Development of Microbial Consortia for Bioremediation of Toxic Synthetic Industrial Dyes

Thesis

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By

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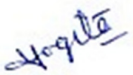

Pantnagar ***(Yogita Martoliya)***
June, 2017 *Authoress*

C E R T I F I C A T E

This is to certify that the thesis entitled **"Development of Microbial Consortia for Bioremediation of Toxic Synthetic Industrial Dyes"** submitted in partial fulfillment of the requirements for the degree of **Master of Science** with major in **Microbiology** of the College of Post-Graduate Studies, G.B. Pant University of Agriculture and Technology, Pantnagar, is a record of *bonafide* research carried out by **Ms. Yogita Martoliya** Id. No. **49379** under my supervision and no part of the thesis has been submitted for any other degree or diploma.

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14/06/2017

Pantnagar
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C E R T I F I C A T E

We, the undersigned, members of the Advisory Committee of **Ms. Yogita Martoliya** Id No**. 49379,** a candidate for the degree of **Master of Science** with major in **Microbiology** agree that the thesis entitled **"Development of Microbial Consortia for Bioremediation of Toxic Synthetic Industrial Dyes"** may be submitted in partial fulfillment of the requirements for the degree.

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Introduction

Chapter 1 INTRODUCTION

Synthetic dyes are colored aromatic organic compounds that are used to impart color to some substrate such as cloths, paper, plastic etc. There are man-made noxious synthetic chemical compounds which are difficult to degrade. Dyes are polyaromatic coloring agents having different functional group and over 10^5 different dyes have been produced worldwide with an annual production of nearly 700000 metric tons **(Lavanya *et al.,* 2004).** It is estimated that around 280,000 tons of textile dyes are discharged in textile industrial effluent every year worldwide **(Laxmi *et al.,* 2015).** In India, dyestuff industries contribute to 50 large scale organizations and 1000 small subunits which produce around 1, 30,000 tones of dyestuffs. Currently these synthetic dyes have vast industrial application and are being widely used in different industries such as textile, Paper and pulp, leather, pharmaceuticals, cosmetics, plastic, colored photography, paper printing etc. Over 10-20 % of the dyestuff used in different industries is lost in the effluent during dyeing process and persists in the environment for a long time. Textile, pulp and paper, tannery industries are the greatest generators of liquid effluent consisting colored pollutants due to use of high quantities of water in the dyeing process **(Kalyani *et al.,* 2009).**

Large scale production and extensive application of synthetic dyes can cause considerable environmental pollution and serious health hazards on human beings and animals. The colored industrial effluents include several toxic chemicals including synthetic dyes (e.g. azo dyes and reactive dye) that affect soil fertility, natural resources and aquatic life **(Mester and Tien, 2000).** Among all known dye stuffs, azo dyes (e.g. Congo red, methyl orange, acid black, direct dyes, etc) constitute the largest group of dyes used in textile and other industries. Azo dyes are highly recalcitrant compounds containing one, two or more azo groups (N=N), their recalcitrance increases with increasing number of azo groups. These are also known for their mutagenic, cytotoxic and carcinogenic effects on flora and fauna (**Weisburger *et al.,* 2002).** Azo dyes are the main constituents of colored water pollution because of their wide industrial applicability and uses. More than 2000 azo dyes are produced annually constituting around 50% of total dyes. Beside these azo dyes, triarymethane dyes (e.g. fuschsine dyes methyl violet, phenol dyes, and malachite green and brilliant green) are also well

known for their carcinogenic effects on humans and animals and hence are banned in many countries **(Gogate and Bhosale, 2013).** Therefore, one of the major worldwide threats of today's era is undoubtedly water pollution especially the colored effluent containing synthetic dyes.

These dyeing industrial effluents when released in main water bodies have an adverse effect on the environment and all the living beings; moreover, some of these degradative byproducts (e.g. amines) can also be highly toxic, mutagenic, and decrease penetration of light and photosynthesis; hence their removal from the environment is urgently required. Although various physical, chemical, and physicochemical methods are used for the remediation of dye waste water but use of most of these technologies is limited due to their high cost, low efficiency, limited versatility and handling of the waste generated, result in the production of large amounts of sludge, which creates the secondary level of land pollution **(Kaushik and Malik, 2009)**. The traditional waste water treatment technologies are used in many industries, it involve three steps of treatment. In primary treatment, physical methods are used to remove the solid waste, for secondary treatment use of microbes and activated sludge are common. In tertiary treatment chemicals are used to purify the liquid waste by charcoal and chlorination,**(Massoud and Nasr, 2009)** but these technologies are markedly ineffective because of the chemical stability of pollutants, therefore development of cost effective environment friendly technologies for bioremediation of dye waste water are of utmost importance **(Couto, 2009).** Therefore, it is important to develop efficient and cost-effective methods for the removal of dyes in effluents and contaminated soil. As a viable alternative, biological process including different taxonomic groups of microorganisms such as bacteria, fungi, yeast, and algae received increasing interest due to their cost effectiveness, ability to produce less sludge, and eco-friendly nature **(Anjali *et al.,* 2007).** Biological methods are generally considered as cost economic, environment friendly as they can lead to complete mineralization of organic dyes at low cost (**Pandey *et al.,* 2007**). Bioremediation process may employ either plant (phytoremediation) or some microorganisms such as algae, fungi, bacteria (microbial remediation). Decolorization of dye by microorganisms may take place either through the adsorption of dye on microbial biomass (biosorption) or enzyme catalysed biodegradation of the dyes by the cells. **(Chakraborty *et al.,* 2013; Sudha *et al.,* 2014).**

Bacteria can achieve a higher degree of degradation and even complete mineralization of dyes under optimum conditions. The biodegradation of azo dyes may occur either aerobically, anaerobic or by a combination of both. The initial step in the bacterial degradation of azo dyes is the reductive cleavage of azo bond by an enzymatic biotransformation **(Singh *et al.,* 2014)**. Bacteria cleave the azo bond by azoreductase enzyme, results in decolorization of dye. Fungi, mainly Basidiomycetes, are capable of decolorizing and mineralizing broad spectrum of different dye structures **(Machado *et al.,* 2006).** The biodegradation of dyes by white rot fungi and their ligninolytic enzyme systems offers an advantage over other processes because of their ability to completely mineralize various dyes to CO2 and H2O. The ligninolytic enzymes produced by white rot fungi are laccase (E.C 1.10.3.2), lignin peroxidase (LiP) (E.C 1.11.1.14), manganese peroxidase (MnP) (E.C. 1.11.1.13), versatile peroxidase, cellobiose dehydrogenase and H_2O_2 producing enzymes **(Christian *et al.,* 2005; Moorthi *et al.,* 2007).** These enzymes are highly non-specific and are able to transform organopollutants that have structural similarities to lignin as well as have been reported to decolorize various dyes **(Harvey *et al.,* 1986; Gold *et al*; 1989; Hammel *et al.,* 1993).** Some of the diazotrophs (filamentous Cyanobacteria) can fix atmospheric nitrogen and thus the utilization of algae for waste water treatment is an efficient and cost effective process **(Saha *et al.,* 2010).** The presence of many different compounds of chemical in the dye effluents may affect the microbial degradation of a single species of microorganisms and thus using different groups of microorganisms as a consortium can be efficient. The microbial consortia are a mixed group of microbes involve many different mechanisms and even various types of enzymes to degrade the dye components completely **(Elumalai *et al.,* 2016).**

To increase the extent of dye decolorization, optimization of culture condition and environmental factors are used in many studies. The effect of pH, temperature, dye concentration, carbon and nitrogen source, on dye decolorization was studied **(Gahlout *et al.,* 2013).** Decolorization of dye by microorganisms may take place either through the adsorption of dye on microbial biomass (biosorption) or enzyme catalysed biodegradation of the dyes by the cells. **(Chakraborty *et al.,* 2013; Sudha *et al.,* 2014).** The enzymatic mechanisms in dye decolorization includes several enzymes like laccse, LiP, MnP etc, hence the polyacrylamide gel electrophoresis is important for the molecular characterization of these enzymes **(Maier *et al.,* 2004**).

The microbial cultures can degrade synthetic dyes but immobilized cells and enzymes can show better results. The immobilization of the cultures or enzymes on the substrate increases the microbial growth and enzyme production. Several substrates which are used for immobilization are charcoal, rubber, starch, alginate beads, glass, wooden chips etc **(Brena and Viera, 2006).** To decolorize the industrial effluent, establishment of large scale waste water treatment process is necessary which requires a bioreactor in which immobilized cultures (solid state fermentation) are used for the dye decolorization. In a bioreactor the growth conditions and microbial activities are maintained **(Diorio *et al.,* 2008).** In the treatment of industrial effluent gravel bed aerated bioreactor and mixed bed aerated bioreactor was used in many countries **(Kumar *et al.,* 2006)**. Different membrane bioreactor was also reported in the treatment of waste water released from the industries **(Vinodha *et al.,* 2013)**.

The degradation of toxic synthetic dyes are very important but it is also very important to check the metabolites which are produced during dye decolorization; either they are toxic or not. For this several techniques are used to monitor the dye degradation and degraded metabolites, which includes UV-Visible spectrophotometer, FTIR analysis, HPLC, GC-MS, LC-MS etc **(Hua *et al.,* 2013; Kolekar *et al.,* 2008).**

Objectives:

In view of above, present investigation was undertaken with following objectives:

1. Selection of potential indigenous dye decolorizing microbial culture(s) and development of microbial consortia (co-culture) for efficient dye decolorization.
2. To study enzymatic and non enzymatic mechanisms involved in dye decolorization by selected isolates.
3. Monitoring of dye decolorization by microbial cultures using analytical techniques.

Review of Literature

Dyes and pigments are most widely used coloring agents that are used to impart color to different materials. Now a day's these dyes are widely used in many industries. Natural dyes and colors derived from plants, animals and minerals are being used since ancient time. In 19th century, first synthetic dye (mauveine) was synthesized by W. H. Perkin in 1856 with the use of aniline and thus the use of synthetic dyes was started. In the beginning of 20th century, the natural dyestuffs were replaced by synthetic dyestuffs **(Whitaker *et al.,* 1949).** The colored compounds which absorb electromagnetic energy in the visible range of ~350-700 nm are known as dyes. Dye molecules contain a chromophore and an auxochrome group. The chromophore group imparts colour to the dye molecule while auxochrome group intensifies the colour of the chromophore by altering the overall energy of the electron system. Usual chromophore groups are C=C, C=N, C=O, N=N, -NO_2 and quinoid rings while auxochromes are -NH_3, -COOH, -SO_3H and -OH **(Zollinger, 1987).**

Synthetic dyes are widely used in many industries, e.g. textile, paper & printing, pharmaceuticals and also in making of other commercial products. These dyes are highly resistant to microbes and have stable structure, very high stability in light and washing; hence their degradation in natural condition as well as their removal from the effluent is very difficult **(Hildenbrand *et al.,* 1999)**.

2.1 Environmental Impact of Synthetic Dyes: Hazardous Effects

Dye containing untreated effluents is continuously being released from the industries in the environment. They persist in the environment for a longer period thus, causing various environmental hazards. The exposure of these toxic dyes and recalcitrant molecules in the environment is harmful for flora and fauna. Different types of dyes such as azo, nitro, disperse, acidic, basic, triphenyl methane, etc; are being used in many industries and very high amount of these dyes enters in our environment.

Some triaryl dyes having carcinogenic and mutagenic properties are known to enhance the tumor growth in fishes. According to **Haiey *et al.* (1975)**; benzedine based azo dyes (congo red) cause carcinogenic and tumorogenic effects in lab animals.

Triphenyl methane dyes are being extensively used as biological stains and also in human & veterinary medicine; but these dyes also cause several harmful effects. It can also persist in nature for a longer time and has an adverse effect on aquatic life as well as on terrestrial life **(Au *et al.,* 1978; Azmi *et al.,* 1998).** The azo dyes have been observed to cause reduction in seed germination efficiency and plant growth. Further, at high concentration it inhibits the shoot and root elongation. Some dyes have also some multiorgan toxic effects, which decrease the growth, fertility, food intake and also cause spleen, kidney, heart and liver damage. Some dye-effluents contain hazardous chemicals which disturb the genetic makeup of an organism **(Vogel, 1982).** Some byproducts of azo dyes (amines) have also been reported to have mutagenic and carcinogenic properties **(Rafii *et al.,* 1995).** These soluble azo dyes enter the body by direct contact through skin or sometimes its inhalation causes allergies, irritation of eyes, cancer, *etc.* **(Nikulina *et al.,* 1995**). **Chen, (2006)** have reported that the sedimentation of reduced azo and nitro compounds in aquatic life gives rise to carcinogenic amines. Melachite green (Triphenylmethane) dye is reported to affect the reproductive and immune system and also being considered as a potential carcinogenic and genotoxic agent **(Srivastava *et al.,* 2004)**. N_hydroxyl amine product of azo and nitro compound was reported to cause DNA damage **(Umbuzein and Arit, 2005).** Hence, all forms of chemical dyes are responsible to cause adverse effect in all forms of life.

2.2 Types of Dyes

2.2.1 Basic dyes

Mauveine, the first man made synthetic dye discovered by Perkin was a basic dye. These dyes are used to color wool and silk from a dye bath containing acid, but only in the presence of a mordant (usually a metallic salt) which increases the affinity of fabric for the dye and leads to dyeing of cotton fibers. Unfortunately, these dyes have very poor light and wash fastness (**Pellew, 1998**).

2.2.2 Direct dyes

These are water soluble dyes having direct affinity for all cellulose fibers including viscose rayon and some of them are also used to dye silk and wool. The addition of salt is mandatory to improve the dye yield and to obtain deeper shades.

Table 2.1: Detailed description and molecular structure of some of the common azo dyes used in different industries.

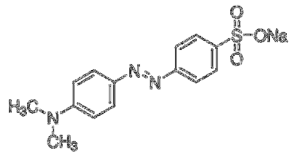
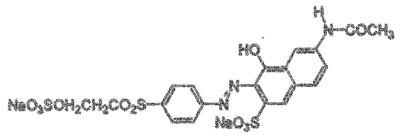
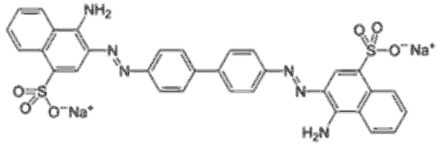
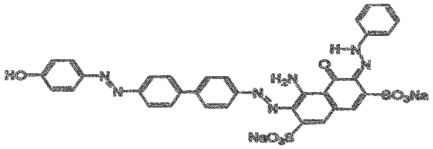
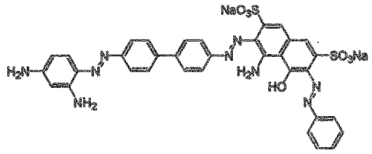
S.No.	Group	Example	Molecular Formula	Molecular Mass	Molecular structure
1.	**Azo dyes (Mono azo Dye)**	Methyl Oragne	$C_{14}H_{14}N_3NaO_3S$	327.334 g/mol	
2.	**Azo dyes (Mono azo Dye)**	Reactive orange	$C_{20}H_{17}N_3Na_2O_{11}S_3$	617.54	
3.	**Azo dyes (Diazo Dye)**	Congo Red	$C_{32}H_{22}N_6Na_2O_6S_2$	696.66 g/mol	
4.	**Azo dye (Diazo Dye)**	Direct Green	$C_{34}H_{23}N_7Na_2O_8S_2$	767.70g/mol.	
5	**Azo dye (Triazo Dye)**	Direct Black	$C_{34}H_{25}N_9Na_2O_7S_2$	781.73 g/mol	

Table 2.2: List of the most commonly used industrial dyes with their absorption maxima

S. No.	Synthetic Dyes	Color	Absorption maxima (λ_{max})	Industrial application
1.	Congo Red	Red	497nm	Textile, Paper & Printing, leather and cosmetics etc.
2.	Direct Green	Green	623nm	Textile, Paper & Printing, leather and cosmetics etc.
3.	Direct Black	Black	580-600nm	Textile, Paper & Printing, leather and cosmetics etc.
4.	Brilliant Green	Sea Green	630nm	Paper & Printing, Textile etc.
5.	Crystal Violet	Dark Violet	588nm	Biological stain, Dyeing Industry etc.
6.	Melachite Green	Sea Green	615nm	Paper & Printing, Food, Plastic and Textile etc.
7.	Methyl Orange	Orange	460-470nm	Textile, Dyeing and Paper & Printing etc.

2.2.3 Vat dyes

The most important member of this group is Indigo which is the oldest dye known to man **(Barker, 2004)**. Indigo is obtained from plant source (*Indigo feratinctoria*). It is insoluble and blue in color in reduced form but is colorless and water soluble when converted to indigo white. These are not soluble in water hence are converted into alkali soluble leuco compounds (colorless) by reducing with sodium hydrosulphite. On exposure to air these dyes get oxidized into the fabrics and become insoluble thus giving blue colour **(Shenai, 1999)**. These dyes are also called as vat dyes because prior to the process reduction of the dye is carried out in wooden vat. These dyes are mainly used to color cotton fibers.

2.2.4 Reactive dyes

In 1956, a new class of dye was introduced in the market. The cold dyeing and hot dyeing types are basically two types of reactive dyes. These dyes react chemically with the fiber when applied and the process is irreversible, i.e. it cannot be removed by

washing or boiling. So, large amount of salt is needed to force its deposition on the fabric as these dyestuffs have low affinity to cellulose **(Shenai, 1997)**.

2.2.5 Azo dyes

It is the largest group of dyes having –N=N- as a chromophore in the aromatic system. These can be mono-azoic, di-azoic, tri-azoic and poly-azoic dyes depending upon the number of azoic groups present. Tetrazine is a synthetic lemon yellow azo dye which is used as a food colouring agent **(Chaube *et al.,* 2010)**.

2.2.6 Sulphur dyes

In 1873, first Sulphur dye was discovered in France. As a result of its outstanding fastness to light, washing and boiling it was the best dye known at that time. These dyes were popular as they were easily available, easy to apply and having good wash-fastness **(Shenai, 1997)**. However, the limitation of the sulphur dyes is that they produce dull shades and lack red colour. Sulphur dyes are water insoluble in their normal state but are readily soluble in sodium sulphide solution, as this form in having high affinity for all the cellulose fibers.

2.2.7 Mordant dyes

These dyes require a mordant like water, light and perspiration to improve the fastness of the dye on the fiber **(Barker, 2004)**. Also, it is very important to choose a good mordant as different mordant can significantly change the final color. Most natural dyes are mordant dyes **(Shenai, 1997)**. The synthetic dyes are also generally mordant dyes (chrome dyes) used for wool. The mordant potassium dichromate is generally applied after the dye treatment.

2.2.8 Acid dyes

These are water soluble anionic dyes which are used to dye silk, wool, nylon and modified acrylic fibers. Salt formation is done among anionic groups and cationic groups in the fiber for the attachment of dye to the fiber. Acid dyes are not substantive to cellulosic fibers **(Barker, 2004)**.

2.2.9 Disperse dyes

These dyes are free of ionizing group. In 1920, a new regenerated cellulose acetate fiber was introduced due to which an entire new range of dyes were developed.

For dyeing, dispersing agent is very necessary for these dyes. These dyes are also known as acetate dyes. Sulphoricin oleic acid (SAR), dispersal and cellitoin are important dispersing agents.

2.2.10 Oxidation dyes

Though these are not exactly dyestuffs but because of their exceptional fastness to light and washing, these as dyestuffs are of great importance. Through oxidation of aniline the most important member of this group is produced and it is used for dyeing of fur and leather goods **(Barker, 2004)**.

2.3 Dye waste water Treatment Process

Textile, paper & printing, cosmetics industries are the greatest generator of toxic waste water. These industrial effluents include dissolved solid waste, toxic dyes, metals and chemicals which make it more toxic for our environment and for the living beings. These industrial effluents suppress the growth of green flora and have carcinogenic and mutagenic effects on animals and humans; hence the treatment of these toxic effluents is very important. There are various treatments for the removal of toxic substances from the industrial effluent such as physicochemical method, chemical method, and biological methods. A detailed description of dye with various advantages / disadvantage of different physical, chemical or biological methods are given in **Table 2.3**. All these methods are very efficient in the removal of toxic chemical from the waste water and all these different methods are being used by many industries to treat their industrial effluents.

2.3.1 Physicochemical Methods

A. Membrane Filtration

For treatment of dye containing effluents the membrane of 10,000 dalton molecular weight cutoffs (MWCO) are used in microfiltration, ultrafiltration, nanofiltration and reverse osmosis for the removal of salt and larger molecules as main process. In this method due to the small pore size, removal of large molecules is not possible **(Cooper, 1993)**. All these four methods are very efficient but RO has an advantage to remove both organic and inorganic substances; hence used in the treatment of drinking water supply. These methods have been reported in dye house waste treatment **(Chen *et al.,* 2007).**

B. Sorption and ion exchange

Sorption method includes natural or synthetic sorbents like activated clay, zeolites, cellulosic and polymeric resins, modified rice husk, starch, fiber etc **(Simkovic *et al.,* 1996).** Their efficiency and price varies from one sorbent to another. In recent years anion exchange resins have been shown as an excellent sorbent for the recovery and removal of reactive dyes **(Karcher *et al.,* 2002)**, but some disadvantages are also there, like slow pore diffusion, low accessible flow rate, high pressure flow and flow channeling. To compensate this limitation, anion exchange membrane has been successfully implemented to remove anionic reactive dyes **(Liu *et al.,* 2007)**.

2.3.2 Chemical Methods

A. Coagulation

This method is used for the removal of color from the industrial effluent in the final process of treatment **(Chekli *et al*., 2017).** Different inorganic coagulants (lime, organic polymer, aluminium, maganesium & iron salts) are used in the coagulation process. In this process large amount of sludge is being produced containing dyes and chemicals which is a big disadvantage for this process. The use of organic polymer as a coagulant produces less amount of sludge; hence it is presently used **(Southern, 1995)**. Moreover, these polymers are harmful for aquatic ecosystem as they inhibit the nitrification process in biological waste water treatment plant **(Churchley, 1995)**. Also, these coagulants added to the waste water cause flock formation which is separated by flotation **(Chung *et al.,* 2000)**.

B. Electrolysis

For dye containing waste water this method has been successfully applied **(Cooper, 1993)**.This technique mainly depends on the anode used and the properties of waste water and its condition **(Anizares *et al.,* 2002)**. This method is an economic, effective, and eco-friendly way of dealing with textile dyeing wastewater at the industrial level as it does not generate any secondary sludge or pollutant and nearly degrade all the pollutants present in the waste water **(Mohan *et al.,* 2007)**. In traditional system electrode of Au, Pt, and C material was used for the oxidation reaction through oxygen transfer with low reaction rate constant. Lead dioxide is one of the most commonly used anodes for electrochemical degradation of many pollutants due to high oxygen potential **(Morsi *et al.,* 2011)**.

C. Ozonation

In chemical oxidation, ozon is a powerful chemical oxidizing agent used to treat the dye waste water having an oxidation potential of 2.07. Ozone reacts either to the double bond in the dye molecule or rapidly form hydroxyl free radicals which react with organic compounds **(Strikland & Perkins, 1995).** Some dyes respond more quickly to ozone oxidation than others. According to **Cooper, (1993)**, ozonation is successful in degradation of waste water containing azoic, sulphur, basic dyes and reactive dyes. High cost of ozone generation and short half life is the major limitation of this technique **(Gogate and Pandit, 2004).**

D. Chemical Reduction

For azo dyes, this process is mainly effective as this causes cleavage of azo bond (N=N) with the generation of small nano-chromophoric aromatic amines. According to **Reife, (1991)** reactive dyes are adsorbed very finely on activated carbon when treated with a reducing agent. This technique reduces color upto 90%. Sodium borohydride, sodium hydrosulphite, thiourea dioxide, tin (II) chloride etc are the common chemical reducing agents used in this technique **(Reife and freemann, 1996).** It is very important to investigate the potential reversal of the reaction on the exposure to oxygen otherwise the color may reappear when discharging treated dye effluent to the environment **(Joshi *et al.,* 2004)**.

2.3.3 Biological Methods: Bioremedial Techniques

As the removal of dyes and chemicals from the industrial effluent by physicochemical and chemical methods is very costly, non eco friendly and produces a large amount of sludge, hence there is a need to find an alternative method which is cost effective and technically effective. To alternate the physicochemical and chemical techniques, microbes can be used to remove the toxic dyes from the industrial waste water **(Mou *et al.,* 1991)**. The process of biological dye removal techniques i.e. bioremedial techniques involve biological agents like plants and microbes. These techniques involve biosorption, biotransformation or biomineralization and bioaugmentation etc.

a. Biosorption

The binding of dye, heavy metals and other chemicals on the biological sorbent like microbial biomass either living or dead in an aqueous solution is a property of biosorption **(Volesky, 1990)**.

b. Biotransformation / Biomineralization

Biotransformation (Chemical modification) of several pollutants (dyes, synthetic chemicals etc) is an easy way to remove contaminants from the environment **(Ali *et al.,* 2009).** Biotransformation is the chemical modification of some pollutants or chemical compounds by the use of microorganisms. When the pollutants are transformed into CO_2, H_2O or NH_4, then it is known as biomineralization.

c. Bioaugmentation

To increase the degradation rate of contaminants, several microorganisms are added to the contaminated site **(Murugesan *et al.,* 2006)**. By this process the breakdown of toxic synthetic chemicals becomes easier. This technique is commonly used in the sludge bioreactor for waste water treatment **(Stephenson and Stephenson, 1992)**.

