yeast extract as carbon and nitrogen sources, respectively under shaking culture conditions. During the degradation of CV dye, an increase in the production of ligninolytic enzymes was found indicating its involvement in the degradation process of CV dye. The bacterial consortium has shown much decolorization in addition to degradation potential in

treatment of synthetic CV dye but the probability of bacterial consortium was found less in the degradation of textile effluent as investigated through GC-MS/MS analysis of consortium treated textile wastewater. The degradation of CV dye was further indicated by the diminution in toxicity of extracted metabolites investigated through phytotoxicity test on *Phaseolus mungo* plant. GC-MS/MS analysis supported the breakdown of native dye structure into three byproducts, Phenol, 2,6-bis(1,1-dimethylethyl) (a), 2',6'-Dihydroxyacetophenone (b) and Benzene (c). The extracted metabolites of degraded CV dye were found to be safer not only for plant germination but also in the reduction of phytotoxicity percentage but also for animal toxicity as tested on *Caenorhabditis elegans*.

Thus, the whole work concludes that the developed bacterial consortium from *Aeromonas hydrophila, Aeromonas caviae* and *Aeromonas media,* could effectively be used as an alternative to physical in addition chemical process employed for the handling of dye containing textile wastewater.

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SCIENTIFIC PUBLICATIONS AND ACHIEVEMENTS

List of Publications

Review/Research

- **Mani, S.**, Bharagava, R.N., 2016. Exposure to Crystal Violet, its toxic, genotoxic and carcinogenic effects on environmental and its degradation and detoxification for environmental safety. Rev. of Environ. Conta. and Toxicol. 237, 71-104.
- **Mani, S.**, Bharagava, R.N., 2017. Isolation, Screening and Biochemical Characterization of Bacteria Capable of Crystal Violet Dye Decolorization. International Journal of Applied and Advanced Scientific Research. 2(2), 70-75.

Chapters (In Book)

- **Sujata,** Bharagava, R.N., 2016. Microbial Degradation and Decolorization of Dyes from Textile Industry Wastewater. Bioremediation of Industrial Pollutants, Pp 53-90.
- Surabhi, Z., Sandhya, **Sujata**, Gaurav, S., Bharagava, R.N., 2016. Microbes an ecofriendly tools for the treatment of industrial wastewaters. Microbes and Environmental Management, Pp 78-103.

Papers and Chapters communicated

- **Mani, S.,** Bharagava, R.N., 2017. Ligninolytic enzyme producing *Aeromonas hydrophila* and its Crystal violet dye decolorization and degradation activity at different optimized conditions in Frontiers In Microbiology. **(Research Paper) (In Revision)**
- **Mani, S.**, Bharagava, R.N., 2017. Textile wastewater Dyes: Toxicity profile and treatment approaches In Green Technology and Environmental Sustainability (In Springer International), Singh and Kumar (EDs). **(Chapter)**

- Chowdhary, P., **Mani, S.**, Reyes, I.P., Bharagava, R.N., 2017. Effects of industrial wastewaters on soil sustainability and environment In Soil Amendments for Sustainability: Challenges and Perspectives (In CRC Press), A. Rakshit, B. Sarkar and P.C. Abhilash (EDs). **(Chapter)**
- Mishra, S., More, N.K., Yadav, A., Zainith, S., **Mani, S.,** Chowdhary, P., Bharagava, R.N., 2017. Heavy metal contamination: an alarming threat to human health & environment has been communicated **(Chapter)**

Workshop & Conferences attended

- Participated in **POSTER PRESENTATION** session of the 55th annual national conference of Association of Microbiologist of India, Organized by department of Agriculture Microbiology, TNA University, Coimbatore, November, 12-14, 2014.
- Attended 2 days workshop on Hands-on-training SEM, FTIR, FPLC and Ion Chromatography Organized by USIC, BBAU, Lucknow.
- Participated in 103rd annual national conference of Indian Science Congress held at University of Mysore, Mysuru, January, 3-7, 2016.
- Participated in **POSTER PRESENTATION** session of the 57th annual Conference of Association of Microbiologist of India, Organized by department of Botany, Gauhati University, Assam, India, November, 24-27,2016.
- Participated in **POSTER PRESENTATION** session of 4th Lucknow Science Congress-2017, Organized by Department of Environmental Microbiology, BBAU, Lucknow, March, 3-4, 2017.