Development of innovative clean-up technology (Biofilters) for effective degradation of textile dye in industrial wastewater effluent

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By

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DECLARATION

I hereby declare that the thesis entitled, **"Development of innovative clean-up technology (Biofilters) for effective degradation of textile dye in industrial wastewater effluent"** submitted for Ph.D. Biotechnology Degree to Department of Biotechnology, Lovely Professional University is entirely original work and all ideas and references have been duly acknowledged. The dissertation has not been formed the basis for the award of any other degree.

Deepika Bhatia
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CERTIFICATE

This is to certify that **Miss. Deepika Bhatia** has completed her Ph.D. thesis in Biotechnology titled **"Development of innovative clean-up technology (Biofilters) for effective degradation of textile dye in industrial wastewater effluent"** under my guidance and supervision. To the best of my knowledge, the present work is the result of her original investigations and study. No part of this thesis has been submitted for any other degree or diploma.

The thesis is fit for the submission of the partial fulfilment of the conditions for the award of degree of Ph.D. in Biotechnology.

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ABSTRACT

Mitigation strategies to contain the deleterious effect of textile industry effluents have captured the interest of researchers. The discharge of untreated textile industry effluent in the environment have negative impacts on the ecology and environment of biosphere. This thesis research resulted in conducting several studies to determine the impact of textile industry on water quality and developing innovative green technologies for effective treatement of textile industry wasterwater. In the first study, the effluents from different textile industries of Ludhiana were collected for one year (2014-15). Effluents being discharged from textile units of Ludhiana are the major sources of water pollution at the receiving drain known as Buddha Nallah. Seasonal variations in physico-chemical characteristics like pH, temperature, TDS, BOD, COD and TSS were observed at two selected sites on Buddha Nallah. Results of this study clearly indicated that the values of these water quality parameters were much higher compared to the permissible limit of WHO for surface water streams. The maximum value of pH was observed in the summer season (8.78$\pm$0.470 and 8.51$\pm$0.411) and lowest pH values were observed during the rainy season (7.38$\pm$0.48 and 7.11$\pm$0.59) for both sites, respectively. Suspended solids were found to be highest in the autumn season for both sites and lowest in summer. The values of BOD and COD of effluent were very high throughout all seasons. pH values as high as 900 mg/L and 580 mg/L were observed for COD and BOD, respectively. The present study highlights the need for treatment of textile effluents before they are discharged into water bodies. Moreover, the systematic study of correlation between various physico-chemical parameters of effluent was also studies, which not only helps us to determine the quality of water but also quantifies the concentration of pollutants in water. The results of this study clearly indicated that strong positive correlation exists between pH and water temperature, TDS, TSS, BOD and COD for both sites in the autumn season. For Site 1, in rainy monsoon season, a strong positive correlation was observed between pH, BOD, COD and water temperature. TDS data showed positive correlation between TSS, BOD and COD. For site 2, in rainy monsoon season, water temperature and TSS exhibited negative correlation with other water quality parameters. In the present study, water temperature also showed positive correlation with TDS. Also, TDS has a weak positive correlation with COD but exhibited strong correlation with BOD for both sites in the winter season. In summer season, for both sites, moderate positive correlation was observed between pH, water temperature and TDS. BOD has a moderate positive correlation with COD and TSS. This study highlights the need for the treatment of textile industry effluents before they discharged into water bodies.