Table 2.3: Advantages and disadvantages of various physical, chemical or biological methods used for dye waste water treatment (Source: Kumar *et al.,* 2012)

	Method	Advantages	Disadvantages
Physical	Adsorption	Good removal of wide variety of dyes	Nonselective to adsorbate
	Membrane filtration	Remove all dye types	Concentrated sludge production
	Ion exchange	Regeneration: no adsorbent loss	Not effective for all dyes
	Irradiation	Effective oxidation at lab scale	Requires a lot of dissolved O_2
	Electro kinetic coagulation	Economically feasible	High sludge production
	Coagulation-flocculation	Good elimination of insoluble dyes	Cost of sludge treatment: garbage dump
	Adsorption on active carbon powder coupled with coagulation process	Matter, organic matter, and low influence on color Fast fouling of suspended matter	Cost of active carbon powder
	RO	Retention of mineral salt and hydrolyzed reactive dyes and auxiliaries	High pressure process, Fouling with high concentrations
	Nanofiltration	Separation of mineral salts, hydrolyzed reactive dyes and auxiliaries	Treatment for complex solution with a high concentration of pollutant
	Ultrafiltration/microfiltration	Low pressure process	Inadequate quality for reused the permeate
Chemical	Fenton's reagent	Effective decolorisation of both soluble and insoluble dyes	Sludge generation
	Ozonation	Good elimination of color	No diminution of COD values Extra costs
	Photochemical NaOCl	No sludge production Initiates and accelerates azo-bond cleavage	Formation of by-product release of aromatic amine
	Electrochemical destruction	Breakdown compounds are	High cost of electricity
Biological	Standard Biological degradation	Efficiency of oxidizable matter 90%	Low biodegradability of dye, the salt concentration stay constant

2.4 Bioremediation

Different groups of microorganisms are capable of degrading the toxic dyes in different environmental conditions, the major classes are bacteria, fungi, algae, actinomycetes, yeast etc **(Paszczynski *et al.,* 1991)**. Cluis in 2004 also reported plants for the remediation of toxic dyes (phytoremediation) with the advantage of cost effectiveness, long term applicability and its application to the polluted sites.

2.4.1 Phytoremediation

For the treatment of industrial waste water, the phytoremedial techniques involve phytoextraction, phytostabilization, phototransformation, phytodegradation, and rhizofiltration **(Vidali, 2001)**. Phytoremediation is the use of plants for the remediation of toxic waste. In this technique, fast growing plants, with fibrous & deep roots are very useful **(Khandare *et al.,* 2011).** In some studies eucalyptus bark was used to remove the reactive dyes from the waste water by adsorption **(Morais *et al.,* 1999)**. *Eichhornia crassipes* (water hyacinth) has a great potential to remove methylene blue and methyl orange dye **(Tan *et al.,* 2016)**.

2.4.2 Microbial Remediation

Different types of microbial remediation techniques employing different bioagents (e.g. algae, fungi, bacteria, yeast etc) have been reported for successful decolorization on dye waste water.

I. Bacterial Remediation

Removal of toxic dyes from industrial waste water with the help of bacteria is known as bacterial remediation. In 1970s, the isolation of dye degrading bacteria was started with a report on *Bacillus subtilis* **(Horitsu *et al.,* 1977)**, followed by other bacteria: *Bacillus cereus* **(Wuhrmann *et al.,* 1980)**, *Pseudomonas* strain **(Kulla, 1981)**, *Proteus mirabilis* **(Chen *et al.,* 1999)**. Many more bacteria were reported in the degradation of several toxic dyes, either aerobic or anaerobic such as *Pseudomonas* sp, *Bacillus subtilis*, *Escherichia coli*, *Enterococcus* sp, *Corneybacterium* sp, *Clostridium* sp, *Acinetobacter* sp, *Geobacillus* sp, *Rhizobium, Lactobacillus, Klebsiellla* sp have been reported in the degradation of azo dyes **(Stolz, 2001; Olukanni *et al.,* 2006; Lin and Leu, 2008)**.

Several strains of actinomycetes have also been reported in the degradation of reactive dyes including azo dyes, anthraquinone, and phthalocyanine through adsorption on biomaas **(Zhou and Zimmermann, 1993)**. In recent years, an actinomycete was reported to degrade reactive yellow dye enzymatically using lignolytic enzymes such as lignin peroxidase, laccase and tyrosinase **(Zabin *et al.,* 2011)**.

II. Fungal Remediation (Mycoremediation)

Fungi in the removal of dye containing effluent have been investigated several times **(Azmi *et al.,* 1998).** Fungi have an advantage over the bacteria as it solubilizes the insoluble substrate by producing extracellular enzymes; the extracellular nature of fungal enzymes is also an advantage to tolerate high concentration of toxic chemicals **(Kaushik and Malik, 2009).**

Several wood rotting fungi have been reported to degrade a wide range of toxic dyes and other chemicals. *Phanerochaete chrysosporium* was the only white rot fungi which were reported to have an ability to degrade some toxic dyes during early days **(Glenn and Gold, 1983).** According to **Elias *et al.* (2000)**, another wood rot fungus *Trametes hirsute* was able to degrade triphenyl methane, indigoid, anthroquinonic and azo dyes with the production of laccase enzymes. Apart from this, *Fungalia trogii*, *Panicillium geastrivous*, *Rhizopus oryzar*, *Pleurotus ostreatus*, *Aspergillus flavus* and *Aspergillus niger* have also been reported in the dye degradation process **(Fu and Viraraghavan, 2001; Wesenberg *et al.,* 2003)**.

III. Algal Remediation (Phycoremediation)

The first report on dye removal by algae came on 1900 for the removal of azo dyes by azoreductase producing algae **(Jinqi and Houtian. 1992).** The degradation of azo dye by *Oscillatoria* and *Chlorella* to its simpler organic compounds or CO_2 has also been reported. A green algae *Spirogyra* was also investigated for biological treatment of azo dyes **(Mohan *et al.,* 2002)**. Some cyanobacteria were also having an ability to degrade azo dyes in waste water **(Dellamatrice *et al.,* 2017)**.

2.5 Mechanisms involved in Microbial Dye Decolorization

Dye Decolorization by various microorganisms is very efficient, eco friendly and cost effective process. Microbial dye decolorization may choose one of the two

process either adsorption or biodegradation of dye by producing several enzymes **(Zhou and Zimmermann, 1993)**. As per **An *et al.* (2002**); the dye waste water was decolorized into colorless liquid after the treatment with microbial cells; whereas the microbial cell either retain the color of dye (adsorption) or remain colorless (biodegradation).

2.5.1 Enzymatic Degradation of dyes

Enzymes are the biocatalysts which accelerate or catalyze the chemical reaction. In dye degradation also several enzymes are involved, that are being produced by various microorganisms either intracellularly or extracellularly. In microbial systems several oxidoreductase enzymes such as laccase, Manganese peroxidase, lignin peroxidase, azoreductase and hydroxylases are extensively used for the dye degradation **(Reddy, 1995)**. As the physicochemical techniques have several disadvantages, hence; the use of these enzymes for dye degradation is now gaining momentum **(Pinheiro *et al.,* 2004)**. **Fig 2.2** and **Fig 2.3** shows the enzymatic degradation of azo dyes.

A. Laccases (E.C.1.10.3.2)

Laccase is an oxidase enzyme having copper metal which acts on the phenolic compounds and converts them into simpler compounds. The enzyme is also commonly found in plants and fungi. The enzyme mainly degrades synthetic dyes through a mechanism of non specific free radical by degrading them into non toxic simpler compounds by the nucleophilic attack on the phenolic group of azo dyes **(Chivukula *et al.,* 1995; Wong and Yu, 1999; Susana *et al.,* 2005)**. Laccase enzyme was also studied in various bacteria such as *Pseudomonas putida*, *E. coli* and *Pseudomonas* sp **(Telke *et al.,* 2009)**.

Laccase enzyme is widely applicable in textile dye bleeching, pulp bleaching, biosensors, effluent detoxification and bioremediation **(Gianfreda *et al.,* 1999; Xu, 1996)**. It is also a potential enzyme for biofuel cells. Crude laccase from the white rot fungi *Lentinus polychrous* was also found in the degradation of several dyes including methyl orange, reactive green and anthraquinonic dyes **(Ratanapongleka and Phetsom, 2014)**.

B. Lignin Peroxidases (EC 1.11.1.14)

Lignin peroxidase (LiP) was first discovered during H2O2-dependent Cα-Cβ cleavage of lignin model compounds and was later on shown to catalyse depolymerisation of methylated lignin *in vitro*, oxidation of non phenolic aromatic lignin compounds and also in the side chain of lignin **(Tien and Kirk, 1983).** These enzymes are glycoprotein having a molecular weight of 38-46 kDa. This enzyme has also been used for the degradation of various recalcitrant aromatic molecules such as polychlorinated biphenyls and toxic synthetic dyes. Third group of peroxidases; versatile peroxidases (VP) has been exposed in species of *Pleurotus* and *Bjerkandera* which can be considered as a hybrid between MnP and LiP, since they can oxidize not only Mn2+ but also phenolic and nonphenolic aromatic compounds including dyes **(Francisco *et al*., 2009)**.

C. Manganese Peroxidases (EC 1.11.1.13)

Manganese Peroxidase (MnP) was first discovered in *Phanerochete crysosporium* **(Pribnow *et al.,* 1989).** MnP is an extracellular hemeprotein that catalyses H_2O_2 dependent oxidation of derivatives of lignin polymer and can oxidize Mn^{2+} to Mn^{3+} **(Gold and Alic, 1993)**. A white rot fungi, *Ganoderma* sp. was also reported to produce high amount of MnP in solid and liquid medium **(Zhou *et al.,* 2013)**.

D. Azoreductase (EC 1.7.1.6)

Azoreductases are the membrane bound enzymes that catalyse the chemical reaction involving cleavage of azo group only in presence of NADH and FADH **(Gingell and Walker, 1971)**. Most of the azoreductases can reduce azo bond (N=N) of azo dyes but are not able to degrade / reduce sulphonated azo dyes to produce colorless amines **(Punj and John, 2009)**. An azoreductase from *Bacillus* sp. B29 has been reported to have an ability to reduce sulphonated azo dyes **(Ooi *et al.,* 2009)**. Azoreductase activity was also observed in several species of bacteria such as *Caulobacter subvibrioides* C7-D, *Xenophilus azovorans* KF46F, *Pigmentiphaga kullae* K24, *Enterobacter agglomerans* and *Enterococcus faecalis* **(Gurulakshmi *et al.,* 2008).**

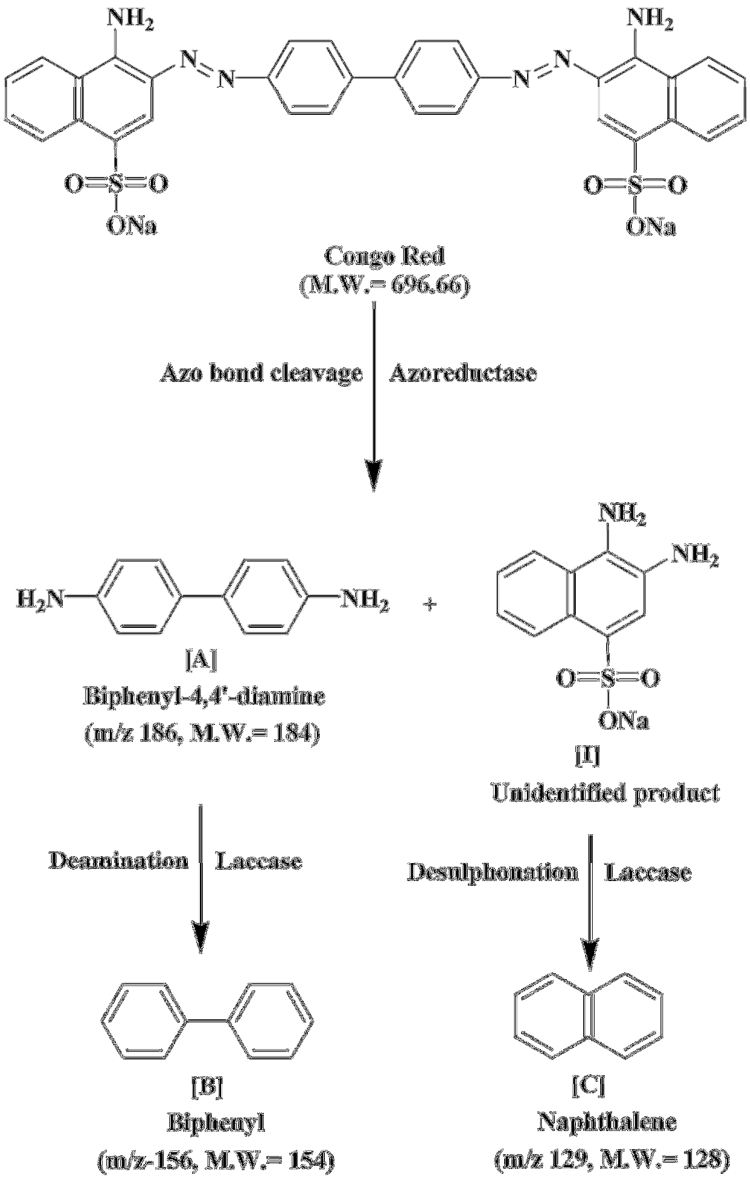


Fig. 2.2 Pathway for enzymatic degradation of congo red dye involving azoreductase and laccase enzyme (Source: Lade *et al.,* 2015).

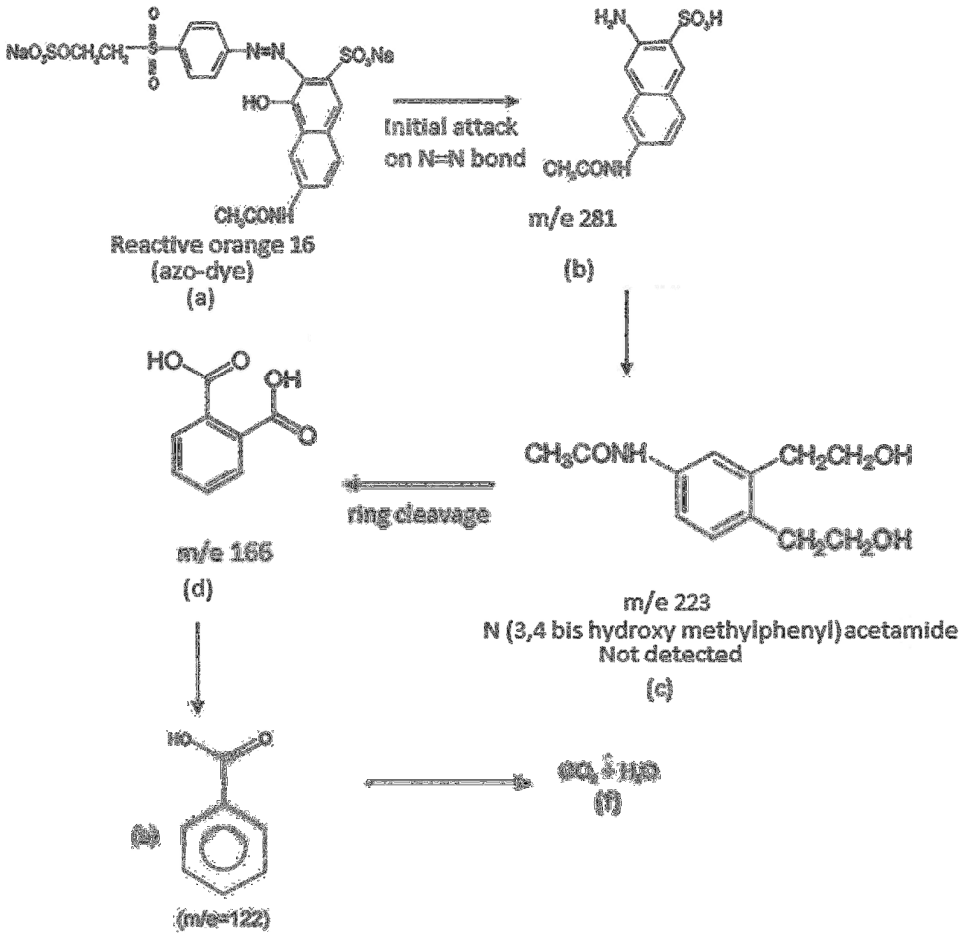


Fig. 2.3 Biodegradation of Reactive orange (Source: Sultana *et al.,* 2015)

2.5.2 Non Enzymatic Mechanisms

Dye decolorizations of synthetic dyes by non enzymatic mechanisms are very common. It involves mainly three processes – biosorption, bioaccumulation and biotransformation.

2.6 Use of Immobilized Cells / Enzymes in Bioremediation of Synthetic Dyes

Immobilization refers to the technique for anchoring the cells or enzymes on the inert support such as charcoal, rubber, starch, alginate beads, glass, wooden chips etc. The immobilization of the cultures or enzymes on the substrate increases the microbial growth and enzyme production **(Brena and Viera., 2006)**. For efficient degradation of dyes, different ways of immobilization have been developed which are used to treat the colored waste water **(Moreno-Garrido, 2008)**. Entrapment is one of the most commonly used methods for immobilizing single-celled microorganisms such as

bacteria and yeast, which can effectively avoid the loss of microorganisms from treatment systems. In this way, the microbial cells are always immobilized in alginate beads or polyvinyl alcohol (PVA) gel pellets **(Samuel *et al.,* 2013; Martínez *et al.,* 2013)**. Some studies have also shown the immobilization of *Pseudomonas aeruginosa* and *Phanerochaete crysosporium* in a solid gel matrix of sodium alginate beads and PVA that helps in the adsorption of reactive dye procion blue 2G **(Saravanan *et al.,* 2013)**. Compared with other ways, though the entrapment methods may be complex to execute, they still offer advantages such as high cell densities, high stability, absence of cell washout and extended reaction times **(Tuttolomondo *et al.,* 2014)**.

2.7 Microbial Consortia and Dye Decolorization

Microbial consortia are the combination of two or more microorganisms that can live synergistically. The microbial consortia were first discovered by Johannes Reinke in 1872 **(Reinke, 1872)**. Microbial Consortia and mixed cultures tend to possess enhanced decolorization activity and are more resistant to extreme pH (acidic or alkaline), temperature (high or low) and high dye concentrations **(Balapure *et al.,* 2015; Lade *et al.,* 2015)**. Microbial consortia have been widely used in cleanup of a number of pollutants in laboratory and field bioremediation studies. It is generally thought to be more effective than pure cultures in biodegradation. This is possible due to its broader enzymatic capacity; the formation of toxic intermediate metabolites is counteracted by the selection of these dead end products formed mainly by co-metabolism processes. Bacteria express their full capacity to degrade the pollutants in optimal conditions, temperature, pH, and supplements have great influence on their growth **(Jain *et al.,* 2012; Mao *et al.,* 2012)**.

Fungal consortia have been considered as better treatment for textile wastewater as compared to conventional biological methods or systems using single fungal strains.

Anastasi *et al.* (2009) have reported the use of a fungal consortium comprising of *Trametes versicolor, Bostra fumosa, Bjerkandera adusta*, along with straw as solid substrate, to detoxify Poly R-478. Highly efficient fungal consortia were also reported for degradation of azo dyes and textile wastewater treatments **(Yang *et al.,* 2009)**. Fungal co-cultures of *Panicillium oxalicum* SAR-3, *Aspergillus niger* SAR-6 and *Aspergillus flavus* SAB-3 has also been reported in the higher degradation of azo dyes **(Saroj *et al.,* 2015)**.

2.8 Factors affecting microbial dye removal

Microorganisms are very susceptible to the presence of some chemicals, such as dye, high salinity, high content of organic compounds and variation in pH *etc*. For the degradation of dye, most suitable microorganisms are those which are isolated from the dye contaminated site as they have the potential to survive in that environment and are well adapted to grow in such extreme conditions. The bioremediation of dye depends on many factors as given below:

A. Effects dye structure

It has been observed that the enzymatic degradation of dyes is highly influenced by its structure. The dyes with electron-withdrawing groups are easier to degrade than those with electron releasing groups, such as –NH–triazine **(Sawhney and Kumar, 2011)**.

B. Influence of carbon and nitrogen sources

Carbon and nitrogen sources have an important influence on the extent of decolorization using microorganisms. Different microbial metabolic characteristics lead to differences in the uptake of these sources, thus affecting dye decolorization **(Moosvi *et al.,* 2007)**. Carbon and energy sources are used for the growth and survival of the microorganisms and as electron donors, which are necessary for the breakage of the azo bond. Carbon and nitrogen sources are accepted differently by different microorganisms and have an important effect on the extent of decolorization.

C. Influence of salinity, dye concentration, pH, temperature and oxygen

The conditions which affect the efficiency of microorganisms to decolorize dyes are the presence of salts, concentration of dyes, pH, temperature and oxygen. Generally, sodium concentration above 3000 ppm causes moderate inhibition of most bacterial activities, thus, dye removal efficiencies under saline conditions gets reduced. The studies show that adsorption and enzymatic activity is also dependent on the pH. The extent of decolorization is influenced by the pH of the media, which also affects the color of the solution and the solubility of the dye. *Candida tropicalis* adsorbs 45% of Basic Violet 3 at pH 3, 85% at pH 4 and 33% at pH 9 **(Das and Das, 2011)**.

2.8 Genetics of Dye Decolorization

The knowledge of genes and enzymes that are involved in the biodegradation of synthetic dyes is restricted but the knowledge of metabolic pathway of its degradation is well understood **(Yatome and Colleagues, 1993)**. Laccase is a major enzyme that is involved in the degradation of dyes hence is required for the process. To obtain large quantity of this enzyme, several laccase genes have been cloned from different fungal source **(Jonsson *et al.,* 1995; Yaver *et al.,* 1996)**, such as *Trichoderma reesei* **(Saloheimo *et al.,* 1991)**, *Pichia pastoris* **(Otterbein *et al.,* 2000)**, *Pichia methanolica* **(Soden *et al.,* 2002)**, *Aspergillus niger* **(Record *et al.,* 2002)** *Saccharomyces cerivisiae* **(Bulter *et al.,* 2003)**, *Aspergillus nidulans* **(Larrondo *et al.,* 2003),** *Yarrowia lipolytica* **(Jolivalt *et al.,* 2005)**, *Aspergillus oryze* **(Hoshida *et al.,* 2005)** and *Kluyveromyces lactis* **(Faraco *et al.,* 2008)** for contribution of laccase over producing recombinant. Previous research on the regulation of laccase gene expression demonstrates that the transcription of laccase gene can be regulated by metal ions **(Collins *et al.,* 1997)**, various aromatic compounds related to lignin or lignin derivatives **(Terron *et al.,* 2004)**, nutrient nitrogen **(Collins *et al.,* 1997)** and carbon **(Soden *et al.,* 2001)**.

2.9 Analytical techniques

For monitoring dye degradation various techniques were used to identify the possible metabolites by the previous researchers. UV-Visible spectroscopy, FTIR analysis, HPLC **(Chakraborty *et al.,* 2013)**, GCMS **(Feng *et al.,* 2000)**, LCMS **(Zille *et al.,* 2005)**, XRD, SEM **(Liu *et al.,* 2011)** have been used to study the mechanisms involved in the dye degradation and also to know the possible intermediate metabolites after the degradation of synthetic dyes **(Chakraborty *et al.,* 2013)**.

Materials and Methods

Chapter 3 MATERIALS AND METHODS

3.1 Synthetic Dyes

Four toxic, carcinogenic synthetic dyes having wide application in textile, pulp and paper, tannery, printing and other industries were used in the present investigation out of which three dyes belong to Azo group (e.g. congo red, direct green and direct black) and one dye belong to triarylphenyl group (e.g. brilliant green). All these dye were of analytical grade and procured from HiMedia / local market.

3.2 Microbial cultures used

A total of twenty eight microbial cultures including bacteria (17), fungi (7), microalgae (3) and Cyanobacteria (1) were used in the study. Sixteen bacterial cultures were isolated from diverse sources viz rhizosphere, root nodule, cow dung, field soil and mushroom fruiting bodies; three white rot fungal cultures were isolated from the fruiting bodies growing on tree trunk / decaying wood; three fungal isolates, three microalgae cultures were previously isolated in our own laboratory, standard culture of *Trichoderma harzianum* rifai, MTCC 792, and a standard culture of Cyanobacteria (*Synecococcus* sp.) were obtained from the culture collection department of microbiology, C.B.S.&H., G.B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand. A detailed description of all the microbial cultures used during present investigation is given in Table 3.1.

3.3 Isolation and Maintenance of microbial cultures

For the isolation of wood rotting fungi (white rot), the fresh mushroom fruiting bodies growing over tree trunk / rotten wood were collected in sterilized polythene bags and brought to the laboratory. The fruiting body samples were first surface sterilized with 75% ethanol for two min, followed by washing with sterilized triple distilled water under aseptic condition. Small pieces were cut, exposed surface was removed and internal biomass was inoculated on to the potato dextrose agar (PDA) plates in triplicates and incubated at 30 $\pm 2^0$C for 5-10 d, the mycelial growth was reinoculated on fresh PDA plates and purified cultures were stored as PDA slants at 4 ^{0}C in refrigerator.

Table 3.1 Detailed description of various microbial cultures isolated from diverse sources.

S. No.	Source	Site of collection	Total no. of isolates	Index of isolates
I. Bacterial isolates				
1.	Maize -rhizosphere	CRC, G.B.P.U.A&T. Pantnagar	1	PMB1
2.	Soyabean nodule	CRC, G.B.P.U.A&T. Pantnagar	3	PSNB1, PSNB2, PSNB3
3.	cow dung	G.B.P.U.A&T. Pantnagar	5	PCB1, PCB2, PCB3, PCB4, PCB5
4.	Non rhizosphere(field) Soil	G.B.P.U.A&T. Pantnagar	1	PSB1
5.	Mushroom Fruiting body 1	Tree trunk G.B.P.U.A&T. Pantnagar	3	PMFB1 , PMFB2, PMFB3
6.	Mushroom Fruiting body 2	Tree trunk G.B.P.U.A&T. Pantnagar	1	PMFB4
7.	Mushroom Fruiting body 3	Decaying wood G.B.P.U.A&T. Pantnagar	1	PMFB5,
8	Mushroom Fruiting body4	Decaying wood G.B.P.U.A&T. Pantnagar	2	PMFB6, PMFB7
II. Fungal isolates				
8	Culture collection, Dept. of Microbiology	*Bambusa vulgaris rhizosphere* G.B.P.U.A&T. Pantnagar	1	PBT-6
9.	Standard culture*, T. harzianum rifai,* MTCC 792	MTCC Chandigarh India	1	*num*
10	Mushroom Fruiting body 1	Tree trunk G.B.P.U.A&T. Pantnagar	1	PMF1
11	Mushroom Fruiting body 2	Tree trunk G.B.P.U.A&T. Pantnagar	1	PMF2
12.	Mushroom Fruiting body 3	Decaying wood G.B.P.U.A&T. Pantnagar	1	PMF3
13	Mushroom Fruiting body4	Decaying wood G.B.P.U.A&T. Pantnagar	2	WFPN1, WDP2
III. Green Microalgae				
14	Culture collection, Dept. of Microbiology	G.B.P.U.A&T. Pantnagar	3	PASM, PHSM, PRSM
IV. Cyanobacteria				
15	Standard culture collection IARI, New Delhi (*Synechococcus*)	Standard culture collection IARI, New Delhi	1	*Synechococcus* sp.

For the isolation of symbiotic nitrogen fixing bacteria (Rhizobia), freshly collected nodules from soya bean root were washed with water, surface sterilized with 75% ethanol for one min, and 0.1 % mercuric chloride ($HgCl_2$) solution for two min followed by washing with 1% KCl solution and 3-5 times washing with sterilized triple distilled water. Then the nodules were aseptically crushed with sterilized glass rod and the nodule exudates were streaked onto congo red yeast extract Mannitol agar (CRYEMA) plates. Inoculated plates were incubated at 30 $\pm 2^0$C till appearance of pink mucoid colonies, which were further purified on the same medium and discrete isolated colonies were selected and preserved as YEMA slants at 4 ^{0}C in refrigerator. For isolation of endophytic bacteria from the mushroom fruiting bodies, the sterilized pieces of the fruiting bodies were crushed in triple distilled water aseptically and then pour plated on nutrient agar (NA) plates. The remaining nitrogen fixing / non nitrogen fixing bacterial cultures were isolated from freshly collected and serially diluted samples (cow dung, maize rhizospheric soil, field soil) on Ashby's agar plates and nutrient agar plates respectively. Inoculated plates were incubated at 30 $\pm 2^0$C till appearance of colonies which were further purified on their respective media. The cultures were maintained and stored as nutrient agar / Ashby's agar slants at 4 ^{0}C. For the isolation of fungal cultures the media was amended with antibiotic (streptopenicillin) while antifungal agent (nystatin) was added to the culture media for isolation of bacteria.

All the purified fungal cultures were maintained on potato dextrose agar (g. L^{-1}: peeled potato 200.0 g, dextrose 20.0g and agar 15.0g, pH 6.2 ±0.2) and active fungal discs (5mm diameter) were used for further experiments. All the microalgae and cyanobacterial cultures were maintained and revived on BG11 mineral salt medium with 12 h dark / light periods at 25 ^{0}C in tissue culture lab (relative humidity 60-80 %), department of biochemistry, G.B.P.U.A&T Pantnagar.

3.4 Screening (*in vitro*) and Selection of Dye Decolorizing Microbes

3.4.1 Qualitative Screening for *in vitro* Dye Decolorization: Plate Assay

Plate assay was performed for preliminary selection of dye decolorizing microbial isolates using mineral salt medium supplemented with synthetic dyes individually (100mg/l). Twenty eight different microbial groups were used for the

screening of dye decolorization. Dye amended MSM plates were inoculated with active fungal mycelial discs (5mm) in the centre or spot inoculated with active bacterial / microalgae cultures and incubated at 30±2^{0}C for 7 d. Appearance of clear decolorized halo zone around the colonies was considered as positive test and the isolates showing decolorized zone around their colonies were selected for further experiments. The extent of decolorization was confirmed by the relative decolorizing indices (I_{dec} = Zone diameter / Colony diameter).

3.4.2 Quantification of Dye Decolorization

Quantitative estimation of dye removal from dye containing media by the microbial isolates was done spectrophotometrically. Batch culture experiments use active fungal mycelial discs (5discs / 5mm each) or active bacterial and microalgae cultures (@ 10%) were performed in 150mL Erlenmeyer flasks containing 50mL of mineral salt medium supplemented with 100mg/L dye. The cultures were incubated at 30±2^{0}C until complete decolorization. Samples were withdrawn up to seven d at a definite interval of 24 h and centrifuged at 10,000 rpm for 10 min. Optical densities (absorbance) of the clear supernatants were measured using uv-visible spectrophotometer (Thermo) at 497nm (λ_{max} for congo red), 630nm (λ_{max} for brilliant green), 623nm (λ_{max} for direct green) and 580nm (λ_{max} for direct black) and % dye decolorization by each culture for each dye was calculated using the following formula **(Daneshvar *et al.,* 2007)**.

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

3.5 Detection and estimation of dye decolorizing enzyme

3.5.1 Qualitative screening for Enzyme detection

I. Laccase Enzyme: Plate Assay

Qualitative screening for *in vitro* laccase production by fungal, bacterial, microalgae and Cyanobacteria cultures were done using 0.01% (v/v) of guaiacol as substrate. Culture media supplemented with guaiacol were inoculated with the microbial mono cultures and incubated at 30±2^{0}C for seven d under dark static conditions. Presence of reddish brown color around the microbial colony was considered as positive result which shows the oxidation of guaiacol by laccase enzyme.

II. Azoreductase Enzyme: Plate Assay

In vitro qualitative screening for azoreducatse enzyme was done using mineral salt agar medium supplemented with azo dye @ 100mg/L (congo red, direct green, direct black). The active microbial cultures were inoculated on to the dye containing plates and incubated at 30±2^{0}C for seven d under dark static conditions. The presence of decolorized around the colonies indicated presence of azoreductase enzyme.

III. Peroxidase (catalase) Test

Peroxidase (catalase) test was done using H_2O_2 as the substrate. In the active broth culture of bacteria / microalgae/ cyanobacteria was mixed with few drops of H_2O_2 and visualized for gas formation. Appearance of bubbles within a minute indicates catalase production.

In case of fungal cultures active mycelial biomass along with the broth was wortexed and mixed with few drops of H_2O_2 and visualized for bubble formation **(Sunitha *et al.,* 2013)**.

3.5.2 Quantitative Estimation of Enzyme

3.5.2.1 Enzyme Production

To study the enzymatic nature and the extracellular enzymes involved in dye decolorization process, the cultures were inoculated in dye containing mineral salt medium. The inoculated cultures along with uninoculated controls were incubated at 30±2 ^{0}C under dark static conditions for 12 d. The aliquots (2.0 mL) were withdrawn periodically at a regular interval of 48 h. The microbial biomass was removed from the samples by centrifugation at 10,000 rpm for 10 min at 4^{0}C. The supernatant was used as source of crude enzyme. In order to elucidate the involvement of the extracellular enzymes, the enzyme (laccase, lignin peroxidase, manganese peroxidase and azoreductase) activities were determined in the culture filtrates during dye decolorization process.

3.5.2.2 Enzyme Activity Measurement

I. Laccase Enzyme Activity

Guaiacol oxidation method as described by **Kalra *et al*., (2013)** was used for quantification of extracellular laccase enzyme. Appearance of reddish brown color

upon oxidation of guaiacol by laccase enzyme is used to determine the enzyme activity at 450 nm. For measuring enzyme activity, the reaction mixture (5ml) was prepared by adding 3mL of 10mM sodium acetate buffer (pH 5.0), 1 mL of 2mM Guaiacol and 1 mL of enzyme solution. An enzyme blank was also prepared in which enzyme was replaced by water. Then the reaction mixture was incubated for 15 min in water bath at 30^0C for the production of reddish brown color; the absorbance was read at 450 nm by UV-Visible spectrophotometer. Enzyme activity was expressed as International Units (IU), where 1 IU is the amount of enzyme required to oxidize 1 µmol of guaiacol per min. Enzyme activity in U/mL was calculated using the following formula-

EA = A * V/ t * e * v

Where, E.A = Enzyme activity;

A = Absorbance;

V = Total volume of reaction mixture (ml);

v = enzyme volume (ml);

t = incubation time;

e = extinction coefficient for guaiacol (0.6740 lM/ cm).

II. Azoreductase Enzyme Activity

Azoreductase activity in culture filtrate during dye decolorization was determined following the method given by **Maier *et al.* (2004)**, with slight modifications. Assays were carried out in cuvettes (pathlength = 1 cm) with a total volume of 1.0 ml. Reaction mixture contained 400 µL of Phosphate buffer, 200µL of enzyme sample and 200 µL of the azo dye (Congo red, 100 mgL^-1), resulting in a final concentration of 16.3 µM dye. The reaction was started by the addition of 200 µl of NADH (7.09 mg mL^{-1}; final concentration, 2 mM) and was monitored spectrophotometrically at 497 nm. The slope of the initial linear,decrease of absorption (ΔA min^{-1}) was used to,calculate azoreductase activity based,on the molar absorption coefficient,of Congo red dye (ε = 6.26 x $10^4 mol^{-1}$ cm^{-1}). One unit of enzyme activity,was defined as the amount of enzyme required to reduce 1.0 µM of azo dye per minute in the presence of NADH. Enzyme activity was expressed as U/mL and calculated using the following formula-

$$\text{U/mL enzyme} = \frac{(\Delta A497/\text{min Test} - \Delta A497/\text{min Blank})}{\varepsilon \text{ x } v}$$

Where, ε = Molar Extinction Cofficient;

v = Total volume of reaction mixture.

III. Lignin Peroxidase Activity

Lignin peroxidase (LiP) activity was determined by measuring the rate of H_2O_2 dependent oxidation of veratryl alcohol to veratryldehyde, spectrophotometrically following the method given by **Tien and Krik (1988)** with slight modification. The reaction mixture (2.05ml) contained 0.8mM veratryl alcohol in 0.1 M citrate buffer (pH 3.0) and 1 mL of crude enzyme. The reaction was started by addition of 150mM H_2O_2 at 30^0C and the linear increase in absorbance was immediately measured spectrophotometrically in 1 min at 310 nm. One unit (U) of LiP activity was defined as "the amount of enzyme required for conversion of 1μM of substrate (veratryl alcohol) into 1μM of product (veratryldehyde) per minute at 310 nm" and was expressed as UmL^{-1} (ϵ=9300M-1 cm^{-1}). The enzyme activity was calculated using the following formula-

$$1 \text{ Unit} = \frac{\Delta E/\Delta T * V}{\epsilon * v * t}$$

Where, ΔE/ΔT = Increase in absorbance per minute;

V = Volume of assay solution in cuvette;

ε = Molar Extinction coefficient of veratryl alcohol;

v = volume of sample;

t = time in minutes.

IV. Manganese peroxidase assay

Manganese Peroxidase (MnP) activity was determined spectrophotometrically at 270 nm by monitoring the oxidation of Mn^{+2} to Mn^{+3} following the method given by **Paszczynski *et al*., (1988)** with slight modifications. The reaction mixture (3.06mL) contained 0.1 mM guaiacol, o.1 mM $MnSO_4$ in 0.1M citrate buffer (pH5.0) and 1 mL of crude enzyme. The reaction was started by addition of 0.1mM H_2O_2. One unit of MnP enzyme activity was defined as "The amount of enzyme required for conversion of 1μM

of substrate into 1 μM of colored product in 1min at 465 nm" (ϵ= 12.1 x 10^3 M^{-1} cm^{-1}), and calculated using the formula as mentioned in 3.5.2.2 (III).

3.7 Development of Microbial Consortia for enhancement of dye decolorization

In order to improve microbial dye decolorizing efficacy through synergistic action, co-cultures / mixed cultures were developed using potential fungal / bacterial cultures. Twenty two microbial consortia for direct black azo dye was developed using 3 potential fungal isolates (PMF2, PMF3, WFPN1), a standard culture of *T. harzianum* and 2 selected bacterial isolates (PMB1 and PFMB4); while for the aryl dye brilliant green decolorization, fifteen microbial consortia were developed using the selected fungal isolates (PMF2 and WDP2), three microalgae isolates (PASM, PHSM and PRSM) and one Cyanobacterial culture (Synecococcus sp). Active fungal cutures of selected isolates were developed on PDA plates and 6 mycelial discs per consortium were inoculated. Active bacterial / microalgae / cyanobacterial cultures (OD- 0.8), of selected isolates were inoculated at the rate of 10 % in each consortium. These co-cultures were grown in mineral salt medium amended with direct black or aryl dye brilliant green @ 100mg/L at 30±2 0C for 4 d. Samples were withdrawn periodically at an interval of 24 h in case of direct black dye and at an interval of 12 h in case of brilliant green dye and analysed spectroscopically for color change at 580nm and 630nm for direct black and brilliant green dye respectively.

3.8 Extracellular protein profiling by Native PAGE

To check the extracellular protein profiling from selected potential microbial cultures native polyacrylamide gel electrophoresis (PAGE) was performed **(Sambrook *et al.*, 2000)**. For gel electrophoresis, crude extracellular protein samples were prepared by aetone precipitation method. Five day old decolorized broth cultures were centrifuged at 8000 rpm (4 ^{0}C) for 10 min. Extracellular proteins were extracted from the supernatant using acetone. The precipitated protein was washed and dissolved with phosphate buffer (pH 7.0) and used for protein profiling.

3.8.1 Stock solution

1. Acrylamide and bisacrylamide stock solutions (30%): Acrylamide (29.2 gm) and N,N'- methylene bis acrylamide (0.8gm) were dissolved in double distilled water (ddw,100ml) using a magnetic stirrer, filtered and stored at 4 0C in dark colored bottle.

Table 3.2 Mixed microbial cultures (consortia) developed using potential isolates fro decolorization of direct black azo dye

Consortia	Microbial cultures used		
	Fungal isolates	**Bacterial isolates**	**Microalgae/ Cyanobacterial isolates**
I	PMF2	PMB1	-
II	PMF3	PMB1	-
III	WFPN1	PMB1	-
IV	*T. harzianum*	PMB1	-
V	PMF2	PMFB4	-
VI	PMF3	PMFB4	-
VII	WFPN1	PMFB4	-
VIII	*T. harzianum*	PMFB4	-
IX	PMF2+PMF3	PMB1	-
X	PMF2+PMF3	PMFB4	-
XI	PMF2+PMF3+WFPN1+*T. harzianum*	-	-
XII	PMF2+PMF3+WFPN1+*T. harzianum*	PMB1	-
XIII	PMF2+PMF3+WFPN1+*T. harzianum*	PMFB4	-
XIV	PMF1+PMF2	-	-
XV	PMF1+PMF2+PMF3	-	-
XVI	PMF2+WDP2	-	-
XVII	PMF1+PMF2+PMF3+WFPN1	-	-
XVIII	PMF2+PMF3	-	-
XIX	PMF2+WFPN1	-	-
XX	PMF2+*T. harzianum*	-	-
XXI	WFPN1+*T. harzianum*	-	-
XXII	WDP2+*T. harzianum*	-	-

Table 3.3 Mixed microbial cultures (consortia) developed using potential isolates fro decolorization of brilliant green dye

S.No.	Microbial Cultures Used		
	Fungal isolates	**Microalgae isolates**	**Cyanobacteria isolates**
I	PMF2	-	*Synechococcus* sp.
II	PMF2	PASM	-
III	PMF2	PHSM	-
IV	PMF2	PRSM	-
V	WDP2	-	*Synechococcus* sp.
VI	WDP2	PASM	-
VII	WDP2	PHSM	-
VIII	WDP2	PRSM	-
IX	-	PASM	*Synechococcus* sp.
X	-	PHSM	*Synechococcus* sp.
XI	-	PRSM	*Synechococcus* sp.
XII	PMF2 + WDP2	-	*Synechococcus* sp.
XIII	PMF2 + WDP2	PASM	*Synechococcus* sp.
XIV	PMF2 + WDP2	PHSM	*Synechococcus* sp.
XV	PMF2 + WDP2	PRSM	*Synechococcus* sp.

2. **Stacking gel buffer:** 0.5 M Tris- HCl (pH 6.8)
3. **Seperating gel buffer:** 1.5 M Tris- HCl (pH 8.8)
4. **Electrophoresis buffer:** Tris- Glycine buffer (Tris base 25.0mM, Glycine 192.0mM, pH 8.3)
5. **Sample buffer2X:** 62.0mM Tris – HCl (pH 6.5), Glycerol 10%, EDTA 0.1 %, Bromophenol blue dye 0.1%.
6. **Fixing solution:** Methanol (50%) and glacial acetic acid (10%)

7. **Staining solution:** CBB- R250 dye (0.25%) dissolved in methanol (50%) and glacial acetic acid (10%)
8. **Destaining solution:** Methanol (10%) and Glacial acetic acid (7%)
9. **Ammonium persulphate (APS, 10% v/v) solution:** APS (0.1 gm dissolved in ddw (1mL))

3.8.2 Preparation of gel

Proteins were analyzed using 5% stacking gel and 10% resolving gel by native PAGE. The following composition of resolving and stacking gels were used for native PAGE analysis.

S.No.	Components	Quantity (mL)	
		Resolving gel (10%)	**Stacking gel (5%)**
1	Acrylamide stock (30%)	8.0 mL	1.3 mL
2	Resolving / stacking gel buffer	5.0 mL	2.0 mL
3	APS (10% w/v)	0.2 mL	0.08 mL
4	TEMED	0.01 mL	0.01 mL
5	Triple distilled water	6.79 mL	4.53 mL
6	Total volume	20.0 mL	8.0 mL

3.8.3 Gel casting

Cleaned, dried gel plates were assembled with the spacers and clams, separating gel solution was poured between the glass plates, overlaid with double distilled water and allowed to polymerasize at room temperature. When the gel was fully set, overlay solution was removed, and then the gel was washed with double distilled water. The stacking gel was then poured and the combs were inserted 1.5 cm above the resolving gel. The gel was allowed to polymeraze, the combs were then removed and the wells were cleaned. The gel plates were assembled in electrophoresis chamber and filled with electrophoresis buffer. Air bubbles were carefully removed through a syringe and the gel was pre run at 80 V for 15 min.

3.8.4 Preparation and loading of sample

For sample preparation, equal volume of each protein sample was mixed with sample buffer (1:1) and directly loaded onto wells with the help of micropipette. The gel was first run at 80 V till the sample was in stacking gel and then the voltage was raised to 100 V. the gel was allowed to run till the dye reaches 0.5cm from the lower edge of the gel (**Karp *et al.,* 2012**).

3.8.5 Staining and destaining of gel

The gel was transferred in the staining solution and left overnight at room temperature. Destaining was done till the background was fairly reduced to make the protein bands visible.

3.9 Zymogram Analysis for Laccase isoforms

Zymogram analysis was done to identify different isoforms of the laccase enzyme, if produced by the selected isolates in presence or absence of azo dyes. After the gel was completely run in Native PAGE, it was washed with triple distilled water and allowed to stand in 50mL of sodium acetate buffer (100mM, pH 4.5) containing 1 mL of 10mM ABTS as substrate for 30-60 min for visualization of laccase activity bands (**Patel *et al.,* 2014**).

3.9 Analytical techniques

To investigate the possible mechanism for synthetic dye removal from the broth cultures by the potential isolates, spectral analysis of the cell free decolorized samples (supernatant) was carried out using UV–Visible spectrophotometer (Thermo) at different wavelengths ranging from 200-700 nm and FT-IR spectroscopy at 400-4000 cm^{-1}.

3.10.1 UV-Visible spectral analysis

For monitoring the extent of dye removal, UV-Visible spectral analysis of selected potential isolates was performed on the decolorized samples along with an uninoculated control. The UV-Visible spectral analysis was done by using the UV-Visible spectrophotometer (Thermo) and change in spectra were recorded at the wavelength (λ) range of 200 to 700 nm.

3.10.2 Fourier Transform Infrared Spectroscopy

In order to check the extent of dye decolorization and to study the mode and mechanisms involved in decolorization process by the selected isolates, FTIR analysis was done using a Thermo-Nicolet Nexus 670 FTIR spectrophotometer with KBr pellets. FTIR was performed on the decolorized sample and an uninoculated control. The spectra were collected at a resolution of $4cm^{-1}$ in the wave number region of 400-4000 cm^{-1}.

3.10 Characterization of selected isolates

The potential isolate WFPN1 was analyzed on the basis of polyphasic approach (morphological, biochemical, physiochemical and molecular characteristics). The strain showed phenotypic resemblances (such as colony/ mycelia growth pattern, structure of fruiting body etc) with the *Ganoderma* species and was further characterized on the basis of 18S ITS r-RNA gene sequence analysis. The polymerase chain reaction (PCR) amplification and sequencing of partial 18S rRNA gene was carried out using the standard primer set ITS1 (forward, 5' TCCGTAGGTGAACCTGCGG3') and ITS4 (reverse, 5'TCCTCCGCTTATTGATATGC3') as described earlier **(Bruns *et al.*, 1991)**. The sequences (18S ITS rRNA gene) were then analysed using basic local alignment tool (BLAST) at NCBI. Phyllogenetic tree based on 18S ITS rRNA gene sequences was constructed using MEGA**7** (N-J method).

3.12 Statistical analysis

All the experiments were performed in triplicates. A completely randomised design (CRD) with one or two factors was used. The experimental data were statistically analyzed by two- way ANNOVA (analysis of variance) using the Statistical software program STPR2 and means were compared. The statistical significance level (p- value) was set at 0.05 and the results were reported in terms of critical difference (CD) and standard error of mean (SEm±).

Results and Discussion

Chapter 4 RESULTS AND DISCUSSION

Due to the use of dyes in various industries, the release of industrial effluent in main water stream is increasing globally. It is reported that textile, paper, printing and other industries release up to 15-20 % of the dye containing effluent in water bodies; hence, it can pollute the nearby water and soil. Synthetic dyes are mainly used in the textile, pharmaceuticals, pulp and paper, printing, leather industries etc. Most of the synthetic dyes are carcinogenic and mutagenic in nature and cause several diseases in flora and fauna. Several physical and chemical techniques are used in the treatment of industrial effluent e.g. membrane filtration, ozonation, coagulation, ionization etc. These physicochemical and chemical techniques are not environment friendly and very costly. Hence, the alternative method which is biological method has vast potential to degrade / remove these synthetic dyes from the waste water. For achieving this target indigenous potential microbial isolates have to be identified and used for the degradation of toxic synthetic dyes. In view of these facts, present investigation was carried out on decolorization of three commonly used azo dyes viz. congo red, direct black and direct green and one triaryl methane dye viz. brilliant green. A total of twenty seven microbial cultures including sixteen bacterial cultures, seven fungal cultures, three microalgae cultures and one cyanobacterial culture were used in the study.