Second study was conducted to collect locally occurring bacteria from a stream receiving the textile industry wastewater in the city of Ludhiana, India. From hundreds of bacteria present in the wastewater; six strains of bacteria were isolated which has the potential to remove dye contaminants from the polluted water. These isolated bacteria were tested for their decolorizing potential of dyes [namely, disperse red 167.1 (rubine BL), disperse red 167 (dark red 2B), disperse blue 60 (blue BG) and disperse red 54 (scarlet RR)] within 24 hours after inoculation at different concentration (50 ppm, 150 ppm and 250 ppm). The biochemical and molecular characterization resulted in the identification of isolated bacteria as *Bacillus subtilis* (KX034562), *Paenochrobactrum glaciei* (KX034561), *Brevibacillus panacihumi* (KX034559), *Bacillus cereus* (KX034566), *Bacillus sphaericus* (KX034564), and *Paenibacillus pocheonensis* (KX034565). Therefore, in this study, an attempt was made to develop the bacterial consortium containing six efficient dye degrading bacteria after investigating their compatibility and synergistic activities among each other. Initially a loop full of individual bacterial pure cultures were inoculated separately in minimal media and incubated for 24 h at 30ºC. For the development of bacterial consortium, 6 h old cultures were then transferred aseptically into nutrient medium followed by 24-hour incubation period at 30ºC. The consortium thus obtained was used as the source of inoculum for decolorization assay with individual bacterial sp. for decolorization of selected azo-disperse dyes. Among these bacteria, *B. cereus* showed the highest potential for decolorization of disperse red 54 (up to 84.17 %) and 76.29% for disperse blue 60 in 24 hours; *P. glaciei* showed 84.80% decolorization rate for disperse red 167 in 24 hours; consortium displayed highest rate of decolorization for disperse red 167.1 (82.76 %). The bacterial consortium also exhibited maximum rate of decolorization for disperse red 54 (83.46%) in 24 hours. Furthermore, enzyme analysis was carried out to ensure decolorization of selected dyes by these bacteria. The results indicated the involvement of extracellular enzymes, azoreducatse and laccase for this purpose. The FTIR and GC-MS analysis of extracts proved the degradation of selected dyes to yield metabolites. The phytotoxicity assay was also carried out to assess the toxicity of parent dye and the extracted metabolites. The results showed a significant difference in the germination index, shoot and root length of *Triticum aestivum* by parent dye as compared to extracted metabolites. The overall result of this study shows clearly that naturally occurring bacteria in wastewater bodies can be used to develop economically viable bio-filters to remove textile industry pollutants from rivers and streams to improve the ecological conditions of polluted rivers and create healthy environmental conditions for marine life.

A third study was conducted develop bio-filters by using low cost agricultural biological materials as a substrate/matrix for the immobilization of consortium for the removal of dyes from aqueous solution. Therefore, the third study was conducted for the immobilization of the bacterial consortium on corncobs for the efficient treatment of disperse red 167.1 (rubine BL). This study helped us to understand and determine, if the design of biofilter and equations developed from lab-based column studies can describe environmental conditions that occur in the "real world."

Affectionately

Dedicated

To

My Beloved Parents and My Loving Sister Sheetal

and My Friend Harsh

Acknowledgement

Strength doesn't come from what you can do, it comes from overcoming the things you once thought you couldn't

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(Deepika Bhatia)

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PREFACE

Ludhiana, is the hub of textile industry in Punjab. It is famous for dyeing and printing of textile fabric. Day by day, an increase in the usage of chemical-based dyestuff and their direct or indirect release into the natural surface water body (Buddha Nallah) is contaminating the environment and posing a great ecological hazard to the people of Ludhiana city. Considering this grave situation, the current study is focused to assess the contamination load of the effluents released by textile industries located in the vicinity of Buddha Nallah. Also, an effort was made to isolate and identify indigenous bacterial strains which are capable of degrading the selected azo-disperse dyes and to develop biofilters. The use of microorganism (bacteria) for the removal of dyes from textile effluent will serve two purposes - a cost effective and an environmental-friendly approach for bio-degradation of textile dyes.

The selected bacterial species were examined to check their potential to degrade the selected dyes. It was considered that the dye was utilized as an energy source for bacteria showing decolorization. The mechanisms underlying the dye degradation by bacteria were also explored. The possible role of dye degrading enzymes and degraded products after dye degradation were examined. Consortium of bacteria has been developed and utilized by immobilizing on adsorbent for removal of dyes, which provided an insight into the execution of the microbe-based methodology aimed at bioremediation of textile wastewaters from “lab to field”.

INTRODUCTION

Chapter 1

INTRODUCTION

1.1 Components of environment and water pollution

Environmental pollution has caused multi-dimensional impacts on the ecology and human health. Water pollution is one of the major concerns faced by the modern world among all environmental problems (Abhilash et al., 2009). According to United Nations water report in 2006, "there is enough water for everyone" but mismanagement and corruption have resulted in limited access to it (UNDP, 2006). Gradually, the human beings are exploiting every natural resource and using them in an uncontrolled manner which is resulting into the addition of new pollutants into the environment i.e. anthrosphere. Currently the anthrosphere regulates the lithosphere, hydrosphere and atmosphere, which eventually affect the biosphere. Good quality water is needed to maintain and sustain a life for all species on this planet including humans. Increasing population and economic development have an accelerating role in environmental pollution. Discharge of untreated industrial and municipal wastewater to surface water bodies is the main cause for surface and groundwater pollution. Contamination of water resources has occurred at an alarming pace since the beginning of industrialization in the 20^{th} century, especially in developing countries like India which has caused environmental pollution affecting not only the human population but also flora and fauna.