4.1 Qualitative Screening of Microorganisms for dye decolorization

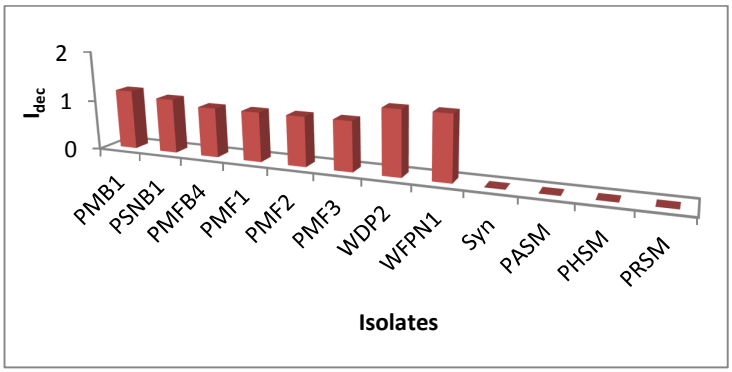
For selecting the dye degrading microbes, twenty eight different microbial isolates were selected and used for *in vitro* dye decolorizing screening. In order to select the potential cultures, a preliminary screening of all microbial cultures was done for their *in vitro* growth potentialities and decolorization abilities in the presence of synthetic dyes on mineral salt medium for 7 d. During *in vitro* dye decolorization screening although all the microbial isolates were able to grow on dye containing mineral salt medium but they showed varied growth and dye decolorizing potentialities. Only few microbial isolates were able to decolorize the synthetic dyes in mineral salt medium. Out of sixteen bacterial isolates, only three isolates (PMB1, PSNB1 and PMFB4) were able to decolorize the azo dyes (congo red, direct green

and direct black), while no bacterial culture showed decolorization of the aryl dye brilliant green. Out of seven fungal isolates, only five (PMF1, PMF2, PMF3, WFPN1and WDP2) were able to decolorize the azo dyes, while only two fungal isolates (PMF2 and WDP2) were able to decolorize brilliant green dye. None of the microalgae/Cyanobacteria could decolorize the azo dyes but all the microalgae (PASM, PHSM and PRSM) and cyanobacteria cultures (*Synechococcus* sp.) were able to decolorize the brilliant green dye. The growth potentialities and decolorization of four synthetic dyes by various microbial groups are given in **Table 4.1**. Decolorization of dyes by the microbial cultures was clearly visible during *in vitro* plate assay **(Plate 4.1)**. The *in vitro* agar plate assay has also been reported as an efficient method for preliminary screening and selection of potential dye decolorizing micro flora **(Murugasen *et al*., 2006)**.

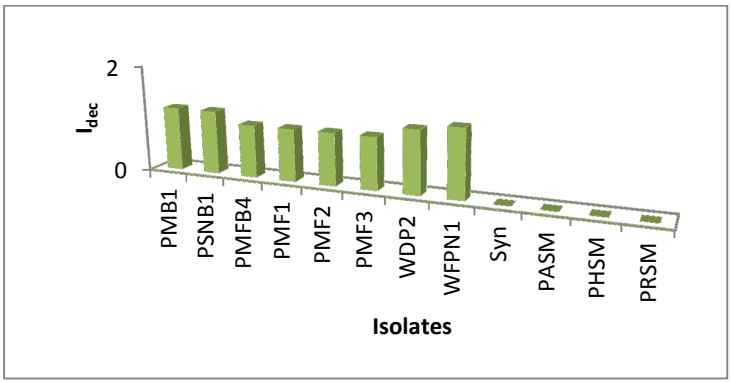
Based on qualitative screening, the relative dye decolorization indices (I_{dec}) for various cultures varied from 0 to 1.3 **(Fig 4.1)**, maximum I_{dec} value of 1.3 was recorded for the fungal isolates WFPN1 and PMF2 in case of congo red dye by, which was significantly higher than the other cultures **(Fig 4.1 a)**. For direct green dye, maximum I_{dec} value (1.3) was recorded for the fungal isolate WFPN1, the value being significantly higher than that for other cultures **(Fig 4.1 b)**. In case of direct black dye, maximum I_{dec} value (1.2) was recorded for the fungal isolate WFPN1, that was significantly higher than the I_{dec} values for other cultures **(Fig 4.1 c)**. In case of brilliant green dye, the maximum I_{dec} value of 1.0 was recorded for all the microalgae / Cyanobacteria cultures while no other bacteria or fungi were able to show decolorizing indices **(Fig 4.1 d)**. The colorless zone formation around the microbial colony indicates the dye decolorizing potential of the culture. Moreover, colorless zone also indicates removal of the dye from that area either through some biosorption mechanisms or through some extracellular enzyme activity. However, no colorless zone was shown by the photoautotrophic microalgae/Cyanobacteria as is evident by the I_{dec} value of 1.0. Thus it may be concluded that qualitative agar plate method cannot be considered as the sole criteria for screening and selection of potential cultures for decolorization of all the synthetic dyes rather quantification of dye removal must be done.

Table 4.1: Qualitative screening of Microbial Isolates for Dye Decolorization and growth potentialities on dye containing mineral salt medium

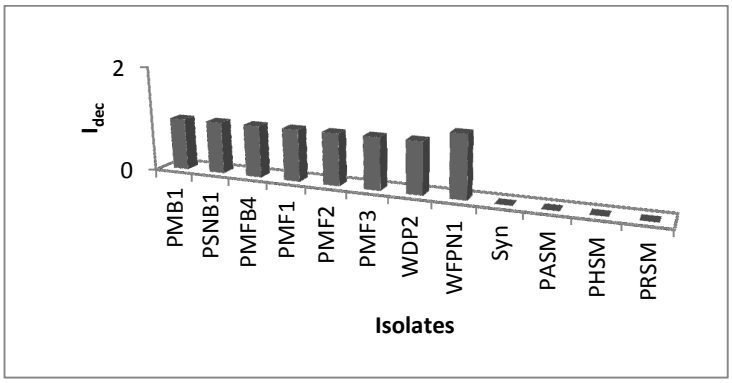
S.No.	Microbial Group	Microbial Isolates	Synthetic dyes							
			Azo dyes						Tri arylmethane Dye	
			Congo Red		Direct Green		Direct Black		Brilliant Green	
			Growth	Decolorization	Growth	Decolorization	Growth	Decolorization	Growth	Decolorization
1	N2 Fixing Bacteria	PMB1	+++	+	+++	+	+	+	+	-
2	N2 Fixing Bacteria	PSNB1	++	+	++	+	+	+	+	-
3	N2 Fixing Bacteria	PSNB2	+	-	+	-	+	-	+	-
4	N2 Fixing Bacteria	PSNB3	+	-	+	-	+	-	+	-
5	Non N2 Fixing Bacteria	PCB1	+	-	+	-	+	-	+	-
6	Non N2 Fixing Bacteria	PCB2	+	-	+	-	+	-	+	-
7	Non N2 Fixing Bacteria	PCB3	+	-	+	-	+	-	+	-
8	Non N2 Fixing Bacteria	PCB4	+	-	+	-	+	-	+	-
9	Non N2 Fixing Bacteria	PCB5	+	-	+	-	+	-	+	-
10	Non N2 Fixing Bacteria	PSB1	+	-	+	-	+	-	+	-
11	Non N2 Fixing Bacteria	PMFB1	+	-	+	-	+	-	+	-
12	Non N2 Fixing Bacteria	PMFB2	+	-	+	-	+	-	+	-
13	Non N2 Fixing Bacteria	PMFB3	+	-	+	-	+	-	+	-
14	Non N2 Fixing Bacteria	PMFB4	+	+	+	+	+	+	+	-
15	Non N2 Fixing Bacteria	PMFB5	+	-	+	-	+	-	+	-
16	Non N2 Fixing Bacteria	PMFB6	+	-	+	-	+	-	+	-
17	Non N2 Fixing Bacteria	PMFB7	+	-	+	-	+	-	+	-
18	Cyanobacteria	*Synechococcus* sp.	+	-	+	-	+	-	+++	+
19	Microalgae	PASM	+	-	+	-	+	-	+++	+
20	Microalgae	PHSM	+	-	+	-	+	-	+++	+
21	Microalgae	PRSM	+	-	+	-	+	-	+++	+
22	Wood Rotting fungi	PMF1	++	+	++	+	++	+	+	-
23	Wood Rotting fungi	PMF2	++++	+	++++	+	++++	+	++	+
24	Wood Rotting fungi	PMF3	++	+	++	+	++	+	+	-
25	Wood Rotting fungi	WFPN1	++++	+	++++	+	++++	+	+	-
26	Wood Rotting fungi	WDP2	++	+	++	+	++	+	++	+
27	Non Wood Rotting Fungi	PBT-6	+++	-	+++	-	+++	-	+	-
28	Non Wood Rotting Fungi	*T. harzianum*	+++	-	+++	-	+++	-	+	-



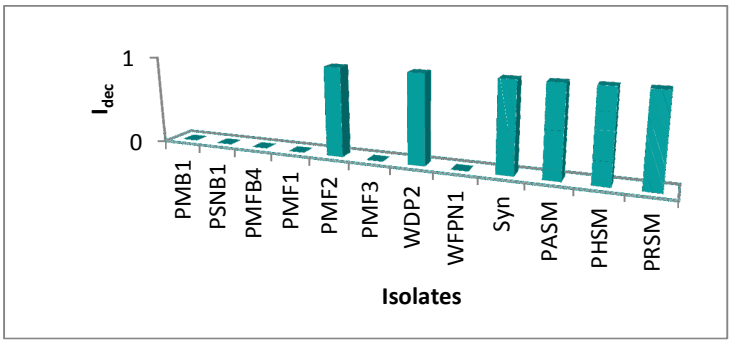
a. congo red



b. direct green



c. direct black



d. brilliant green

Fig 4.1 Comparative evaluations of fungal cultures based on relative dye decolorizing indices (I_{dec}) for congo red (a); direct green (b); direct black (c); direct black (d).

4.2 Quantification of dye decolorization

The microbial cultures showing heavy growth with or without colorless zone on dye containing mineral salt medium with relative decolorizing efficiency of ≥ 1.0, were selected. The extent of removal of test dyes (congo red, direct green, direct black and brilliant green) in broth cultures was further quantified spectrophotometrically and expressed as % decolorization. The decolorization in broth cultures is clearly visible in **Plate 4.2**. The extent of decolorization was monitored by measuring absorbance at 497nm for congo red, at 623nm for direct green, at 630nm for brilliant green and at 580nm for direct black. The % decolorization of azo dyes by various microbial cultures varied from 87.5% - 100%. Decolorization of brilliant green dye varied from 75.81% - 100 %. The bacterial isolates PMB1 and PMFB4; fungal isolates PMF2, WFPN1 and *T. harzianum* showed 100% decolorization for congo red in 5-d incubation period **(Table 4.2)**, which is significantly higher than all other microbial cultures. For direct green dye all selected microbial isolates (Bacteria/fungi) shows 100% decolorization in 5 d incubation period **(Table 4.3)**. Due to the incomplete dissolution of the dye in the media, this dye was not used for further experiments. In case of direct black dye, 100% decolorization was shown in fungal isolates PMF2, WFPN1 and *T. harzianum*, followed by PMF3 (94.6%).The fungal cultures shows significantly higher % dye decolorization in comparison to bacterial isolates **(Table 4.4)**. During the decolorization process, the residual concentration of the dye in culture filtrates was also monitored. It was found that with increasing incubation period and % decolorization, the corresponding residual concentration is decreases in each case. In case of brilliant green dye, all the four microalgae / Cyanobacteria showed 100% decolorization, which is significantly higher than the fungal isolates PMF2 and WDP2 **(Table 4.5)**. The extent of dye decolorization (%) was accompanied with a gradual decline in the residual concentration of the dye. **Fig 4.2** shows the relation between % dye decolorization and % residual concentration in the culture filtrate of WFPN1 (congo red and direct black) and *Synechococcus* sp. (brilliant green). The change in color of the dye in broth cultures proceeds through a change from dark red color to light pinkish in case of congo red; from dark green to light green color in case of direct green; from sea green to greenish white for brilliant green and dark black color to faded black color in case of direct black and then eventually becoming colorless. Removal of dye from the liquid broth was accompanied by its accumulation in the microbial biomass.

Table 4.2 Quantitative Estimation of Dye Decolorization by Selected Monocultures on congo red

S.No.	Microbial Group	Microbial Isolates	% Decolorization of CR						
			0d	1d	2d	3d	4d	5d	6d
1	Control	Control	0	0	0	0	0	0	0
2	N2 Fixing Bacteria	PMB1	0	48.5	74.88	89.3	95.41	100	100
3	N2 Fixing Bacteria	PSNB1	0	42.92	72.37	83.79	93.01	98.52	100
4	Non N2 Fixing Bacteria	PMFB4	0	39.21	74.08	87.3	99.02	100	100
5	Wood Rotting fungi	PMF1	0	40.21	81.29	86.9	80.29	82.65	100
6	Wood Rotting fungi	PMF2	0	49.2	81.29	89.3	97.72	100	100
7	Wood Rotting fungi	PMF3	0	52.54	80.89	85.26	86.98	89.35	100
8	Wood Rotting fungi	WFPN1	0	55.14	90.4	92.31	99.79	100	100
9	Wood Rotting fungi	WDP2	0	44.72	70.1	82.99	83.65	87.49	100
10	Wood Rotting fungi	*T. harzianum*	0	41.2	72.77	88.9	96.92	100	100
SEm±: a (culture)= 0.076; b (incubation period)= 0.064; c (a*b)= 0.171									
CD (5%): a (culture)= 0.21; b (incubation period)= 0.18; c (a*b)= 0.48									

Table 4.3 Quantitative Estimation of Dye Decolorization by Selected Monocultures on direct green

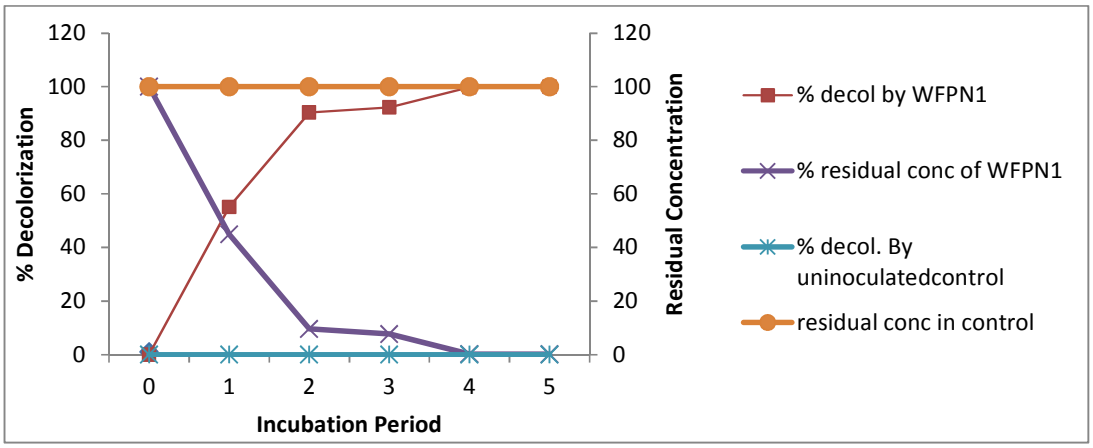
S.No.	Microbial Group	Microbial Isolates	% Decolorization of DG						
			0d	1d	2d	3d	4d	5d	6d
1	Control	Control	0	0	0	0	0	0	0
2	N2 Fixing Bacteria	PMB1	0	41.91	51.141	78.31	87.01	100	100
3	N2 Fixing Bacteria	PSNB1	0	28.7	62.3	70.2	79.1	100	100
4	Non N2 Fixing Bacteria	PMFB4	0	39.8	73.2	79.3	83.9	100	100
5	Wood Rotting fungi	PMF1	0	45.1	63.47	70.6	88	100	100
6	Wood Rotting fungi	PMF2	0	42	59.12	66.1	84.1	100	100
7	Wood Rotting fungi	PMF3	0	45.67	55.56	78.5	89.6	100	100
8	Wood Rotting fungi	WFPN1	0	48.64	70.79	74.15	93.4	100	100
9	Wood Rotting fungi	WDP2	0	40.53	57.6	65.7	81.9	100	100
10	Wood Rotting fungi	*T. harzianum*	0	40.93	62.1	75	83.3	100	100
SEm±: a (culture)= 0.064; b (incubation period)= 0.054; c (a*b)= 0.14									
CD (5%): a (culture)= 0.18; b (incubation period)= 0.15; c (a*b)= 0.40									

Table 4.4 Quantitative Estimation of Dye Decolorization by Selected Monocultures on direct black

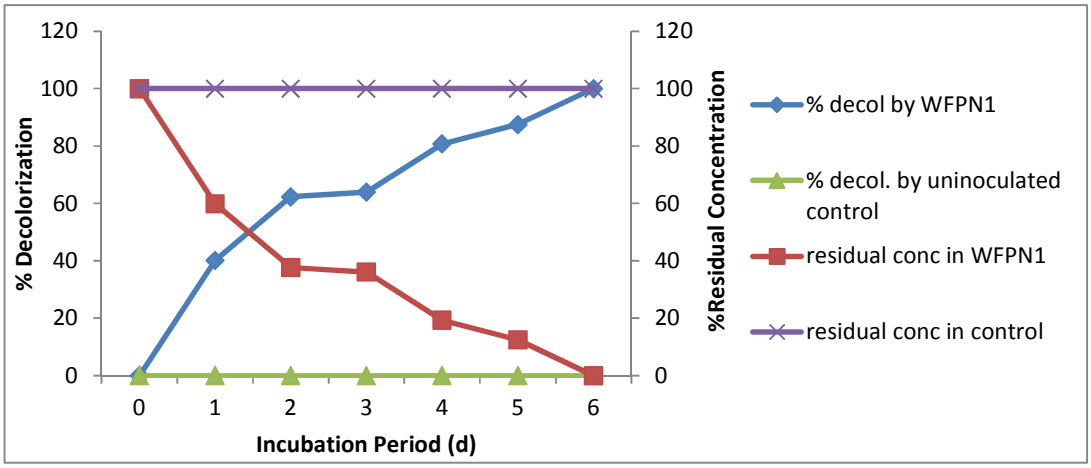
S.No.	Microbial Group	Microbial Isolates	% Decolorization of DB						
			0d	1d	2d	3d	4d	5d	6d
1	Control	Control	0	0	0	0	0	0	0
2	N2 Fixing Bacteria	PMB1	0	45.3	73.9	83.3	88.1	89.7	91.6
3	N2 Fixing Bacteria	PSNB1	0	39.3	65.4	71.93	72.5	86.3	90.8
4	Non N2 Fixing Bacteria	PMFB4	0	46.84	60.1	71.08	78.6	83.3	87.5
5	Wood Rotting fungi	PMF1	0	43.1	71.8	73	80.6	89	92.2
6	Wood Rotting fungi	PMF2	0	39.3	59.3	65.42	75.3	89.2	100
7	Wood Rotting fungi	PMF3	0	46.9	63.8	67.13	72.8	86.9	94.6
8	Wood Rotting fungi	WFPN1	0	40.1	63.9	62.3	80.7	87.5	100
9	Wood Rotting fungi	WDP2	0	45.8	60.2	70.6	71.7	90.1	93.4
10	Wood Rotting fungi	*T. harzianum*	0	45.4	68.5	75.2	77.9	90	100
SEm±: a (culture)= 0.069; b (incubation period)= 0.061; c (a*b)= 0.119 CD (5%): a (culture)= 0.16; b (incubation period)= 0.19; c (a*b)= 0.43									

Table 4.5 Quantitative Estimation of Dye Decolorization by Selected Monocultures on brilliant green

S.No.	Microbial Group	Microbial Isolates	% Decolorization of BG				
			0d	1d	2d	3d	4d
1	Control (BG11)	Control (BG11)	0	0	0	0	0
2	Cyanobacteria	*Synechococcus* sp.	0	65.5	86.8	93	100
3	Microalgae	PASM	0	66	85.6	92.6	100
4	Microalgae	PHSM	0	65.6	91.9	93.9	100
5	Microalgae	PRSM	0	55.7	89.6	93.3	100
6	Wood rot fungus	PMF2	0	61.86	93.14	93.89	94.23
7	Wood rot fungus	WDP2	0	57.64	75.17	75.48	75.81
SEm±: a (culture)= 0.205; b (incubation period)= 0.11; c (a*b)= 0.290 CD (5%): a (culture)= 0.59; b (incubation period)= 0.34; c (a*b)= 0.84							



(a)



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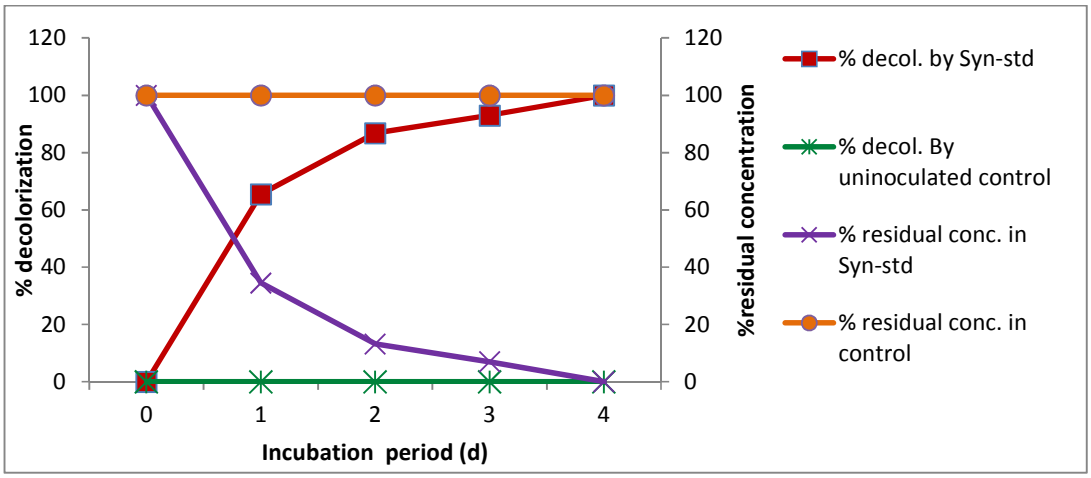


(c)

Fig 4.2 Percent dye removal from dye containing MSM medium along with the % residual concentration of the dye after treatment: a- Congo Red by WFPN1; b- Direct Black by WFPN1; c- Brilliant green by *Synechococcus* sp.

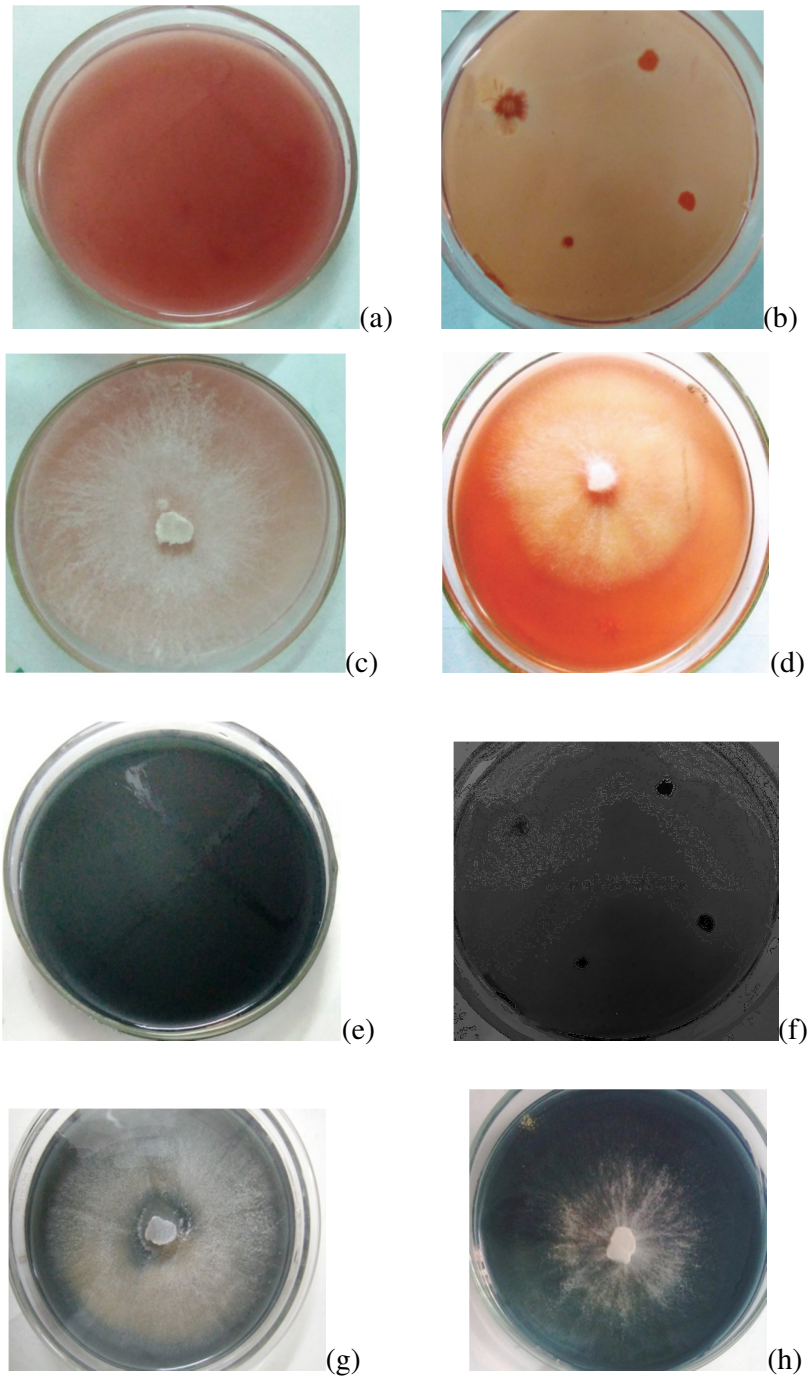
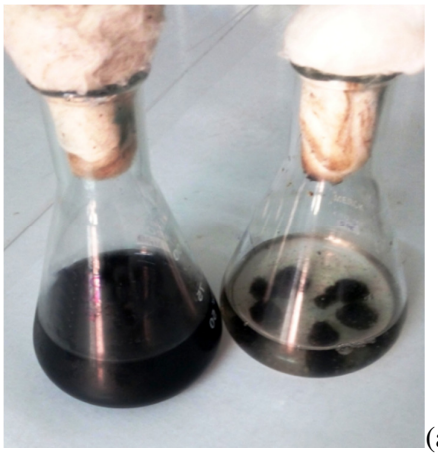


Plate 4.1 Decolorization of congo red and direct black by various bacteria and fungi; (a) congo red control; (b) bacteria; (c) PMF2; (d) WFPN1; (e) direct black control; (f) bacteria; (g) PMF2; (h) WFPN1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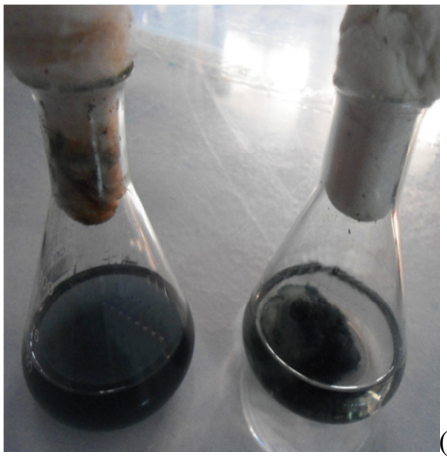
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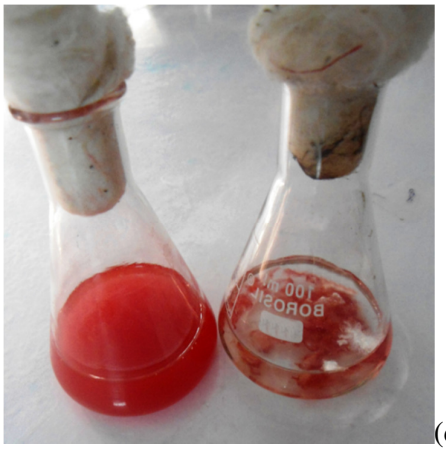
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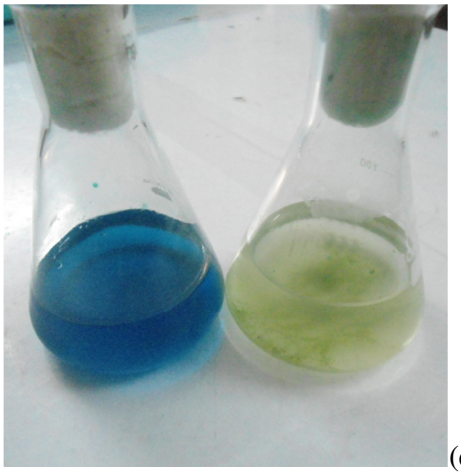
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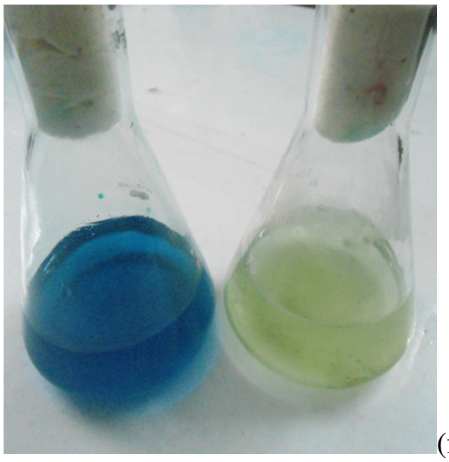

(f)

Plate 4.2 Decolorization of synthetic dyes by fungal and microalgae/ Cyanobacteria culyures: (a, c) WFPN1; (b, d) PMF2; (e) *Synechococcus* sp. (f) PASM

Biosorption of the dye by fungal biomass is clearly evident in the photomicrographs **(Plate 4.3)**. The initial removal of dye from liquid medium through microbial biosorption process as observed by us has also been reported earlier by several workers **(Sivasamy and Sundarabal, 2011)**.

Microorganisms have been reported to play an efficient role in the removal of certain noxious chemical compounds from the environment thereby making the biological treatment process as more effective and eco friendly method. Different microbial groups may employ different methods for the removal of such compounds (e.g. dyes) such as biosorption, biotransformation and biomineralization. Biosorption of dyes with microorganisms as latent sorbent for removal of dyes from industrial effluent has gained considerable attention. Lignolytic white rot fungi are the most widely studied dye decolorizing fungal agents **(Chao and Lee, 1994)**. Azo dyes have most wide industrial application and are used with different number of azo groups i.e. mono-azo, di-azo or multi-azo dyes. Although, several reports are available on microbial removal of mono-/di-azo dyes such as congo red but very few reports are available on biodegradation of more recalcitrant multi-azo dye (direct black). Decolorization of certain dyes may take place in two ways: either by biosorption or its biodegradation / mineralization **(Zohu and Zimmermann, 1993)**. Biosorption of the azo dyes by fungal biomass could also be visualized through phase contrast microscopy **(Plate 4.3)**. Biosorption of congo red by a newly isolated white rot fungus *Ceriporia lacerate* has also been reported recently **(Wang *et al.,* 2017)**. Some earlier authors showed decolorization of reactive dyes by various fungus cultures **(Saranraj *et al.,* 2010)**.

4.4 Enzymology of dye decolorization

Since various lignolytic enzymes have also been reported to play an important role in the dye removal/degradation process, therefore the main extracellular lignin degrading enzymes such as laccase, peroxidase (LiP and MnP) and azoreductase were tested in the filtrates of the selected cultures growing in presence/absence of synthetic dyes.

4.4.1 Qulatitative Detection

In order to know the enzymes involved in dye decolorization process of the the test microbial cultures, the extracellular enzyme activities for laccase, azoreductase and peroxidase were studied.

a. Laccase enzyme

To check the presence of laccase enzyme by selected microbial groups, guaiacol is used as the substrate for the laccase enzyme production. The reddish-brown zone around the colonies indicated the presence of laccase enzyme **(Gao *et al.,* 2011)**. During present investigation, only four fungal isolates (PMF2, PMF3, WFPN1 and WDP2) were able to produce laccase enzyme qualitatively; reddish brown zone around the colonies confirmed the laccase production **(Plate 4.4)**. None of the bacteria, microalgae or Cyanobacteria culture could produce reddish brown zone around the colonies indicating their inability to produce laccase enzyme. Literature search also indicates that laccase production by bacteria/Cyanobacteria or microalgae are not a prominent feature. While most of the wood rotting lignolytic fungi have been well reported extracellular laccase enzyme as was observed by us during present invetigaton **(Wesenberg *et al.,* 2003)**.

b. Azoreductase enzyme

Extracellular azoreductase enzyme activities of the test cultures were qualitatively detected by plate assay using congo red/direct black azo dye amended mineral salt medium. Formation of decolorized zone around the colonies of the bacterial isolates (PMB1, PSNB1 and PMFB4) and fungal isolates (PMF1, PMF2, PMF3, WFPN1 and WDP2) indicated the production of extracellular azoreductase enzyme by these fungal and bacterial isolates **(Plate 4.1)**. However azoreductase enzyme activity could not be recorded in any of the Cyanobacteria / microalgae. Several workers have also reported involvement of azoreductase enzyme in the biodegradation of several azo compounds including different types of azo dyes **(Maier *et al.,* 2004)**.

c. Peroxidase/Catalase test

Gas bubble formation upon addition of H_2O_2 in the liquid broth cultures indicates presence of peroxidase enzymes. During present investigation, only one nitrogen fixing bacterial isolate (PMB1) and two wood rot fungal isolates (PMF2 and WFPN1) showed qualitatively the peroxidase activity. This is an agreement with the study of **Goszczynski *et al.* (1994),** which also shows the involvement of extracellular peroxidase enzymes in the degradation of several azo dyes.

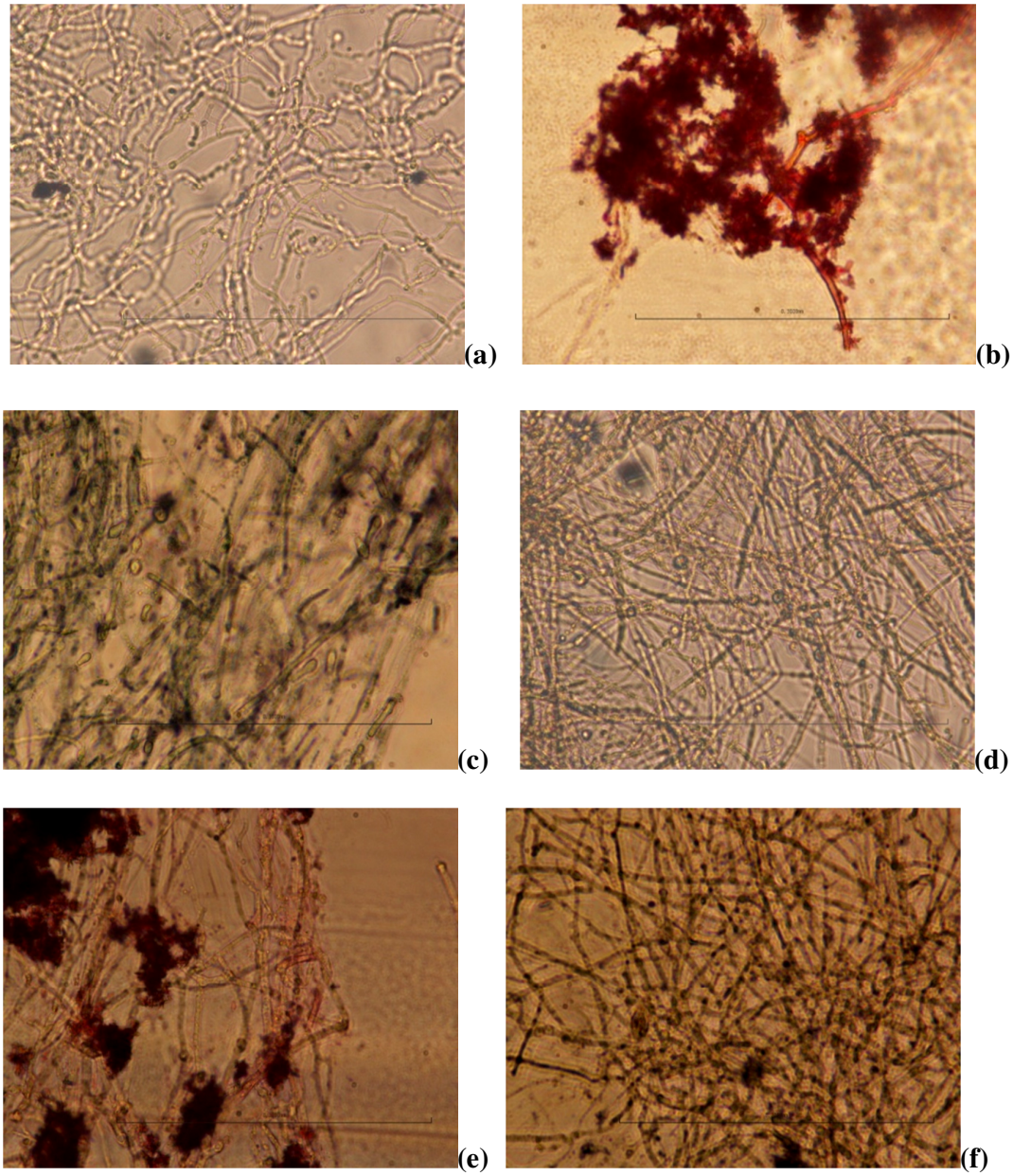
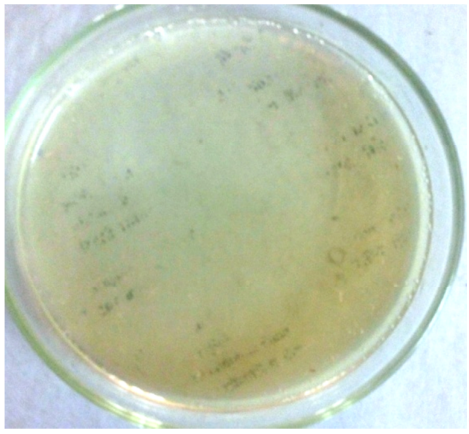
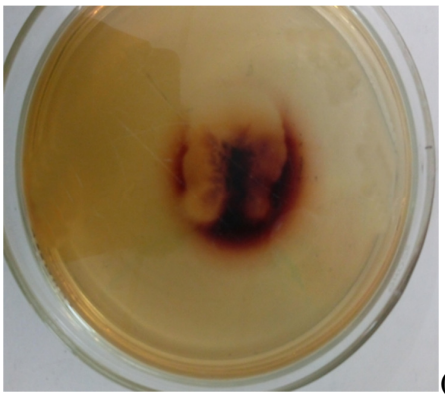


Plate 4.3 Photomicrograph showing biosorption in the fungal mycelia: (a) Fungal isolate PMF2 control; (b) Congo red absorbed PMF2; (c) Direct black absorbed PMF2; (d) Fungal isolate WFPN1 control; (e) Congo red absorbed WFPN1; (f) Direct black absorbed WFPN1.



(a)



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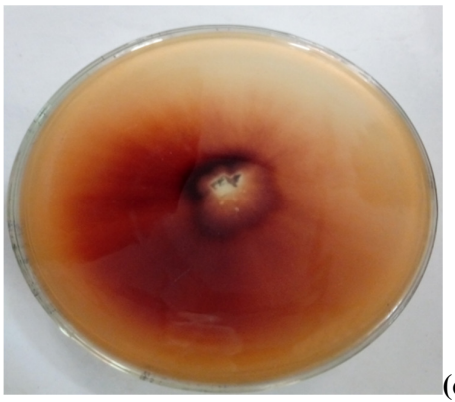

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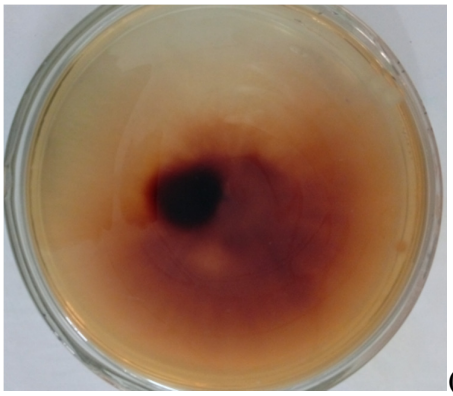

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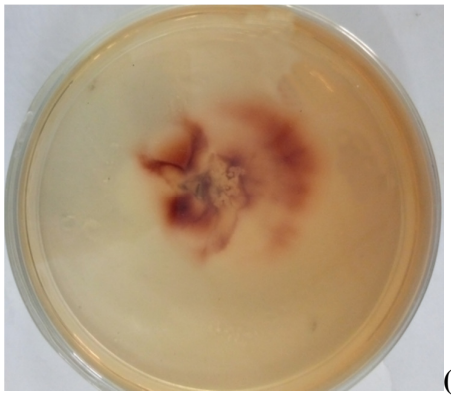


(e)

Plate 4.4 Laccase productions by various wood rot fungal isolates: (a) Control; (b) WFPN1; (c) PMF2; (d) PMF3; (e) WDP2

4.4.2 Extracellular enzyme activities: Quantitative measure

There are many reports indicating enzymatic mineralization and efficient degradation of synthetic dyes by bacteria **(Zabin *et al.,* 2011)**; wood rot fungi **(Robinson *et al.,* 2001)**; and algae **(Jinqi and Houtian, 1992)** using certain extracellular lignolytic oxidoreductase enzymes such as laccase, Lignin peroxidase (LiP), Manganese peroxidase (MnP), polyphenol oxidases. Certain membrane bound enzymes, e.g. azoreductases that are variably expressed by microorganisms have also been reported in the mineralization or degradation process for several azo compounds inclucind azo dyes. Dye mineralization process may involve either some of the constitutively (naturally) synthesized enzymes or the enzymes that are specifically induced only in the presence of some substrate such as dye **(Godhake *et al.,* 2009)**. Therefore, in order to explore the enzymatic process for dye mineralization, the selected bacterial (PMB1), fungal (PMF2, WFPN1 and *T. harzianum*) isolates were further examined for the synthesis of the oxidoreductase extracellular enzymes (Laccase, LiP, MnP and Azoreductase) in the presence or absence of dyes. During quantitative estimation the selected bacterial isolate (PMB1) did not show any laccase, LiP, MnP and azoreductase enzyme activities. Similarly almost negligible/ or no MnP and Lip activities could be recorded for all the three selected fungal isolates **(Table 4.6 and Table 4.7)**. It is clearly evident from the results that in the absence of any of the two azo dyes (congo red, direct black) no enzyme activity was observed in any case, while in presence of congo red or direct black very little MnP or Lip enzyme activity was recorded only for the two wood rot fungal isolates (PMF2 and WFPN1) while no peroxidase activity (MnP/LiP) could be recorded up to 12 d in the culture filtrate of *T. harzianum*. The isolate WFPN1 showed slightly higher enzyme activity varying from 0.0003-0.007 U/mL within 8-12 d. Thus it may be concluded from the results that peroxidase enzymes have almost negligible role in the azo dye degradation, although the enzyme was slightly induced by the two dyes. Moreover peroxidases of the test microbial cultures are not the constitutive enzyme as they are not synthesized in the normal medium without dye. However the inducible nature of the peroxidases indicates that the activity may be further enhanced for the two test cultures (PMF2 and WFPN1) by optimizing the culture condition. Some of the earlier workers have reported peroxidase activity for certain microbial isolates but during

present investigation the test culture were not able to synthesize peroxidase enzymes; it might be due to less or no involvement of these enzyme in dye mineralization process showing strain/genotypic variability.

Extracellular azoreductase activity was measured in the culture filtrates of the four selected microbial cultures in the mineral salt medium either amended with congo red or direct black dye or without any dye up to 12 d fermentation period **(Table 4.8)**. No enzyme activity could be recorded in any case in the absence of any dye even when incubated up to 12 d period.

In the presence of both the azo dyes, no activity was recorded at 0 d i.e. at the time of inoculation. The bacterial isolates PMB1 showed no azoreductase activity even in the presence of congo red dye up to 12 d period, while a little enzyme activity (1.15 U/mL) was recorded in the presence of direct black at 4 d period. The isolate PMF2 showed no azoreductase activity in the absence of dye while a varied enzyme activity ranging from 0.1-0.3 U/mL for congo red and 0.2 – 0.8 U/mL activity in presence of direct black. The standard culture of *T. harzianum* did not show any azoreductase activity in presence or absence of any of the two azo dyes. In case of the fungal isolate WFPN1, the azoreductase activity varied from 0.6 U/mL at 4 d to 14.91 U/mL at 8 d in presence of congo red, while the azoreductase activities in presence of direct blak dye for the isolate WFPN1 varied form 0.15-20 U/mL but showing no activity in the absence of azo dye. It may be concluded from the results that the azoreductase enzyme of the three microbial cultures (PMB1, PMF2 and WFPN1) is an inducible enzyme that is not synthesized at all in the absence of dye. *T. harzianum* was unable to synthesized azoreductase enzyme as no activity could be recorded in any case. The results reveal that based on azo dye induced synthesis of azoreductase enzyme, the test microbial isolates may be kept in following order WFPN1>PMF2>PMB1.

T. harzianum does not involve azoreductase in its dye removing process while the isolate WFPN1 was found most potential isolates showing maximum (20 U/mL and 14.91 U/mL) azoreductase activities in presence of congo red and direct black dyes respectively. Higher azoreductase activity during azo dye mineralization by white rot fungi as observed by us has also been reported by previous workers.

The potential fungal isolates (PMF2 and WFPN1) showed varied enzyme expression capabilities in response to different azo dyes. Extracellular laccase activity

was measured in the culture filtrates of the four test microbial isolates (PMG1, PMF2, WFPN1 and *T. harzianum*) in presence or absence of the two selected azo dyes up to 12 d fermentation period **(Table 4.9)**. The bacterial isolate PMB1 and fungal culture *T. harzianum* did not show any extracellular laccase activity in culture filtrates in presence or absence of azo dye. The results show clearly that in the absence of dye, both the fungal isolates showed laccase activity 19.45 U/mL (PMF2) and 25.96 U/mL (WFPN1) at 4 d incubation period after that the activity in both the cases decreased gradually up to 12 d. In the case of direct black dye, the isolate PMF2 showed the laccase activity varied from 46.78 to 101.74 U/mL and maximum activity was recorded at 8 d, while in case of isolate WFPN1, maximum laccase activity (64 U/mL) was recorded at 4 d thereafter it decreased gradually attaining minimum activity (2.1 U/mL) at 12 d. while for congo red dye, maximum laccase activity was recorded for WFPN1 (30 U/mL) followed by PMF2 (25.61 U/mL).

It is evident from the results that among the enzymes studied, the isolates (WFPN1 and PMF2) showed maximum expression of laccase enzyme followed by that of azoreductase while almost negligible peroxidase expression. It may be concluded that PMF2 is the most potential laccase producer showing maximum laccase activity (101.74 U/mL) followed by WFPN1 showing maximum laccase activity (64 U/mL). Direct black appears to have more laccase inducing capacity as compared to that of congo red dye. It is also evident from the results that the extracellular laccase enzymes from the two potential laccase producing fungal isolates (PMF2 and WFPN1) are constitutive enzymes that are being expressed in the absence of the dye also but there expression is further induced in the presence of azo dyes.

Based on these results it may be concluded that laccase is a constitutively expressed enzyme while azoreductase is induced only in the presence of dye. **Gorska *et al.* (2014)** also reported the production of laccase enzyme by white rot fungi, which were also found in present study. **Murugesan *et al.* (2006)** also shows the decolorization of reactive dyes and similar study was also carried out by **Kaur *et al.* (2015)** for synthetic dye decolorization and laccase production using *Cordycep militaris* MTCC 3936.