Historical trend shows that for the last several decades, industrialization and urbanization has contributed mainly in polluting water bodies. Various forms of pollutants mingle in water causing aesthetic as well as adverse effect on both aquatic and human life. Different kinds of contaminants present in wastewaters from agro-industries, factories and municipal plants have been polluting water bodies for decades, resulting in the growth of disease-causing pathogens, bacteria, hormones, heavy metals, dyes, sediments, pesticides and other organic and inorganic compounds (Acuner and Dilek, 2004). Industries like textile industries consume tons of water each year and discharge large volumes of wastewater in the water bodies (Puvaneswari et al., 2006).

1.2 Overview of textile industries

Textile industries are the major consumers of the dyes. 7×10^5 metric tons of dyes from over 10,000 dyes are available commercially in market today (Chung, 2016). In India, the textile industry is the leading industry which plays a major role in Indian economy, providing the maximum employment after agriculture sector. It is one of the earliest industries to be established and today it is the second biggest industry in China globally. This industry is also an important part of the industrial sector of Ludhiana city of Punjab and produces highly polluted discharge water (Garg et al., 2010), thereby posing a serious threat to people in that region. Pollution of water bodies by different kinds of textile dyes has been found a big health hazard (Puvaneswari et al., 2006). According to a study, out of 56,565 biopsies, 4730 cases of cancer were diagnosed in the Ludhiana belt (Malhotra et al., 2001) raising an accusation on the industries that are responsible for discharging tons of pollutants in the nearby water bodies. The polluted water also affects the soil health and agricultural production with harmal chemicals. The contaminated soil affects the germination and growth of crops (Dos Santos et al., 2006). Textile industries as compared to other industries have a major impact on the environment that should be considered. They consume tons of volumes of water and produce wastewater of enormous quantity from the procedural steps involved in textile processing and dyeing. Approximately 52 gallons of water are required for the processing of 1 Kg of textile. Various chemicals also make up for the large part in the composition of the wastewater due to their role in textile processing. Desizing, scouring, bleaching, mercerizing and dyeing are the most common processing steps in any textile industries (Figure 1) (Dos Santos et al., 2006).

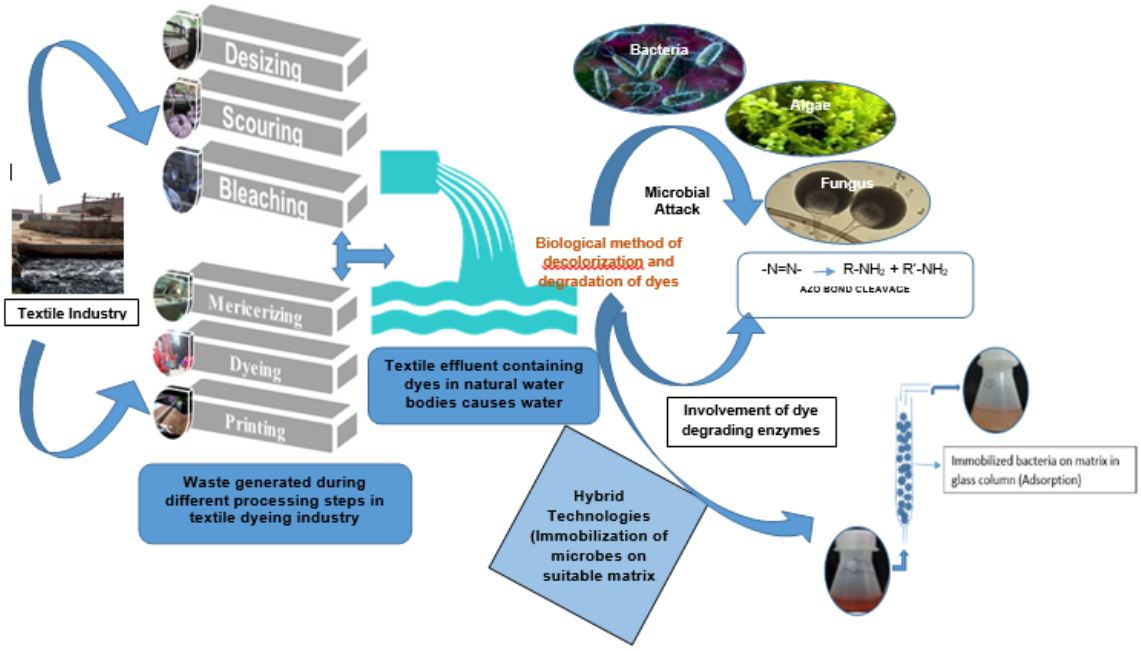


Figure-1: An overview of the waste generated by processing steps in textile industries and various biological methods for effluent treatment