Table 4.6 Extracellular lignin peroxidase (LiP) activities in the culture filtrates of selected fungal/bacterial isolates in dye amended mineral salt medium

S.No.	Isolates	Extracellular LiP activity (U/mL) in presence/absence of azo dyes at different time intervals											
		Without dye				In presence of dye							
						Congo red				Direct black			
		0 d	4 d	8 d	12 d	0 d	4 d	8 d	12 d	0 d	4 d	8 d	12 d
1	PMB1	0	0	0	0	0	0	0	0	0	0	0	0
2	PMF2	0	0	0	0	0	0.0001	0.009	0.0004	0	0.0004	0.001	0.0004
3	WFPN1	0	0	0	0	0	0.002	0.004	0.0009	0	0.002	0.0004	0.0001
4	*T. harzianum*	0	0	0	0	0	0	0	0	0	0	0	0
SEm±: a (culture)= 0.0009; b (incubation period)= 0.0005; c (a*b)= 0.001 CD (5%): a (culture)= 0.0006; b (incubation period)= 0.0004; c (a*b)= 0.0009													

Table 4.7 Extracellular manganese peroxidase (MnP) activities in the culture filtrates of selected fungal/bacterial isolates in dye amended mineral salt medium

S.No.	Isolates	Extracellular MnP activity (U/mL) in presence/absence of azo dyes at different time intervals											
		Without dye				In presence of dye							
						Congo red				Direct black			
		0 d	4 d	8 d	12 d	0 d	4 d	8 d	12 d	0 d	4 d	8 d	12 d
1	PMB1	0	0	0	0	0	0	0	0	0	0	0	0
2	PMF2	0	0	0	0	0	0.0008	0.0004	0.0001	0	0.0005	0.003	0.0008
3	WFPN1	0	0	0	0	0	0.003	0.007	0.0003	0	0.001	0.0007	0.0001
4	*T. harzianum*	0	0	0	0	0	0	0	0	0	0	0	0
SEm±: a (culture)= 0.0008; b (incubation period)= 0.0004; c (a*b)= 0.0014 CD (5%): a (culture)= 0.0007; b (incubation period)= 0.0005; c (a*b)= 0.0008													

Table 4.8 Extracellular azoreductase activities in the culture filtrates of selected fungal/bacterial isolates in dye amended mineral salt medium

S.No.	Isolates	Extracellular azoreductase activity (U/mL) in presence/absence of azo dyes at different time intervals											
		Without dye				In presence of dye							
						Congo red				Direct black			
		0 d	4 d	8 d	12 d	0 d	4 d	8 d	12 d	0 d	4 d	8 d	12 d
1	PMB1	0	0	0	0	0	0	0	0	0	1.15	0	0
2	PMF2	0	0	0	0	0	0.3	0.3	0.1	0	0.2	0.8	0
3	WFPN1	0	0	0	0	0	0.6	14.91	5.3	0	1.15	20	0.15
4	*T. harzianum*	0	0	0	0	0	0	0	0	0	0	0	0

SEm±: a (culture)= 0.003; b (incubation period)= 0.001; c (a*b)= 0.004
CD (5%): a (culture)= 0.009; b (incubation period)= 0.005; c (a*b)= 0.013

Table 4.9 Extracellular laccase activities in the culture filtrates of selected fungal/bacterial isolates in dye amended mineralsalt medium

S.No.	Isolates	Extracellular laccase activity (U/mL) in presence/absence of azo dyes at different time intervals (d)											
		Without dye				In presence of dye							
						Congo red				Direct black			
		0 d	4 d	8 d	12 d	0 d	4 d	8 d	12 d	0 d	4 d	8 d	12 d
1	PMB1	0	0	0	0	0	0	0	0	0	0	0	0
2	PMF2	0	19.45	11.13	3.18	0	25.61	3.26	0	0	46.98	101.74	46.78
3	WFPN1	0	25.96	13.25	3.36	0	30	1.48	0.348	0	64	17.47	2.1
4	*T. harzianum*	0	0	0	0	0	0	0	0	0	0	0	0

SEm±: a (culture)= 0.006; b (incubation period)= 0.007; c (a*b)= 0.01
CD (5%): a (culture)= 0.017; b (incubation period)= 0.021; c (a*b)= 0.031

4.5 Dye decolorization using microbial consortia

Most of the Synthetic dyes used widely in various industries are recalcitrant compounds with complex molecular structure. Recalcitrance of the dye increases with the complexicity of the dye molecules and its biodegradation through microbial systems varies with microbial group, moreover strain variability is also there due to differential gene expression. Therefore, dye degradation by a single culture becomes more and more difficult while microbial consortia comprising of two or more microbial cultures may be expected to have better biodegradable activity if the individual partners acts synergistically in the consortia. In view of this assumption 22 different microbial consortia were developed for the decolorization of tri-azo dye direct black and 15 consortia were developed for decolorization of more recalcitrant triaryl dye (brilliant green). For in vitro removal of direct black from broth culture, the consortia were developed using potential dye decolorizing fungal and bacterial isolates while for brilliant green dye, microbial consortia were developed using microalgae/ Cyanobacteria and only two potential wood rot fungal isolates (PMF2 and WDP2) in mineral salt medium amended with the dye and evaluated up tom the complete removal of the dye from culture filtrates.

As shown in **Table 4.10** 22 microbial consortia prepared for direct black removal were comprised of 10 potential Nitrogen fixing/non nitrogen fixing bacteria, wood rot fungi and a standard culture of *T. harzianum*. These isolates in their mono-cultures showed 39.3% to 46.9% removal of direct black dye in 24h while their consortia were found to have higher dye decolorizing potential showing 39.6% to 76.99 % removal of direct black with in 24 h. in monocultures as well as in consortia the dye decolorizing capability was increased gradually with incubation period. In case of mono-cultutes complete dye removal (100%) was achieved with three fungal cultures (PMF2, WFPN1 and *T. harzianum*) in 6 d. while decolorization results with consortia indicated 100% or complete dye removal in 4 d by 5 different consortia (consortia IV, consortia XI, consortia XII, conasortia XVII and consortia XX), the values being significantly higher than those for rest of the consortia. Thus it is apparent from the results that the microbial consortia developed for direct black decolorization showed an enhanced rate of dye removal (100%

removal in 4 d) as compared to their respective mono-cultures, where complete dye removal was achieved in longer time (6 d). Thus the developed microbial consortia were found to have a rapid dye decolorization potential indicating the synergistic action of its microbial components. **Saroj *et al.* (2015)** have been reported that mixed co-culture of fungi shows efficient decolorization of azo dyes. Some previous workers also reported the dye decolorization by microbial consortia up to 85% **(Mahmood *et al.,* 2015)**.

For removal of triaryl dye (brilliant green) from the culture filtrates seven microbial cultures: three microalgae (PASM, PHSM and PRSM), one Cyanobacteria (*Synechococcus* sp.) and two wood rot fungal isolates (PMF2 and WDP2) showing higher potential for dye removal in mono-cultures was selected for developing microbial consortia. % dye decolorization of brilliant green by the 15 developed microbial consortia varied from 72.4% to 91.8% in 24 h while the component isolates in their mono-cultures could remove only 55.7% to 66% brilliant green dye from the culture filtrate in the same period (24 h). In all the cases (mono-cultures/co-cultures) % dye removal increased gradually with increasing incubation period attaining 100% dye removal by cyanobacteria and microalgae isolates (PASM,PHSM and PRSM) in 4 d while two microbial consortia (Consortia IX and XI) showed 100% removal of the dye in 2 d only **(Table 4.11)**. It is apparent from the data that the microbial consortia (consortia IX and XI) comprising of *Synechococcus* sp. and microalgae isolate showed significantly higher dye removing capacity as compared to other microbial consortia as well as their mono-cultures. It is apparent from the data that microalgae (PASM / PHSM) and Cyanobacteria (*Synechococcus* sp.) acted synergistically in the co-cultures reducing the time require for complete removal of the dye in within 2 d. Earlier workers have also been reported the efficient dye decolorization by consortia, they have also reported that microbial consortia were more resistant to several environment factors e.g. temperature, pH, salinity etc **(Lade *et al.,* 2015)**. The consortia of Cyanobacteria and microalgae have also been reported in the detoxification of waste water **(Subashchandrabose *et al.,* 2011)**.

Table 4.10 % dye decolorization on direct black dye by various consortia in different time interval

Microbial consortia	Consortia members	Decolorization (%) of direct black dye				
		0d	1d	2d	3d	4d
I	PMF2 + PMB1	0	74.19	74.64	86.64	89.57
II	PMF3 + PMB1	0	67.45	79.12	87.88	88.35
III	WFPN1 + PMB1	0	74.64	74.19	88.89	90.59
IV	*T. harzianum* + PMB1	0	73.51	73.06	90.24	100.00*
V	PMF2 + PMB4	0	64.42	66.22	76.99	82.35
VI	PMfF-3 + PMB4	0	63.52	65.77	81.14	84.69
VII	WFPN1 + PMB4	0	68.01	69.36	81.82	87.49
VIII	*T. harzianum* + PMB4	0	55.22	68.69	81.14	86.34
IX	PMF2 + PMB1 + PMF3	0	76.99	77.67	90.01	95.37
X	PMF2 + PMF3 + PMB4	0	70.15	70.59	84.40	89.99
XI	PMF2 + PMF3 + WFPN1 + *T. harzianum*	0	49.05	53.76	89.00	100.00*
XII	PMF2 + PMF3 + WFPN1 + *T. harzianum* + PMB1	0	72.39	74.64	89.79	100.00*
XIII	PMF2 + PMF3 + WFPN1 + *T. harzianum* + PMB4	0	68.13	69.36	85.30	87.21
XIV	PMF1 + PMF2	0	61.23	75.94	80.50	89.68
XV	PMF1 + PMF2 + PMF3	0	62.35	79.22	90.09	92.72
XVI	PMF2 + WDP2	0	59.79	68.75	80.26	86.01
XVII	PMF1 + PMF2 + PMF3 + WFPN1	0	65.15	78.34	92.89	100.00*
XVIII	PMF2 + PMF3	0	62.83	73.54	77.06	86.97
XIX	PMF2 + WFPN1	0	40.4	50.1	71.4	99.65
XX	PMF2 + *T. harzianum*	0	39.6	58.5	80.6	100.00*
XXI	WFPN1 + *T. harzianum*	0	43.9	68.8	80.5	98.9
XXII	PMfF-3 + *T. harzianum*	0	42.6	49.1	56.7	70.5
SEm±: a (culture)= 0.052; b (incubation period)= 0.022; c (a*b)= 0.105 CD (5%): a (culture)= 0.14; b (incubation period)= 0.062; c (a*b)= 0.294						

Table 4.11 % dye decolorization of brilliant green dye by various consortia in different time interval

Microbial consortia	Consortia member	Decolorization (%) of Brilliant Green		
		0 d	1 d	2 d
I	PMF2 + *Synechococcus* sp.	0	78.7	81.2
II	PMF2 + PASM	0	85.6	88.8
III	PMF2 + PHSM	0	72.4	81.8
IV	PMF2 + PRSM	0	86.2	89.9
V	WDP2 + *Synechococcus* sp.	0	84.55	85.8
VI	WDP2 + PASM	0	84.6	87.2
VII	WDP2 + PHSM	0	84.2	85.6
VIII	WDP2 + PRSM	0	91	91.9
IX	*Synechococcus* sp. + PASM	0	90.1	100*
X	*Synechococcus* sp. + PRSM	0	87.7	90.7
XI	*Synechococcus* sp. + PHSM	0	91.8	100*
XII	PMF2 + *Synechococcus* sp. + WDP2	0	84.4	89.3
XIII	PMF2 + *Synechococcus* sp. + WDP2 + PASM	0	83.4	87.1
XIV	PMF2 + *Synechococcus* sp. + WDP2 + PHSM	0	83.4	85
XV	PMF2 + *Synechococcus* sp. + WDP2 + PRSM	0	74.3	84.4

SEm±: a (culture)= 0.013; b (incubation period)= 0.0048; c (a*b)= 0.018

CD (5%): a (culture)= 0.37; b (incubation period)= 0.013; c (a*b)= 0.052

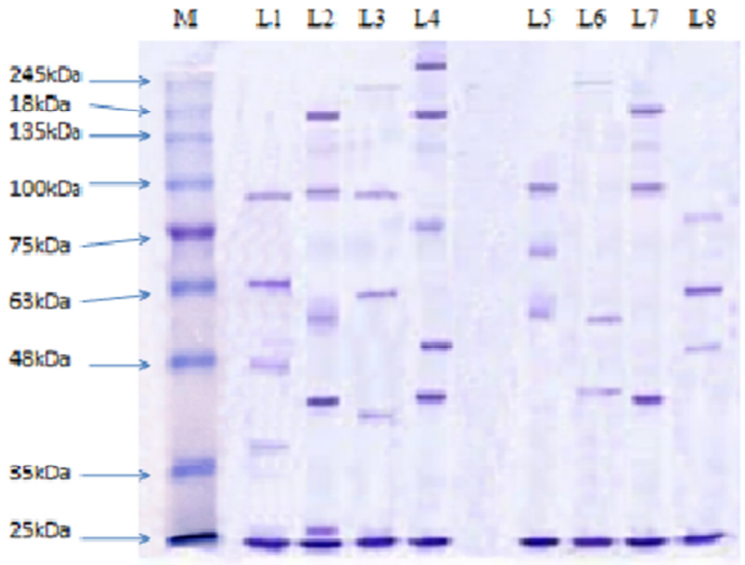
4.6 Extracellular protein profiling

4.6.1 Native-Poly Acrylamide Gel Electrophoresis (PAGE)

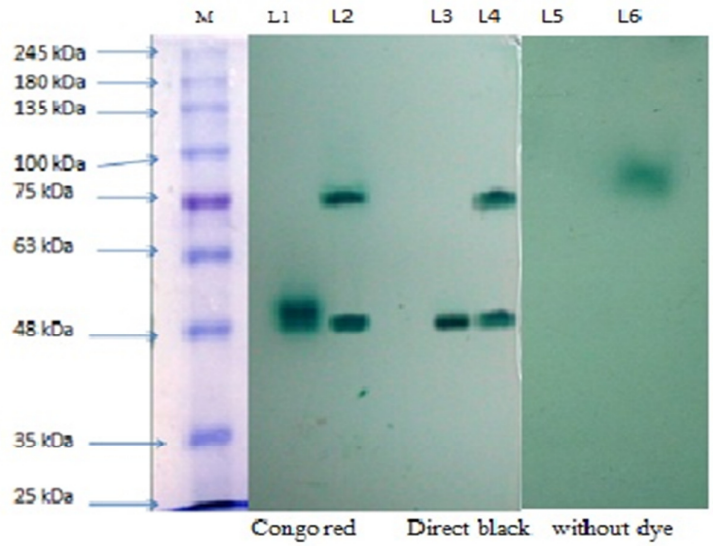
Extracellular protein profiling was done to identify the extracellular proteins synthesized in presence/absence of dye by the selected isolates and their molecular weights. Three efficient microbial cultures (bacterial isolate –PMB1, two fungal isolates -WFPN1 and PMF2 and one Cyanobacteria -*Synechococcus* sp.) grown in mineral salt medium with / without the selected dyes direct black or brilliant green dye were selected for comparative evaluation of the extracellular proteins induced in the presence of any one of the two dyes. The extracellular enzymes were partially purified using acetone precipitation method **(Nejadi *et al.,* 2014)**. The molecular weight of various extracellular proteins was found in the range of 25-245 kDa **(Plate 4.5a)**. The extracellular protein profiling showed different protein bands in the native-gel, which indicated the presence of extracellular proteins. Maximum bands were found in the presence of direct black in case of fungal and bacterial cultures while in case of Cyanobacteria maximum bands were observed in presence of brilliant green dye as compared to the bands observed in absence of dye in either case **(Plate 4.5a, Lane L1 – L8)**. Maximum numbers of bands (5) were found in case of bacterial isolate PMB1 and fungal isolate PMF2, in the presence of direct black dye **(Plate 4.5a, Lane L2, L4)**. This shows that in stress condition (presence of dye), more extracellular proteins are produced. The results revealed clearly that some of the bands were common in presence or absence of dye indicating their constitutive synthesis by the culture. Contrary to it few additional protein bands visualized in the presence of synthetic dye indicates that the synthesis of these proteins is induced only in the presence of dye. It appears that these dye induced proteins might be involved in their mineralization process by the microorganisms.

4.6.2 Laccase isoform analysis

Among the four extracellular oxidase and oxidoreductase enzymes studied during enzyme quantification, maximum activities were recorded for laccase enzyme in dye containing medium indicating its possible involvement in the biodegradation process. Therefore, laccase enzyme from the two fungal isolates (WFPN1and PMF2) showing maximum laccase activity was functionally characterized. Zymogram analysis was done under non denaturing conditions (Native PAGE) through activity staining to



(a)



(b)

Plate 4.5 **(a) Extracellular protein profiling through Native-PAGE; Lane L1 (WFPN1), Lane L2 (PMF2), Lane L3 (*Synechococcus* sp.), Lane L4 (PMB1): in presence of dye; Lane L5 (WFPN1), Lane L6 (PMF2), Lane L7 (*Synechococcus* sp.), Lane L8(PMB1) : in absence of dye; Lane (M) marker.**

(b) Zymogram analysis of extracellular laccase enzyme produced by selected fungal isolates. Lane (M) protein marker; Lane L1, L3 (PMF2); Lane L2, L4 (WFPN1) in presence of dye; and Lane L5 (PMF2), Lane L6 (WFPN1) in absence of dye.

identify different isoforms of the laccase enzyme, if produced by the selected fungal isolates in the presence or absence of two azo dyes (congo red and direct black). The laccase isoforms were identified for their molecular weight using pre stained molecular weight marker **(Plate 4.5b)**. Zymogram analysis revealed two laccase isoforms with molecular weight ~50 kDa and ~75 kDa, synthesized by the isolate WFPN1 in the presence of both the azo dyes **(Plate 4.5b Lane L2 and L4)**. However, in the dye free medium, only one laccase band (~75 kDa) was visualized **(Plate 4.3b Lane L6)**. The results indicate clearly that one lacccase isoform (~75kDa) from WFPN1 was constitutively expressed in the medium irrespective of presence / absence of dye while the other isoform with molecular weight ~50kDa was induced by the azo dyes. For the other fungal isolate PMF2, zymogram study showed only one laccase isoform (~50 kDa) in presence of both the azo dyes **(Plate 4.5b Lane L1 and L3)**. While in dye free medium, no laccase band was observed **(Plate 4.5b Lane L4)**, which showed the inducible nature of laccase enzyme from the fungal isolate PMF2. The results of laccase zymogram analysis are also in accordance with the enzyme (laccase) activities. Earlier workers have also identified the laccase activity bands with molecular weight ranging from 50-100 kDa by native- PAGE. Several workers have also reported the lignolytic wood rot fungi as potential laccase producer and involvement of their laccase enzyme in dye decolorization process as was also found during the present investigation **(Imran *et al.,* 2012)**. A novel laccase enzyme from the white rot fungi has also been reported recently **(Zheng *et al.,* 2017)**.

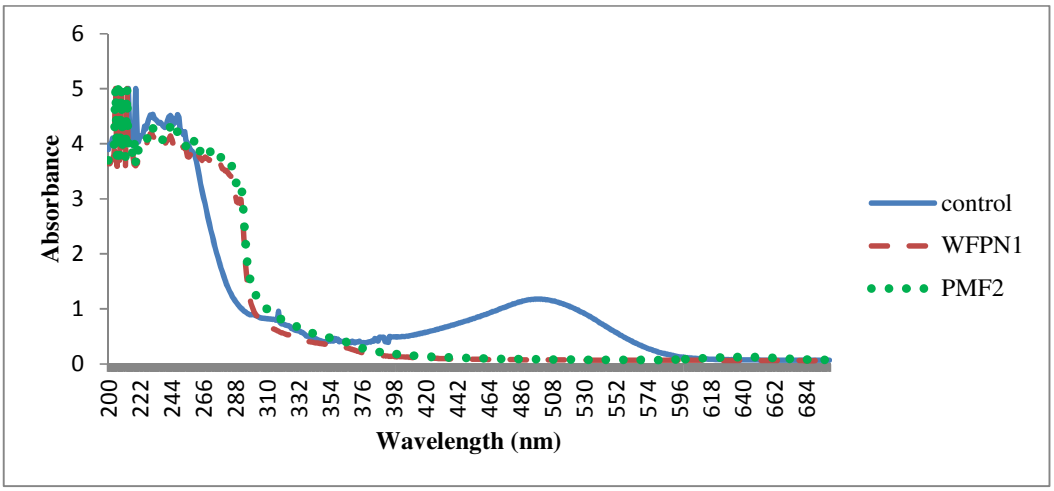
4.7 Monitoring of dye degradation

Microbial cultures have been reported to decolorize or remove the colored synthetic dyes from culture media either by non enzymatic mechanism, i.e. biosorption or by enzymatic mineralization of the original dye molecules into colorless products or by both the mechanisms. Therefore to investigate the possible mineralization mechanism for synthetic dyes and its removal from the broth cultures by the potential fungal isolates (WFPN1 and PMF2) and Cyanobacteria (*Synechococcus* sp.), spectral analysis of the cell free decolorized samples (supernatant) was carried out using UV–Visible spectrophotometer (Thermo) at different wavelengths ranging from 200-700 nm and FT-IR spectroscopy at 400-4000 cm^{-1}.

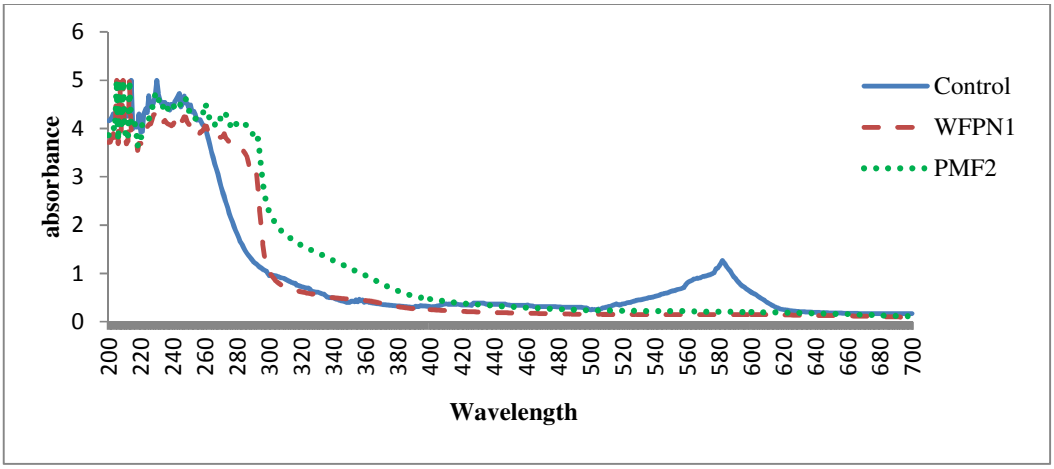
4.7.1 UV-Visible spectral analysis

UV-Visible spectroscopy was used to measure interaction of waves and matter. In UV-Visble spectroscopy, the changes in electronic energy levels within the molecules arising due to electron transfer are detected **(Schmid, 2001)**. It commonly provides the knowledge about conjugated unsaturations, aromatic compounds and conjugated non-bonding electron systems etc. In UV-Vis spectra of the untreated (control) congo red dye sample, a sharp peak was observed near visible region at wavelength of 497nm, which confirmed the λ_{max} of congo red dye at 497-498 nm. The CR peaks observed at 498 nm in the visible region were attributed to azo bonds, N=N **(Hua *et al.,* 2013)**. For untreated (control) direct black dye, a sharp peak was observed at wavelength of 580 nm which is the λ_{max} of direct black. The UV-Vis spectra of the fungal (WFPN1 and PMF2) treated decolorized samples showed a drastic decline in the intensities of the major peaks for the two dyes as compared to their respective untreated control samples in six d **(Fig 4.3a, b)**. The λ-scanning of both the treated (decolorized) dye samples revealed disappearance of the main peaks for both the dyes at their λ_{max} (497 nm and 580nm respectively) that was present as a prominent peak in the untreated control samples. In case of brilliant green dye, the spectra of untreated (control) dye sample showed a sharp peak near visible region at wavelength of 630nm (λ_{max} of brilliant green). While disappearance of the main peak for treated brilliant green dye samples was visualized in the cyanobacteria (*Synechococcus* sp.) treated decolorized samples **(Fig 4.3c).**

This indicates the successful removal of both the azo dyes from the fungal treated samples. **Li *et al.* (2015)** have reported the biodegradation of congo red dye by a bacterial culture *Acinetobacter baumannii* that removed up to 98% of the dye as was analysed through UV-Vis spectrophotometer. They have also reported the azo bond peak of congo red at 498 nm in the control sample and gradual decline in the intensity of peak **(Saratale *et al.,* 2009)**.



(a)



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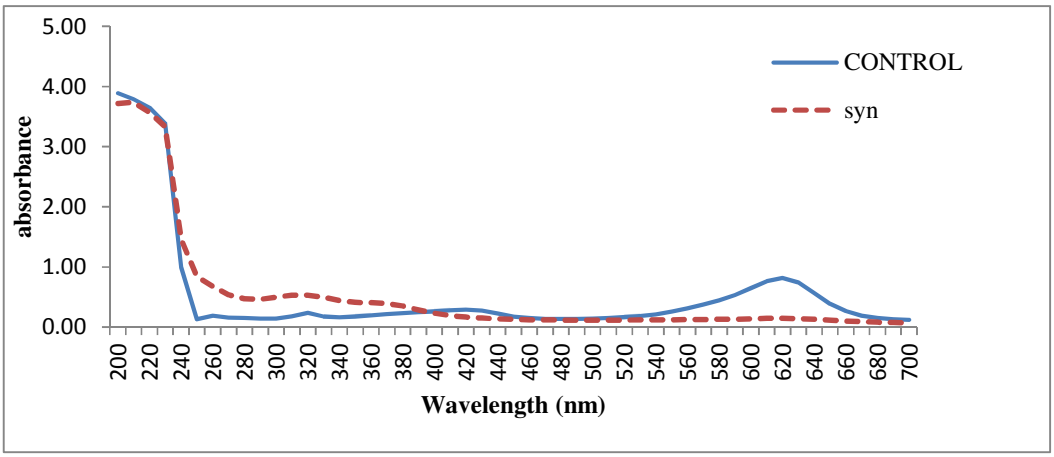


(c)

Fig 4.3 UV-Vis spectra showing decrement in the peak by selected isolates (WFPN1, PMF2 and Syn) in congo red (a), direct black (b) and brilliant green (c).

4.7.2 FTIR Analysis

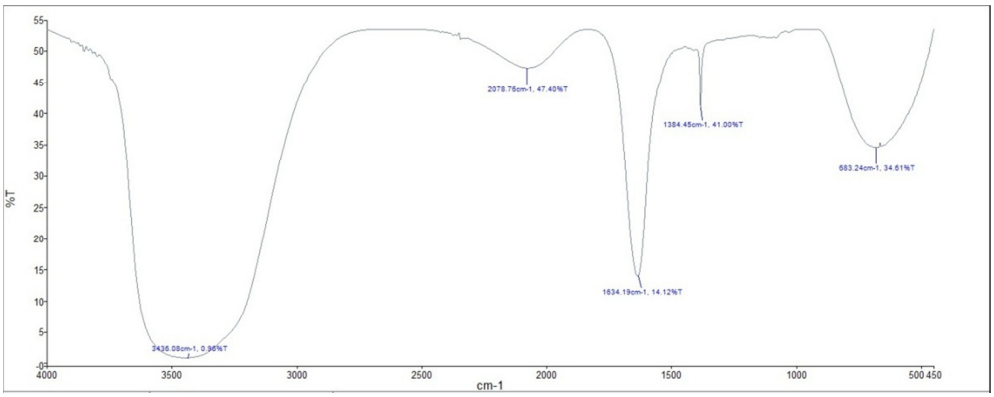
FTIR spectral-analysis is one of the major techniques used to investigate the biodegradable products of some noxious chemical compounds using whole cell microbial catalysts or enzymatic preparations. In the present study, therefore, FTIR analysis was used to determine the structural changes in the dye before and after the microbial treatment. For FTIR spectroscopy two fungal cultures (WFPN1 and PMF2) and one cyanobacteria (*Synechococcus* sp.) were selected due to their high dye decolorizing potential. The peaks in the IR spectrum of untreated congo red dye sample represented the stretching vibrations of N–H bond (secondary amides) at 3436.08 cm^{-1}, C–H stretching of alkanes at 2078.76 cm^{-1}, N=N stretching of azo compounds at 1634.19 cm^{-1}, N–H trans stretching of secondary amides at 1384.85 cm^{-1} and C–H out of plane bending at 683.24 cm^{-1} **(Fig. 4.4a) (Li *et al.,* 2015)**. The FTIR spectra of fungal treated dye samples showed a remarkable decrease in the peak intensity as is evident by narrowing of the peaks also. Several small fragments of peaks were also visualized that indicated clearly, mineralization of the synthetic dyes. The FTIR spectrum of WFPN1 treated congo red dye sample represented shift of the peaks and decreases in the intensities of peaks for O-H stretching at 3732.13 cm^{-1} for the hydroxylation of the product, stretching vibration of N-H bond at 3440.72 cm^{-1}, C-H stretching of alkanes at 2922.65 cm^{-1} and 2846.12 cm^{-1}, N=N stretching at 1642.19 cm^{-1}, stretching vibration of C=N at 1559.77 cm^{-1}, stretching vibration of benzene at 1512.67 cm^{-1}, C-O stretching vibration at 1386.10 cm^{-1} and 1109.40 cm^{-1} and the peak of C-C-C out of plane deformation at 540.04 cm^{-1} and 406.65 cm^{-1} **(Fig. 4.4b)**. The same results were also found in case of PMF2 treated dye sample, the change in peak intensity was visualized clearly during spectroscopy. The spectra represented the change and shift in the peaks of stretching vibrations of N–H bond (secondary amides) at 3454.00 cm^{-1}, C–H stretching of alkanes at 2089.47 cm^{-1}, N=N stretching at 1636.76 cm^{-1}, C-O stretching vibration at 1384.20 cm^{-1} and C-H out of plane bending at 621.32 cm^{-1} **(Fig 4.4c)**.

Besides this, for untreated direct black dye the IR peaks were found as the stretching vibrations of N–H bond (secondary amides) at 3436.46 cm^{-1}, N=N stretching of azo compounds at 1632.85 cm^{-1}, N–H trans stretching of secondary amides at

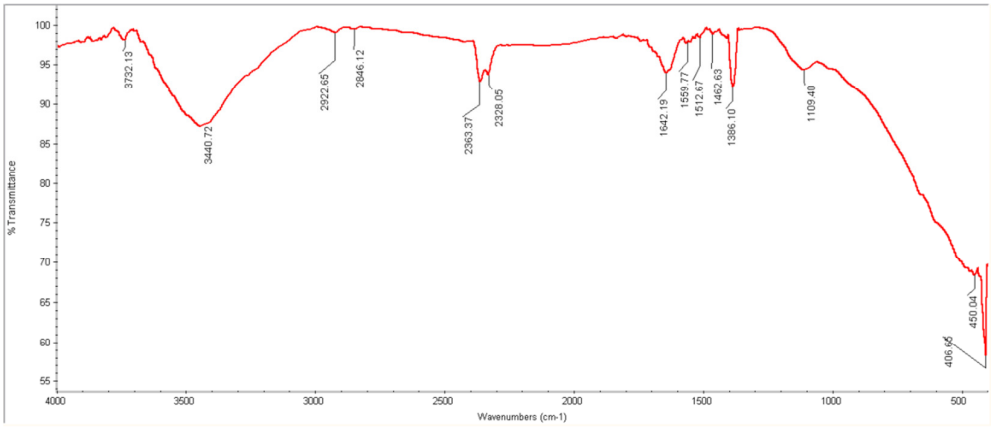
1384.32 cm^{-1} and C–H out of plane bending at 616.75 cm^{-1} **(Fig. 4.5a)**. The FTIR spectrum of WFPN1 treated direct black dye sample represents the change and shift in peaks for stretching vibration of N-H bond at 3466.78 cm^{-1}, N-H stretching at 2363.37 and 2328.05 cm^{-1}, N=N stretching at 1639.66 cm^{-1}, C-O stretching vibration at 1383.16 cm^{-1}, vibrations of C-H at 602.79 cm^{-1} and the peak of C-C-C out of plane deformation at 432.38 cm-1 **(Fig. 4.5b)**. While in case of fungal isolate PMF2, the change and shift in peaks represent the stretching vibrations of N–H bond (secondary amides) at 3454.43 cm^{-1}, C–H stretching of alkanes at 2078.69 cm^{-1}, N=N stretching of azo compounds at 1637.46 cm^{-1}, N–H trans stretching of secondary amides at 1384.24 cm^{-1} and C–H out of plane bending at 653.73 cm^{-1} **(Fig. 4.5c)**.

In case of untreated brilliant green dye, the IR spectra represented stretching vibrations of N–H bond (secondary amides) at 3455.62 cm^{-1}, C–H stretching of alkanes at 2077.07 cm^{-1}, C=C stretching of alkenes at 1637.02 cm^{-1} and C-H out of plane bending at 686.87 cm^{-1} **(Fig 4.6a)**. While the *Synechococcus* sp. treated sample showed change and shift in the peaks as compared to the untreated control. The spectra represented stretching vibrations of N–H bond (secondary amides) at 3463.63 cm^{-1}, C–H stretching of alkanes at 2092.91 cm^{-1}, C=C stretching of alkenes at 1638.58 cm^{-1}, C-O stretching at 1108.53 cm^{-1} and C-H out of plane bending at 621.71 cm^{-1} **(Fig 4.6b)**.

Increase in % transmittance of all the peaks in both the fungal treated dye samples in comparison to their respective untreated control samples was recorded. This increased transmittance shows disappearance/removal of colored dye molecules from the treated sample; at the same time appearance of new peaks in the treated samples might be correlated with the mineralization of the dye into some other colorless mineralized products. Several workers have reported FTIR as an efficient analytical technique to monitor the degradation/transformation of dye by microbial cultures **(Saratale *et al.*, 2009)**. Many previous workers have also done FTIR analysis of the fungal/bacterial treated dye samples to monitor the degradation/mineralization of the dye and reported the change and shift of the peaks in the spectra as was also observed during present investigation **(Srinivasan *et al.,* 2005, Kolekar *et al.,* 2008, Jadhav *et al.,* 2010)**.



(a)



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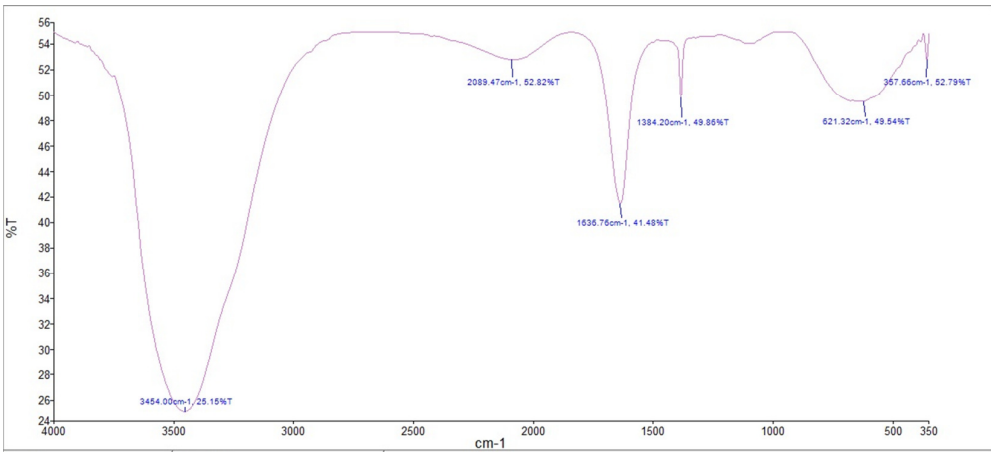
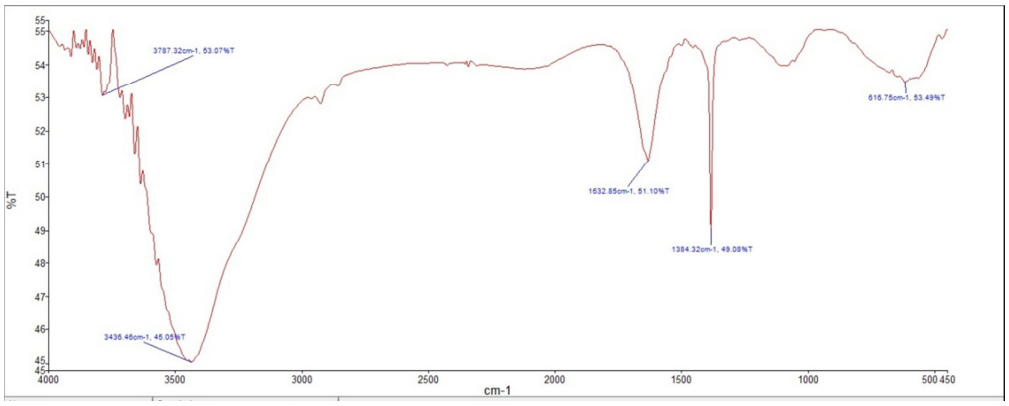
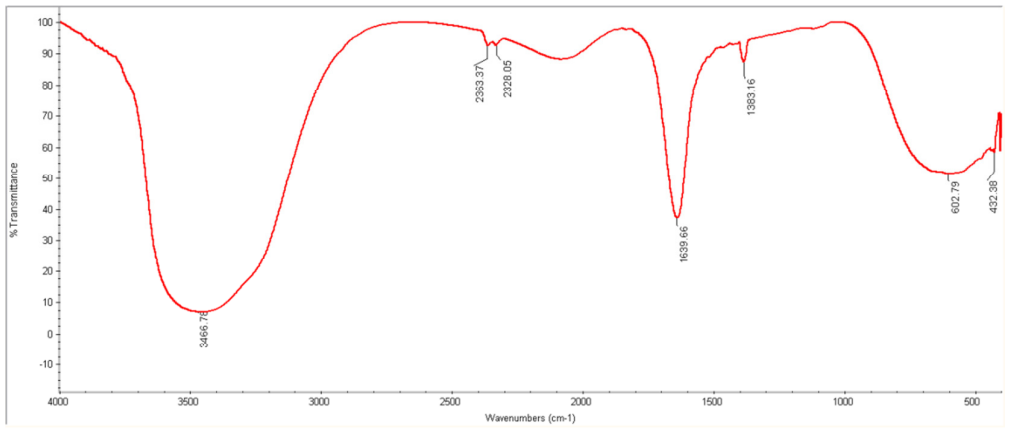


(c)

Fig 4.4 FTIR spectra showing biodegradation of congo red dye: (a) congo red control; (b) WFPN1 treated and (c) PMF2 treated decolorized samples.



(a)



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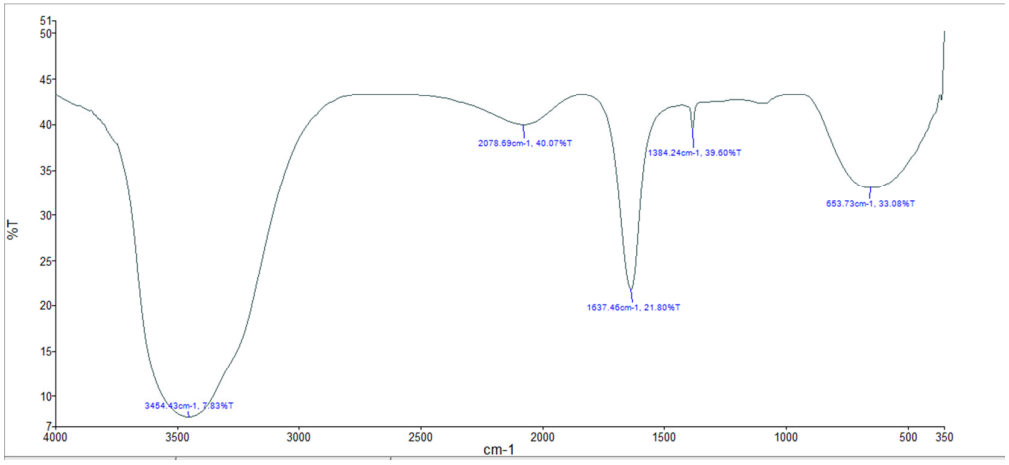
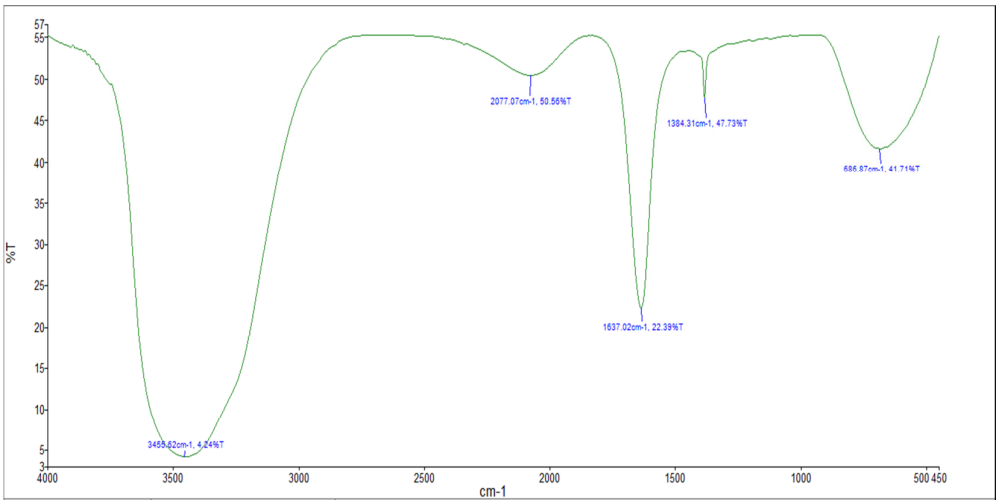
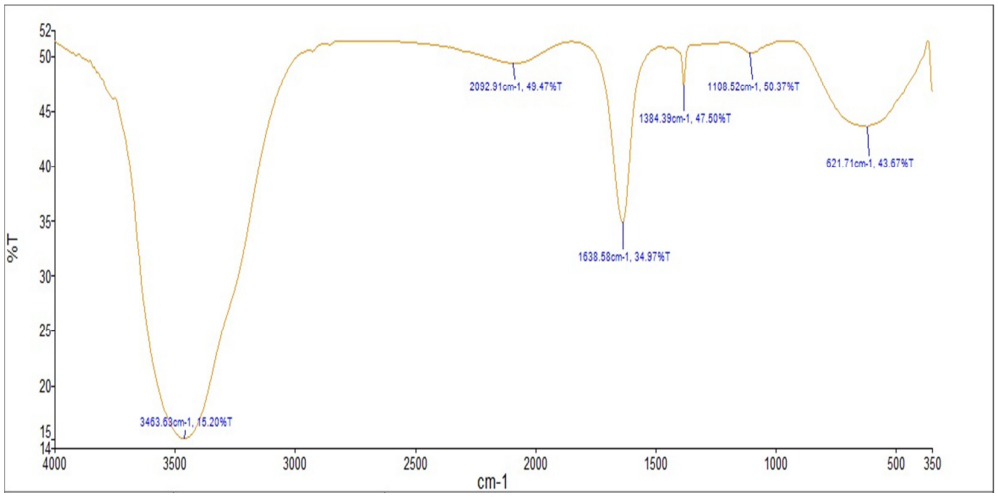


(c)

Fig 4.5 FTIR spectra showing biodegradation of direct black dye: (a) direct black control; (b) WFPN1 treated and (c) PMF2 treated decolorized samples.



(a)



(b)

Fig 4.6 FTIR spectra showing biodegradation of brilliant green dye: (a) brilliant green control; (b) *Synechococcus* sp. (*Synechococcus* sp.) treated decolorized sample.

4.8 Characterization of potential fungal isolate WFPN1

Maintenance of pure microbial cultures (quality control) throughout the process is very important for successful dye decolorization. To achieve it, the culture should be routinely checked and sub-cultured. Therefore, for quality measure, potential microbial cultures should be properly characterized using phenotypic, biochemical and molecular markers and identified using modern tools and techniques. Therefore the selected potential fungal isolate WFPN1 was characterized at phenotypic, biochemical and molecular levels using molecular markers.

4.8.1 Phenotypic characterization

The selected fungal fruiting bodies from their natural habitat were collected in airtight sterile poly-bags to minimize the chance of further contamination and moisture loss and brought to laboratory for further work. The fruiting body of the fungal isolate WFPN1 was kidney shaped and with dark brown stalk. On PDA plates, the isolate WFPN1 shows radial mycelial growth with typical creamy white, regular flat, smooth and filamentous colony **(Table 4.12)**. Our results are in agreement with those of previous finding that indicated most of the similar fruiting body and agar plate characterization of wood rotting fungi **(Westhuizen, 1958; Liu *et al.,* 2009)**. Based on the structure of fruiting body and mycelial growth on PDA, the WFPN1 isolate was preliminary characterized as *Ganoderma* sp. in consultation with the scientists at Mushroom Research Centre, G. B. Pant University of Agriculture and Technology, Pantnagar.

4.8.2 Molecular characterization and identification

The potential isolate WFPN1 was characterized based on 18S ITS r-RNA gene sequence analysis. The polymerase chain reaction (PCR) amplification and sequencing of partial 18S rRNA gene was carried out using the standard primer set ITS1 (forward, 5' TCCGTAGGTGAACCTGCGG3') and ITS4 (reverse, 5'TCCTCCGCTTATTGATATGC3'). The PCR amplicon was purified and sequenced through Yaazh Xenomics Pvt. Ltd. Chennei, India.

I. *In silico* Analysis

The 18S ITS rRNA gene sequence of the fungal isolate WFPN1 was analysed using basic local alignment search tool (BLAST) and gene sequence available in the

National Centre for Biotechnology Information (NCBI) database. Using BLAST search among 18S ITS rRNA gene sequence of potential wood rot laccase producing fungi (WFPN1) and other closely related fungal genera existing in database, percent identify were determined **(Table 4.13)**. It is apparent from the table that the wood rot fungal isolate WFPN1 belongs to the genus *Ganoderma*.

II. Phyllogenetic tree construction

For preparation of phyllogenetic tree to study the evolutionary relatedness of selected wood rot fungal isolate with other wood rot fungal cultures, the retrieved sequences from BLAST search were aligned using MEGA 7.0 bioinformatics tool. It is apparent from the phyllogenetic tree constructed for WFPN1 by neighbor joining method showed maximum identity (99%) with *Ganoderma lucidum* CAW-1 **(Fig 4.7)**. The program MEGA7.0 was used by previous workers for sequence alignment **(Kumar *et al.,* 2016)**. The wood rot fungi, producing morphologically similar fruiting bodies as observed in case of present fungal isolate WFPN1 were also found to belong to the genus *Ganoderma* sp. **(Hapuarachchi *et al.,* 2015)**.

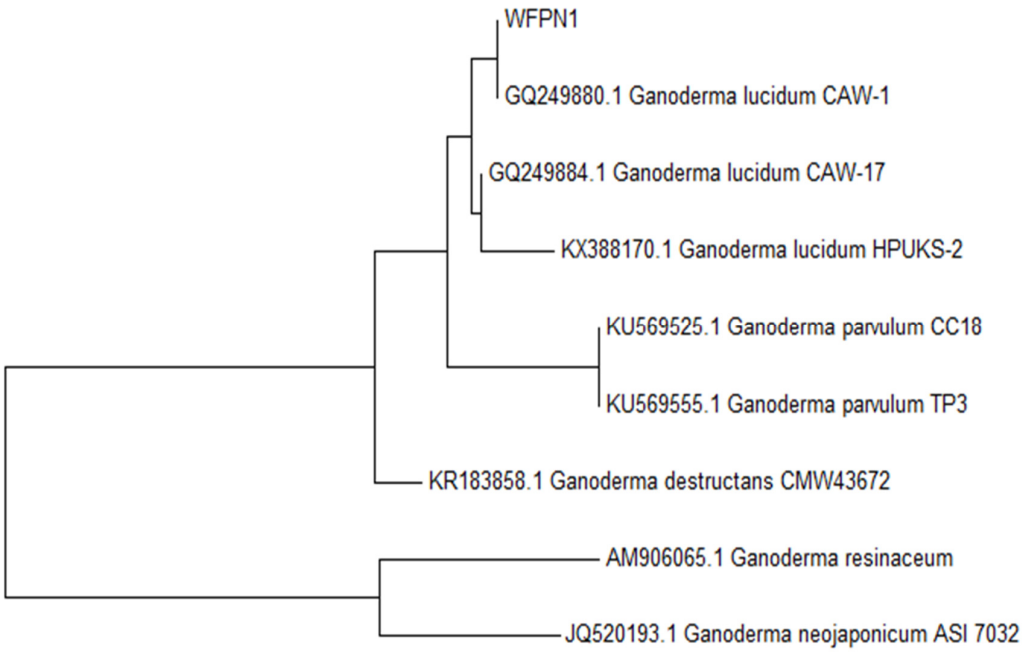


Fig 4.7 Neighbor joining tree based on 18S rRNA gene sequence showing the relationship between WFPN1 and other related type strains of *Ganoderma*.

Table 4.12 Phenotypic characteristics of potential wood rot fungal isolate WFPN1

S.No.	Characteristics	Fungal isolate WFPN1
I.	Fruiting body -Appearance	Dark brown, kidney shaped, stalked, non porous
II.	Colony Characteristics	
i.	Chromogenesis	Creamy white, radial growth
ii.	Shape	Rregular
iii.	Elevation	Flat
iv.	Surface	Smooth
v.	Form	Filamentous
vi.	Margin	Filiform
III.	Cellular Characterstics	
vii.	Mycelial structure	Compact mycelia with septate hyphae
viii.	Asexual spore formation during mycelial growth	-
ix.	Sexual spores	Basidiospores within fruiting body

Table 4.13 18S r-RNA gene sequence of wood rot fungal cultures retrieved from NCBI database showing identity (%) with that of selected fungal isolate WFPN1.

S. No.	Accession No.	Related Strains	Maximum Score	Total Score	Query Cover	E Value	Identity (%)
1	GQ249880.1	*Ganoderma lucidum* CAW-1	1190	1329	77	0.00	99%
2	GQ249884.1	*G. lucidum* CAW-17	1186	1315	74	0.00	98%
3	KX388170.1	*G. lucidum*	1164	1350	77	0.00	97%
4	JQ781874.1	*G. multipileum*	1149	1149	69	0.00	98%
5	KF494997.1	*G. multipileum* 9447	1149	1149	69	0.00	98%
6	LC149613.1	*G. multipileum*	1142	1142	69	0.00	98%
7	KU569555.1	*G. parvulum* TP3	1107	1107	68	0.00	98%
8	KU569525.1	*G. parvulum* CC18	1105	1105	67	0.00	98%
9	KR183858.1	*G. destructans* CMW43674	1098	1098	65	0.00	97%
10	JQ520193.1	*G. neojaponicum*	965	965	68	0.00	94%
11	AM906065	*G. resinaceum*	926	926	67	0.00	93%

Summary and Conclusions

Chapter 5 SUMMARY AND CONCLUSION

Various synthetic dyes used in many industries (textile, paper printing, pulp and paper, wool, tanning, etc.), photo electrochemical cells, hair coloring and cosmetics are highly toxic and carcinogenic in nature. Among all synthetic dyes, azo dyes are the most widely used industrial dyes, representing almost 70% of the textile dyestuff produced. Large scale production and extensive application of synthetic dyes can cause considerable environmental pollution and serious health hazards on human beings and animals. These dyeing industrial effluents when released in main water bodies have an adverse effect on the environment and all the living beings; moreover, some of their degradative byproducts (e.g. amines) can also be highly toxic, mutagenic, and decrease penetration of light and photosynthesis; hence their removal from the environment is urgently required. Considering these facts bio-remedial studies were conducted for selection of dye decolorizing microbial cultures as discussed below:

1. A survey was carried out on twenty eight microbial strains (17 bacteria, 7 fungi, 3 microalgae and 1 cyanobacteria), for *in-vitro* dye decolorization of synthetic dyes by plate assay using dye amended mineral salt agar medium. The cultures were evaluated for their *in-vitro* growth abilities and dye tolerance potentialities for the four synthetic dyes viz. congo red, brilliant green, direct black and direct green in dye containing agar medium. Six fungal cultures and three bacterial isolates was selected for the azo dye decolorization while three microalgae and one Cyanobacteria (*Synechococcus* sp.) cultures and 2 fungal isolates were selected for brilliant green dye for further investigation.