The main pollutants of textile wastewater originate from the dyeing and finishing steps. These steps involve dyeing of the fibers with the permanent color as per desire which is further processed into final commercial products (Kim et al., 2003). Frequent fluctuations in different water quality parameters have been observed in water bodies due to the discharge of textile wastes with high chemical oxygen demand (COD), biochemical oxygen demand (BOD), pH, color, and salinity (Hari et al., 1994). Different organic compounds, dyes and chemicals used in dry and wet processing steps in industries define the waste water composition (Senthilkumar et al., 2011). Sizing agent is applied prior to fabric production which eases the weaving process and later removed from woven fabric by wetting process. This removing process known as desizing is add up to 50% of organic loading in wastewater discharge from the industry (Feitkenhauer, 2003).

1.3 Present scenario of Punjab and wastewater drain in Ludhiana

Punjab is the leading state for agricultural production and planned physical infrastructure and high human development index is one among the developed states of India. Downside of it is that, it is now battling with severe water pollution problems. Increasing urbanization and industrialization have severely deteriorated quality of water resources in major cities of Punjab. The only surface water body of Ludhiana city in Punjab, Budha Nallah, an unlined canal, is the main receptor of city's domestic and industrial sewage. As a result of perennial flow of these sources of sewage into Budha Nallah, it has become the most polluted water body in northern India (Cheema and Virk, 2012).

Extent of pollution of Buddha Nallah is such that natural process of self purification does not take place along all its length. Various studies have been conducted on the quality of Buddha Nallah water and reports were presented on Kala Sanghian from time to time (Cheema and Virk, 2012). All these studies have tried to highlight the level of pollution being caused by the wastes disposed into the Nallah. However, certain steps had been taken to eliminate this problem but nothing concrete has come out as yet. Several streams get polluted day by day thereby increasing the problem of pollution in the State of Punjab. There are hundreds of dyeing industries on both the banks of the Buddha Nallah drain and they throw the untreated wastewater into the drain adding to the pollution at an alarming scale. The effluent from textile mills forms highly concentrated wastewater that is highly colored and consist of various types of dyes used by industries. This wastewater when discharged into lower streams; rivers and lakes not only destroy the aesthetic of the pristine water bodies but also changes the photochemical activities by hindering the sunlight transmission (Aksu et al., 2007). Apart from destroying aesthetic, this wastewater which can impart toxicity, mutagenesis and carcinogenicity (Aksu et al., 2007).

1.4 Classification of textile dyes

Industrial dyes can be classified according to several ways based on their source, chemical structure and nature of chromophoric group (Figure 2), which is more appropriate for dye chemists and technologists, based on their nuclear structure and it may be further classified based on their applications. The approach based on the chemical structure has certain

advantages towards the dye chemists as they reveal the family of dye belonging to the same group based on chemical properties. On the other hand, the approach based on the usage of the dyes is adopted by the Color Index and involves the nomenclature of the dyes (Singh and Arora, 2011).

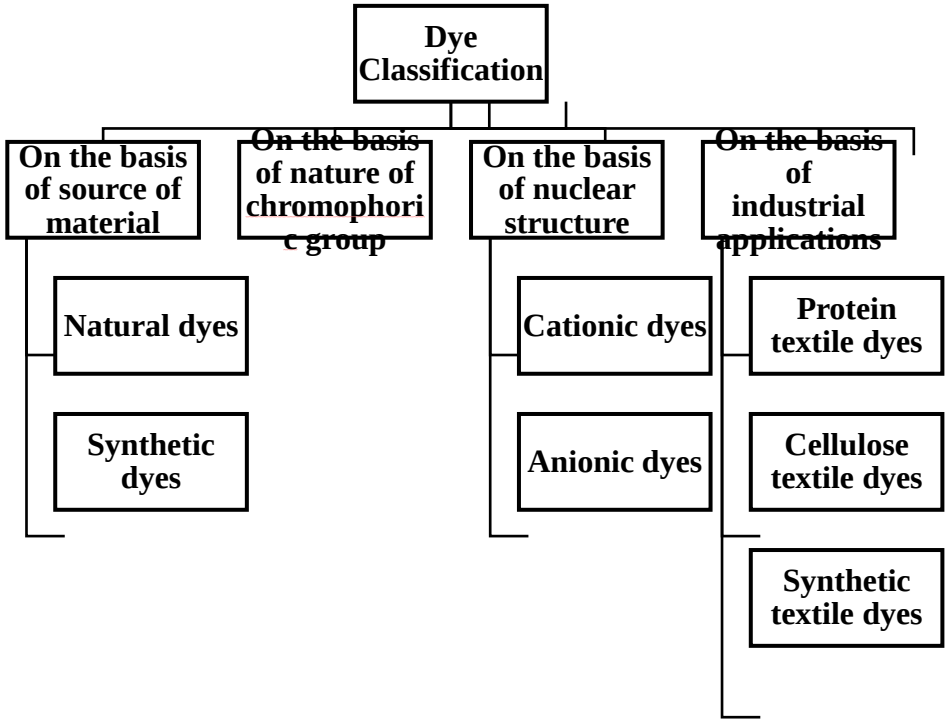


Figure-2: Prominent way of classification of dyes

Natural dyes: These dyes are obtained from plants, animals or minerals and normally a complex mixture of compounds. They also can be classified as a vat or acidic dyes and can also be mordanted. Compared to synthetic dyes, their color brilliance and intensity is poor and these are used only for natural or regenerated fibers. Disperse dye are a class of textile dyes used extensively in textile industries for dyeing secondary cellulose acetate fiber and

polyesters, which is an important group of synthetic fibers. They are non-ionic, insoluble dye with substituted azo, anthraquinone or diphenylamine groups. Among them, azo disperse dyes are the widely used dyes and account for about 50% use in textile industries around the world. Its tinctorial strength and low cost of manufacturing makes it an alternative for other derivative disperse dyes making them the most common group of synthetic dyes that are discharged in the environment (Saratale et al., 2011). Even 1 ppm of the dye can make a significant impact in the visibility of water. Toxicological studies of these dyes have shown toxic effects on daphnids suggesting its toxic potential in other living organisms too (Bae and Freeman, 2007). Some of the azo disperse dyes remains unaltered when treated in sewage treatment plant (Forgacs et al., 2004) and other studies carried out have showed that 85% of these dyes remain undegraded even after activated sludge process (De Aragao et al., 2005).

Synthetic dyes: The classification of synthetic dyes is shown in Figure 3. Easy applicability and cost effectiveness in synthesis along with diversity of available colors as compared to that of other natural dyes are few reasons why synthetic dyes have been used widely. The uses of synthetic dyes have been widely found in tanneries, paper and pulp industry, cosmetic industries etc.

A separate classification which addresses the method of dyes application and to a certain extent the chemical structure of the dyes has been developed. This classification system is as follows:

Acid dyes: These dyes are generally known for large molecular structures. They have one or more carboxylic salts or sulfonic acid functional groups. Fibers are dyed with acidic dyes in acidic solution which develop a positive charge in the fiber and help in the binding of the dye. Cotton, wools are the kinds of fiber that develop a positive charge in acidic solutions. Acidic dyes are generally colorfast for light and laundering. But mordanting improves the overall fastness of acidic dye. They can be further divided into three groups based on their application and wet fastness. Leveling acid dyes are small molecular dyes with better color fastness and require highly acidic dye bath. Milling acidic dyes have better

wash fastness with dull shades and with lesser leveling quality. They are applied from dilute acetic acid solution. Their molecules are larger than leveling dyes. Super milling dyes have high color fastness and wet fastness. They have poor leveling ability. Their molecules are larger and require neutral dye bath solution.

Direct dyes: These are the special class of dyes used for dyeing cellulosic fibers due to their high affinity and their smaller molecular size. Their molecules are long, narrow and flat, as a result, they can easily diffuse into the fiber. Direct dyes often contain one or more azo groups attached to the aromatic chromophore. Since the charge of the fiber is not a factor in dyeing with direct dyes, they require basic dye bath for dyeing. In basic dye bath cellulosic fibers shows better stability. These dyes are categorized into 3 categories. Class A dyes have better leveling ability compared to class B and class C even in higher salt concentration. Class B and C have poor leveling ability but can be improved by adjusting the salt concentration or raising the dye bath temperature as in the case of class C direct dyes. Though direct dyes are colorfast to cellulosic fibers but it can be made better by mordanting.