2. The selected cultures were then screened for quantification of dye decolorization in broth cultures using mineral salt medium. For bioremedial potential, the broth cultures were grown in the mineral salt medium containing glucose (0.1%) and supplemented with the test dye (100 mg/L) and selected on the basis of growth ability as well as decolorization capacity. The % decolorization of azo dyes by various microbial cultures varied from 87.5% - 100% in 5-6 d. Decolorization of brilliant green dye varied from 75.81% - 100 % in 4 d. The bacterial isolates PMB1 and PMFB4; fungal isolates PMF2, WFPN1 and *T. harzianum* showed 100% decolorization for congo red in 5 d incubation period. For direct green dye

all selected microbial isolates (Bacteria/fungi) showed 100% decolorization in 5 d incubation period. In case of direct black dye, 100% decolorization was shown by fungal isolates PMF2, WFPN1 and *T. harzianum*, followed by PMF3 (94.6%).The fungal cultures showed significantly higher % dye decolorization in comparison to bacterial isolates. In case of brilliant green dye, all the four microalgae / Cyanobacteria cultures showed 100% decolorization in 4 d, that is significantly higher than the decolorization of the same dye shown by the two selected fungal isolates PMF2 and WDP2.

4. The 10 selected microbial cultures showing decolorization of all the dyes were screened qualitatively for production of various extracellular oxidoreductase enzymes by plate assay. Only four fungal isolates (PMF2, PMF3, WFPN1 and WDP2) were able to produce reddish brown zone around colony indicating laccase production. Production of decolorized zone around colony on azo dye containing mineral medium showed synthesis of azoreductase enzyme for the bacterial isolates (PMB1, PSNB1 and PMFB4) and fungal isolates (PMF1, PMF2, PMF3, WFPN1 and WDP2) during qualitative plate assay. Only one nitrogen fixing bacterial isolate (PMB1) and two wood rot fungal isolates (PMF2 and WFPN1) showed peroxidase activity qualitatively.

5. The selected bacterial (PMB1) and fungal (PMF2, WFPN1 and *T. harzianum*) isolates were further examined spectrophotometrically for the synthesis of oxidoreductase extracellular enzymes (Laccase, LiP, MnP and azoreductase) in the presence or absence of dyes. During quantitative estimation the selected bacterial isolate PMB1 and the fungal culture (*T. harzianum*) did not show any laccase, LiP, MnP and azoreductase enzyme activities both in absence / presence of dye. While the fungal isolates (PMF2 and WFPN1) showed negligible MnP and LiP activity in presence of dye only. In dye free medium, these two fungal isolates could not show any peroxidase activity. In case of the fungal isolate WFPN1, the azoreductase activity varied from 0.6 U/mL (4 d) to 14.91 U/mL (8 d) in presence of congo red dye, while it varied from form 0.15-20 U/mL in presence of direct black dye, but it did not show any azoreductase activity in the absence of dye. The isolate PMF2 showed no azoreductase activity in the absence of dye while a varied enzyme activity ranging from 0.1-0.3 U/mL for congo red and 0.2 – 0.8 U/mL activity in presence of direct black. In the absence of dye, the isolates PMF2 and

WFPN1 showed 19.45 U/mL (PMF2) and 25.96 U/mL laccase activities respectively at 4 d incubation period after that the activity in both the cases decreased gradually up to 12 d. In case of direct black dye, the isolate PMF2 showed varied laccase activity ranging from 46.78 to 101.74 U/mL (at 8 d), while for the isolate WFPN1, maximum laccase activity (64 U/mL) was recorded at 4 d thereafter it decreased gradually attaining minimum activity (2.1 U/mL) at 12-d.

6. The results of decolorization of direct black with the co-cultures (consortia) of the selected isolates indicated 100% or complete dye removal in 4 d by 5 different consortia (consortia IV, consortia XI, consortia XII, consortia XVII and consortia XX); these consortia showed better dye decolorizing potential for direct black as compared to their respective mono-cultures showing 100% removal in 4 d. For brilliant green dye, the two microbial consortia (Consortia IX and XI) showed 100% removal of the dye in 2 d only that is significantly higher than the decolorization of the same dye obtained with their mono-cultures.

7. During extracellular protein profiling by native-PAGE, maximum number of protein bands were found in case of bacterial isolate PMB1 and fungal isolate PMF2 (5 bands), in the presence of direct black dye. Zymogram analysis revealed synthesis of two laccase isoforms with molecular weight ranging from 48 – 80 kDa by WFPN1 in presence of azo dyes. However, in the dye free medium, only one laccase band (~75 kDa) was visualized. For the fungal isolate PMF2, zymogram study showed only one laccase isoform (~50 kDa) in presence of azo dyes, while in dye free medium, no laccase band was observed.

8. In order to ensure the mechanisms adopted by the selected fungal cultures for *in vitro* removal or decolorization of the synthetic dyes from their culture medium, various techniques like UV-Visible analysis and FTIR analysis of the treated dye were employed and compared with their untreated samples.

Thus, the results of present investigation indicate that the potent fungal isolates WFPN1 and PMF2 are capable of bioremediation of toxic azo dyes while Cyanobacteria (*Synechococcus* sp.) and microalgae (PASM, PHSM and PRSM) are more potential for the bioremediation of brilliant green dye. The findings thus highlight the application of the potential fungal and microalgae/cyanobacteria cultures for treatment of dye wastewater/Industrial effluent.

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Appendices

APPENDIX-I

1. Nutrient Agar	gl^{-1}
Beef extract	3.0
Peptone	5.0
Agar	20.0
pH	7.2±0.2

2. Potato DextroseAagar	gl^{-1}
Peeled Potato	200
Dextrose	20
Agar	15

3. BG 11 Medium

A. Media	gl^{-1}
$NaNO_3$	1.5
K_2HPO_4	0.04
$MgSO_4.7H_2O$	0.075
$CaCl_2.2H_2O$	0.036
Citric Acid	0.006
Ferric Ammonium Citrate	0.006
EDTA	0.001
Agar	20
Trace Metal Solution	1ml

B. Trace Metal Solution	gl^{-1}
Boric Acid	0.0286
$MnCl_2.4H_2O$	0.018
$CuSO_4.7H_2O$	0.002
$NaMoO_4.2H_2O$	0.0039
$CuSO_4.5H_2O$	0.0007
$Co(NO_3)_2.6H_2O$	0.00004

4.	Ashby's Medium	gl^{-1}
	Mannitol	20
	K_2HPO_4	0.2
	$MgSO_4.7H_2O$	0.2
	NaCl	0.2
	K_2SO_4	0.1
	CaCO	5.0
	Agar	15

5.	Congo Red Yeast Extract Mannitol Agar	gl^{-1}
	Mannitol	10
	K_2HPO_4	0.5
	NaCl	0.1
	K_2SO_4	0.2
	CaCO	5.0
	Yeast Extract	1.0
	Congo Red	1%
	Agar	20

6.	Mineral Salt Medium	gl^{-1}
	Glucose	1.0
	K_2HPO_4	1.5
	$MgSO_4.7H_2O$	2.0
	$CaCl_2$	1.5
	NH_4Cl	0.15
	Agar	16.0
	Dye	100mg

APPENDIX- II

GRAM STAINING-

1. Crystal violet

Solution A	Crystal violet	2g
Ethyl alcohol (95%)		20 ml
Solution B	Ammonium oxalate	0.8 g
Distilled water		80 ml

2. Gram's iodine

Iodine	1g
Potassium iodide	2g
Distilled water	300 ml
Sodium bicarbonate	3g

3. Destaining solution

Ethyl alcohol	95 ml
Gram's iodine / Distilled water	5 ml

4. Saffranin

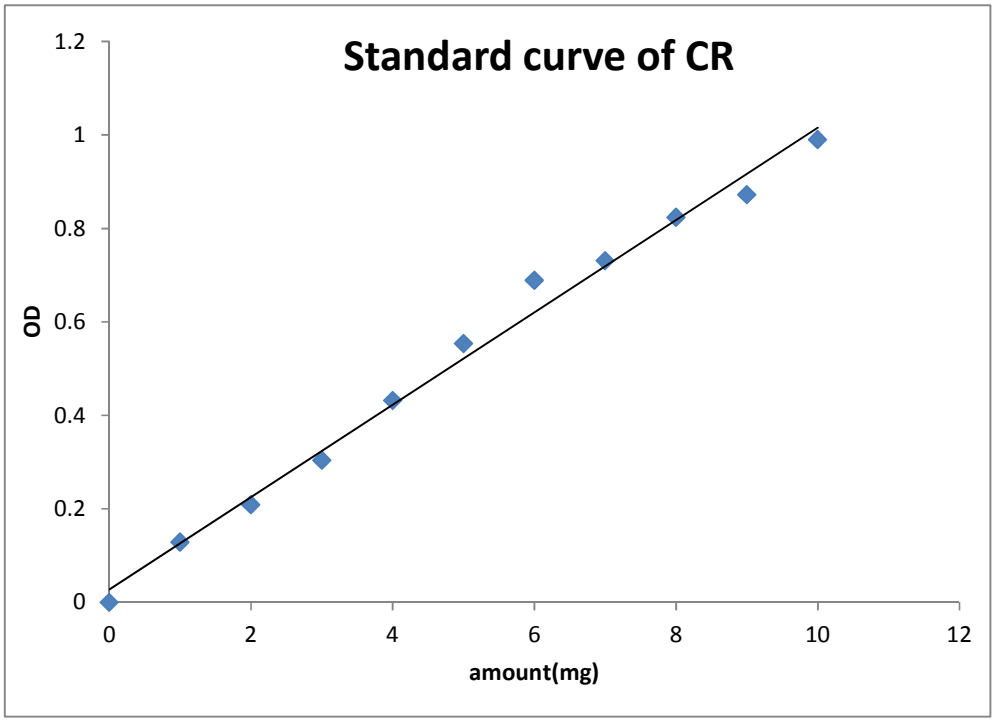
Saffranin	10 ml
(2.5% solution in 100 ml ethyl alcohol)	
Distilled water	90 ml

APPENDIX- III

Standard Curve of Congo Red Dye

For congo red

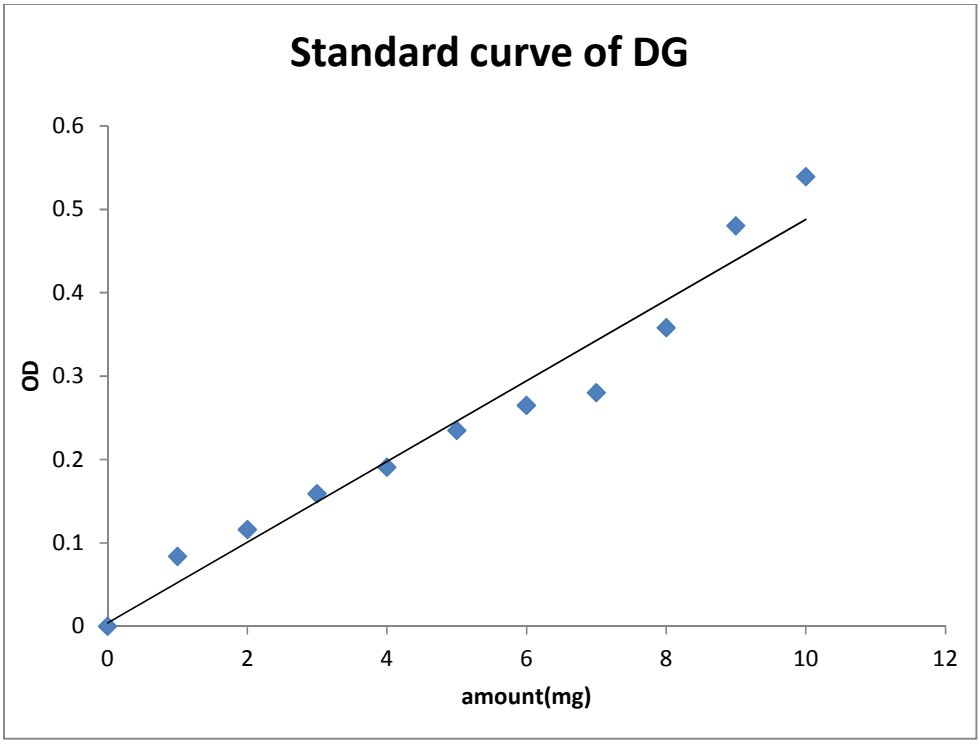
amount(mg)	OD
0	0
1	0.129
2	0.209
3	0.304
4	0.432
5	0.554
6	0.689
7	0.731
8	0.824
9	0.872
10	0.99



Standard Curve of Brilliant Green Dye

For direct green

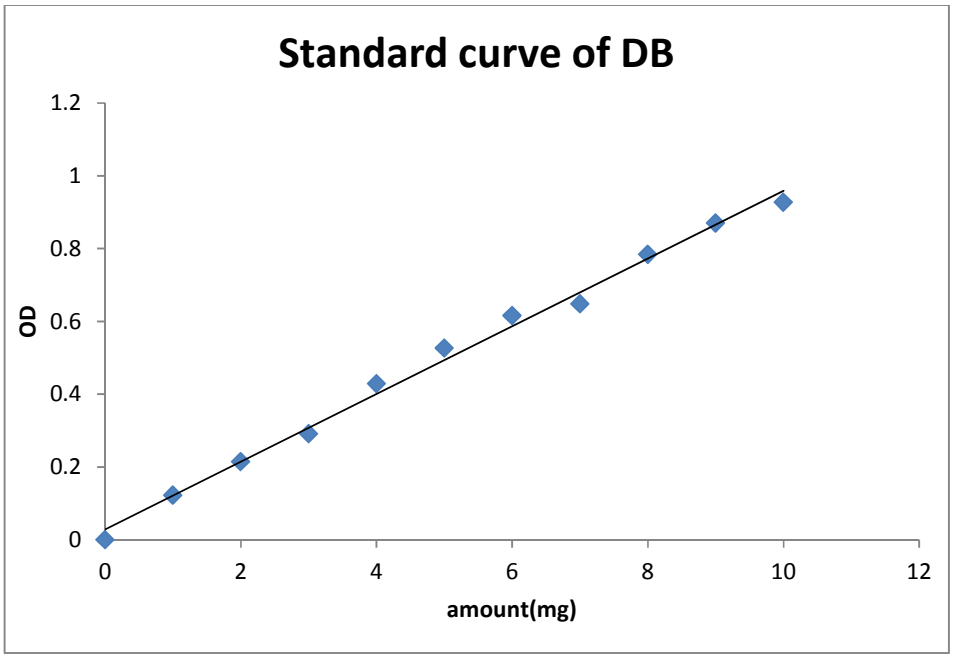
amount(mg)	OD
0	0
1	0.084
2	0.116
3	0.159
4	0.191
5	0.235
6	0.265
7	0.28
8	0.358
9	0.48
10	0.539



Standard Curve of Direct Black Dye

For direct black

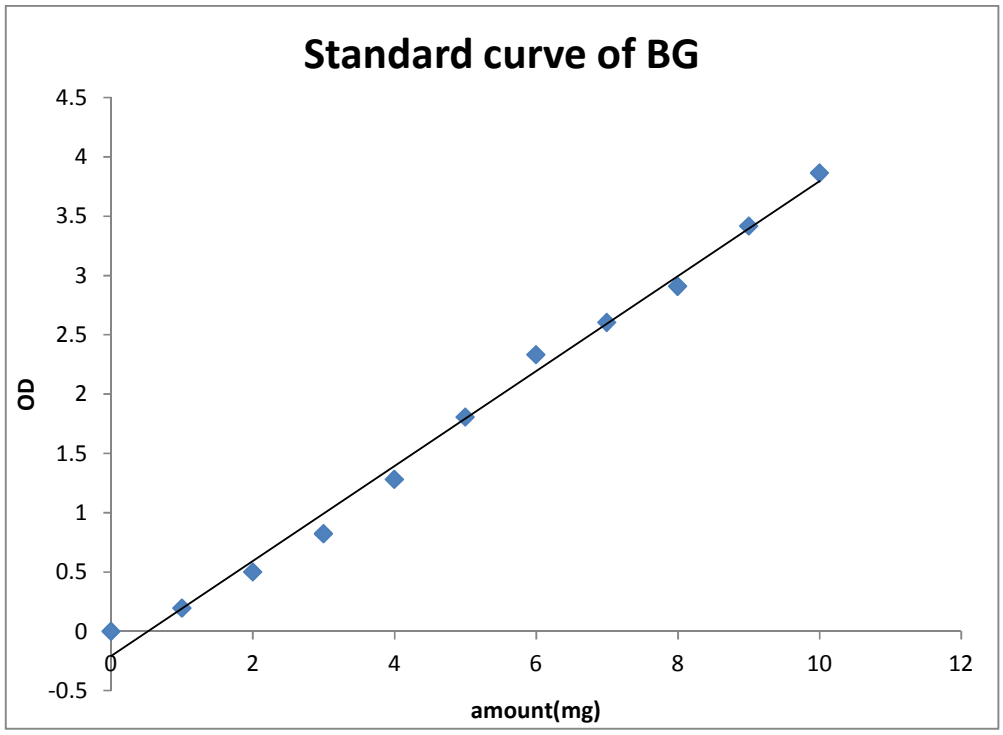
amount(mg)	OD
0	0
1	0.122
2	0.214
3	0.291
4	0.429
5	0.526
6	0.616
7	0.648
8	0.784
9	0.87
10	0.927



Standard Curve of Brilliant Green Dye

For brilliant green

amount(mg)	OD
0	0
1	0.196
2	0.501
3	0.824
4	1.281
5	1.806
6	2.332
7	2.603
8	2.908
9	3.417
10	3.865



VITA

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ABSTRACT

For rapid and cost effective biological removal of synthetic industrial dyes (congo red, direct green, direct black and brilliant green), twenty eight microbial cultures were isolated from different sources. Seventeen bacterial isolates, seven fungal isolates, three microalgae and one cyanobacterium were screened qualitatively for their *in vitro* dye decolorization efficiency. Selected isolates were then evaluated spectrophotometrically for quantification of dye decolorization. The bacterial isolate PMB1, fungal isolates PMF2, WFPN1 and *T. harzianum* showed 100% decolorization potential for azo dyes in 5-6 d, while the three microalgae (PASM, PHSM and PRSM) isolates and the cyanobacteria (*Synechococcus* sp.) showed 100% decolorization for brilliant green dye in 4 d. These selected isolates were then checked for extracellular enzyme activities (laccase, lignin peroxidase (LiP), manganese peroxidase (MnP) and azoreductase) in culture filtrates. Maximum enzyme activities by the selected fungal isolates PMF2 and WFPN1 were recorded for laccase enzyme followed by azoreductase enzyme, while no MnP and LiP activities were observed. The fungal isolate PMF2 showed maximum laccase activity (101.74 U/mL) followed by WFPN1 (64 U/mL) in presence of direct black dye, while for congo red dye, maximum laccase activity was recorded for WFPN1 (30 U/mL) followed by PMF2 (25.61 U/mL). In the absence of dye the maximum laccase activity was recorded in WFPN1 (25.96 U/mL) followed by PMF2 (19.45 U/mL). Maximum azoreductase activity was observed for the fungal isolate WFPN1 (20 U/mL and 14.91 U/mL) for direct black and congo red dye respectively. For increasing dye decolorization efficiency, 22 microbial consortia were developed for direct black dye using fungal and bacterial isolates and 15 consortia for brilliant green dye by using microalgae, Cyanobacteria and fungal isolates. Some of the consortia were found better as compared to their mono-cultures for decolorization of direct black and brilliant green dyes showing 100% removal of direct black and brilliant green dyes in 4 d and 2 d respectively. Zymogram analysis by activity staining through native PAGE indicated induction of certain extracellular laccase isoforms (~50 kDa - ~75 kDa) in the presence of azo dyes. Spectral analysis (UV-Visible spectroscopy and FTIR) also confirmed degradation / removal of these dye by the potential isolates. The isolate WFPN1 showing maximum dye decolorization and enzyme production was found as most potential strain and identified as *Ganoderma lucidum* based on 18S r-RNA gene sequence analysis.



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शोध ग्रंथ शीर्षक	:	''हानिकारक कृत्रिम औद्योगिक रंजको के जैविक उपचार हेतु सूक्ष्मजीवों के कनसोरसिया का विकास''			
सलाहकार	:	डॉ0 लक्ष्मी तिवारी			

सारांश

कृत्रिम औद्योगिक रंजको (कोंगो रेड, डाइरेक्ट ग्रीन, डाइरेक्ट ब्लैक और ब्रिलियंट ग्रीन) के तेजी और लागत प्रभावी जैविक अपघटन के लिये 28 सूक्ष्म जीवों को अलग–अलग स्रोतो से पृथ्क किया गया। सत्रह जीवाणु, सात कवक, तीन सूक्ष्म शैवाल और एक साइनोबैक्टीरिया को उनके रंग विवर्ण करने की दक्षता के लिये गुणात्मक रूप से जांच की गयी। चयनित आइसोलेट्स की रंग विवर्णता जांचने के लिए स्पैक्ट्रोफोटोमेट्रिक रूप से मूल्यांगन किया गया। जीवाणु पी.एम.बी. 1, कवक पी.एम.एफ. 2, डब्ल्यू.एफ.पी.एन. 1 और *ट्राइकोडर्मा हारजियानम* में एजो रंगों को 5–6 दिन में विवर्ण करने की क्षमता का पता चला, जबकि तीन सूक्ष्म शैवाल (पी. ए.एस.एम., पी.एच.एस.एम. और पी.आर.एस.एम.) तथा साइनोबैक्टीरिया ने ब्रिलियंट ग्रीन डाई को 4 दिन में 100 प्रतिशत विवर्ण किया। इन चुने हुए आइसोलेट्स को बाह्य एंजायम गतिविधियों (लैक्केज, लिग्निन परआक्सीडेस, मैंगनीज परऑक्सीडेज और एजोरिडक्टेज) के लिए जांचा गया। चयनित आइसोलेट्स में से अधिकतम एंजायम की गतिविधियां पी.एम.एफ. 2 व डब्ल्यू.एफ.पी.एन. 1 में लैक्केज एंजायम के लिए पायी गयी, जबकि किसी भी आसोलेट में मैंग्नीज परऑक्सीडेज और लिग्निन परऑक्सीडेज गतिविधिया नही पायी गयी। कवक पी.एम.एफ. 2 में अधिकतम लैक्केज एजांयम (101.74 यू/एमएल) और डब्ल्यू.एफ.पी.एन. 1 में (64 यू/एमएल लैक्केज ऐंजायम डाइरेक्ट ब्लैक की उपस्थिति में प्राप्त किया गया। रंजको की अनुपस्थिति में भी लैक्केज एंजायम प्राप्त किया गया। अधिकतम ऐजोरिडक्टेज एंजायम, कवक डब्ल्यू.एफ.पी.एन. 1 में (20 यू/एमएल) डाइरेक्ट ब्लैक रंजक की उपस्थिति में पायी गयी। रंजकों की विवर्णता को बढ़ाने के लिए 22 कनसोरसिया डाइरेक्ट ब्लैक रंजक में विकसित किये गये। 15 कनसोरसिया को ब्रिलिंयट ग्रीन रंजक के लिये विकसित किया गया। 22 में से 5 कनसोरसिया में 100 प्रतिशत डाइरेक्ट ब्लैक रंजक विवर्ण करने की क्षमता पायी गयी। ब्रिलियंट ग्रीन रंजक के लिये 15 में से 2 कनसोरसिया ने 100 प्रतिशत रंग विवर्ण करने की क्षमता पायी गयी। सबसे संभावित आइसोलेट्स लैक्केज जाइमोग्राम विश्लेषण किया गया। रंजक विवर्णता और रंजकों के अपघटन को जांचने के लिये यू.वी. विजिबल और एफ.टी.आई.आर स्पेक्ट्रल विश्लेषण किया गया। डब्ल्यू.एफ. पी.एन. 1 को सबसे ज्यादा प्रबल पाया गया ओर 18 एस.आर.आर.एन.ए. जीन अनुक्रम विश्लेषण के आधार पर *गेनोडर्मा ल्यूसिडम* के रूप में पहचान की गयी।

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