Mordant dyes: These dyes are acid dyes with some extra sites that react with metal salt mordant. These dyes have also got acid salt anion groups just like acid dyes. These dyes are engineered to chelate with metal ions forming an organometallic complex with higher color fastness and lesser solubility. The mordanting step can be applied with various combinations to achieve dyeing with mordant dye. The mordanting can be applied after dyeing, or before dyeing or along with dyeing. Though post-mordanting is found to be more effective.

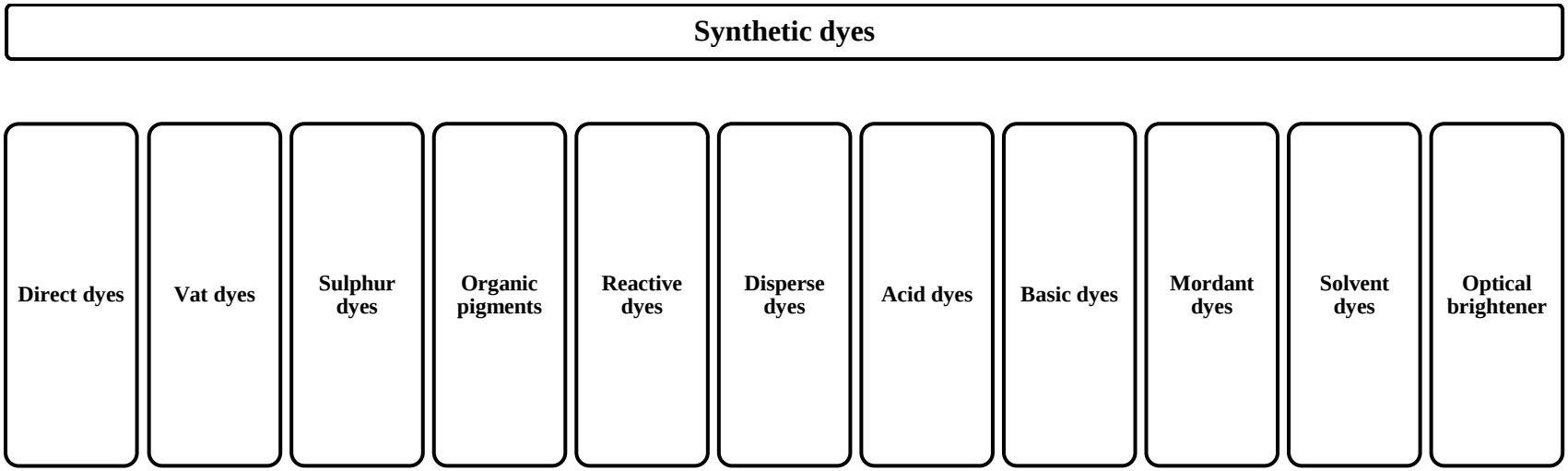


Figure-3: Classification of synthetic dyes (derived from inorganic/organic compounds)

Salts like aluminum, chromium, iron, copper are used commonly as mordants.

Reactive dyes: The reactive dyes contain the basic structure of mordant, acid or direct dye with an additional reactive group, capable of binding covalently with the fiber. Due to their specific reactivity property, these dyes are limited some of the fibers like cellulosic fiber, nylon or protein fibers. These dyes have excellent fastness due to their reactivity. These dyes require slightly acidic or basic conditions for rapid and successful reaction with the fiber. Reactive dyes with a world market share of 60–70% are widely used in industries (Campos et al., 2001).

Basic dyes: These are the colored cationic salts of amine derivatives. Since they are cationic in nature, they migrate towards the negatively charged portion of the fiber. Though the color brilliance and intensity of the dye is excellent but the color fastness is very poor. Mordanting with complexing agent improves the colorfastness of the dye. They are applied in mildly acidic or neutral solutions. They are applied to fibers like cellulosic, protein; nylon specially modified synthetic fibers or acrylic fibers.

Vat dyes: These dyes are usually water-insoluble. These dyes have to be solubilised by reducing them in the presence of a base and then apply to fibers. The dyes are usually anthraquinone or indigoids derivatives. These are applied at low temperatures. After application of the reduced dye, the dye has to be re-oxidized using air or treatment with mild oxidizing agents. Removal of loosely held surface dyes can provide good color fastness. These can also be applied readily to synthetic and cellulosic fibers but are avoided in protein fibers due to their high basicity.

Sulfur dyes: These dyes are a complex of aromatic compounds with sodium polysulfide. Sulfur dyes, just like vat dyes are reduced in the presence of bases prior to their application to the fibers and are re-oxidized after dyeing. Either air or oxidizing agents like hydrogen peroxides are used for the re-oxidizing purpose. They are also found in solubilised leuco form like vat dyes. They are inexpensive and show moderate color fastness.

Azoic dyes: Azoic dyes or naphthol dyes are made by a coupling reaction of an aromatic alcohol or amine with diazonium salts. These contain the azo group.

The fabric is dyed by applying the coupling component like aromatic alcohol or amines which are immersed in a solution containing diazonium salts. Due to its instability, the process is carried out at the near room temperature. These dyes have moderate fastnesses which can be improved by mordanting. The presence of one or more R1-N=N-R2 bonds is the characteristic property of azo dye. Azo dyes are synthetic organic colorants that are widely used in the industries relating to textiles, foods, printing, tattooing, and cosmetics and in clinical settings (Chen et al., 2005). These become pollutants once they are released into the environment (Suzuki et al., 2001). Azo dyes are usually recalcitrant to conventional wastewater treatment. Several methods such as adsorption, chemical treatment, and electrochemical processes have been adopted and proved to be costly which also produce large volume of sludge. More studies are now focused on the methods involving biological treatments (Chen, 2006). Many microorganisms such as bacteria, fungi, and yeast have been observed to decolorize azo dyes by biosorption and biodegradation (Gou et al., 2009). Therefore, the biologically degrading dyes have potential advantages in developing decolorizing bio-treatment of wastewater (Suzuki et al., 2001).

Disperse dyes: Disperse dyes were developed to dye fabrics having hydrophobic properties like acetate, nylon, polyester and other synthetic fibers. They are small polar molecules containing either anthraquinone or azo groups. They do not have charged anionic or cationic groups in their structure. They are partially soluble in water and hence are dispersed with surfactants in the dye bath. The dyes are solubilized in small steps and the fiber is dyed until all the dyes get exhausted. These dyes have good light and wash-fastness but have fume fading problem. Some of the disperse dyes can be used to dye thermoplastic fibers by sublimation method due to their appreciable vapor pressure.

Solvent dyes: These dyes resemble other dye classes but their uniqueness lies in their solubility in alcohols and chlorinated hydrocarbons due to the presence of certain groups in their structure. They are applied to fibers which have poor dyeability in aqueous solutions.

Pigments: These are the class of insoluble color compounds which cannot bind to fibers readily and need to be fixed by using an adhesive compound that locks it down in the fiber material. The pigments dyes have moderate to poor fastness as they are just surface treated.

Nature of chromophoric group						
Acridine dyes	**Anthraquinone dyes**	**Nitroso dyes**	**Arylmethane dyes; Azo dyes; Dizonium dyes; Nitro dyes**	**Xanthene dyes**	**Azine dyes**	**Quinone-imine dyes**
-C=N- and -C=C	-C=O and -C=C	-N=O	-N=N-	-O-C_6H_4-O	-C-N=C- and -C-N-C	derivates of quinine group

Figure-4: Classification of dyes based on the nature of their chromophoric groups

The initial fiber materials are prone to breakage and hence are treated with sizing agents like carboxymethyl cellulose, polyvinyl alcohol, starch which involve the sizing step. Desizing step comes following to remove all the sizing agents. Scouring steps involve the removal of impurities by alkali treatment by chemicals like sodium hydroxide breaking down any fats, natural oils, surfactants and waxes emulsifying them in the scouring bath. Removal of unwanted colors from the fibers is ensured by the use of bleaching technique, in which sodium hypochlorite and hydrogen peroxide are used. Mercerizing steps prepare the fiber for dyeing. It increases the dye ability of the fiber along with its luster and fiber appearance by chemical treatment with the concentrated

alkali solution and acid solutions. This step especially requires large volumes of water, both for dying in the dye bath and rinsing step. For enhancing the dye absorption by the fiber, many chemicals like metals, sulfide, formaldehyde surfactants and salts are used. The chemicals required can range from inorganic compounds to organic products. More than 7000 chemicals are involved in the dyeing process. These chemicals, if untreated before discharge in the wastewater, can cause harmful effects. Of all the steps involved, wet processing steps produce the highest amount of wastewater containing the diverse chemicals and most importantly the synthetic dyes of different chromophoric groups like azo, anthraquinone, triaryl methane etc (Figure 4). Out of the 800,000 tons dyes used per year, 60-70% of them belong to the azo class of dye. None of the synthetic dyes provide a 100% fixation and as a result, a good amount of the unfixed dye make their way into the wastewater which is estimated to be present in concentrations of up to 500mg/ml (Pearce et al., 2003).

At the end of 19th century, synthetic dyes almost completely replaced the natural dyes since its accidental discovery by an English chemist W.H. Perkin in 1856. Since then thousands of synthetic dyes are developed and manufactured (Wesenberg et al., 2003). Synthetic fibers like polyester and nylon posed a challenge to the natural dyestuffs leading to their replacement and mass production of synthetic dyestuffs. The discovery of chromogen during the early textile industrial set up led to the discovery of almost all types of textile dyes which used today. The annual production of synthetic dye exceeds a production of 700,000 metric tons each year (Toh et. al., 2003). Some of the countries like Russia, India, China, and South Korea are the major producers of synthetic dyes with China leading, producing 28% of the global dye need annually (Wesenberg et al., 2003). In the year 1999, the synthetic dye industry was worth USD 6.6 Billion markets with Asia holding 42% of the share (Torres et al., 2003). Among all the dyestuffs produced globally, Azo dyes are estimated to make up about 70% by weight (Pinheiro et al., 2004). Thus, azo dyes make up for the largest group of synthetic colorant and of huge environmental significance. Due to their relative ease of manufacturing and other suitable characteristics like stability to light degradation, temperature, varying color availability, oxidizing agents, as well as microbial attack, they are the considered dye

of choice for several dying industries like textile industries, papermaking industries, printing industries etc (Torres et al., 2003).

Dyes are resistant against light, water as well as oxidizing agents and as a result these are very reluctant to remove from environment once they are released. Therefore, its treatment by any conventional treatment process becomes pretty much useless (Shaul, 1991). Treatment of wastewater containing dyes is very cumbersome process and difficult as a result of wide ranges of pH, salt concentration and chemicals present in the water.

1.5 Treatment of textile effluents

Variety of effective physical and chemical treatment methods are commercially available but most of them are either expensive or not adaptable to a wide range of dyes, as a result these do not completely solve the problem of complete decolorization of dyes in industrial effluents.

The methods which are involved in removing the colors before discharging into the environment are adsorption, flocculation, coagulation etc. These methods, due to high cost and large sludge production, pose a serious limitation. Therefore, economical and environment-friendly techniques are required for the removal or degradation of dyes from the wastewater. Biological methods which have capabilities of bioconversion of organic pollutants to stable nontoxic product (Kuhad et al., 2004) have currently been viewed as alternative methods. These methods are environmental friendly, less energy intensive and effective.

1.6 Importance of biological methods (Bioremediation)

Bioremediation of wastewater involves the use of living organisms, primarily microorganisms, to degrade the environmental contaminants into less toxic forms. These naturally occurring bacteria and fungi or plants that are locally available to degrade or detoxify substances, hazardous to human health and/or the environment. The microorganisms may be indigenous to a contaminated area or they may be isolated from elsewhere and brought to the contaminated site. Contaminanting compounds are transformed by living organisms through reactions that take place as a part of their metabolic processes. Biodegradation of a compound is often a result of the actions of

multiple organisms. When microorganisms are imported to a contaminated site to enhance degradation, that process is known as bio-augmentation. Bacteria are most commonly used microorganism for bioremediation processes. For decolorization of dyes in wastewaters a *Phanerochaete chrysosporium* which is a white rot fungus has been used extensively (Kirk et al., 1992) which is also correlated with its ability to synthesize lignin degrading exoenzymes such as lignin and manganese peroxidases (MnP) (Harazono et al., 2003) or Laccases (Sharma et al., 2009).

It is found that biodegradation of dyes is less effective via activated sludge treatment unit. But further, many microbes such as algae, bacteria, fungi and yeasts have been reported which have the ability to decolorize and mineralize the dyes under favourable environment conditions. Significant literature is available which discuss about the microbiological and physiochemical approached for decolorization of dyes (Banat et al., 1996). The treatment by microbes facilitate as the cheap and eco-friendly approach for removal of toxic dyes and metals from textile effluent. Due to diverse nature of microbes, they serve as the invaluable tool for biotreatment of effluent (Olukanni et al., 2006). Different biotechnology approaches have been considered by researchers as a potent approach for remediation of pollutant in an eco-friendly manner (Robinson et al., 2001).

Many reports have been documented which discuss the ability of bacteria to decolorize the dye, either in its pure form or in consortium (Robinson et al., 2001). The microbial consortium shows significant advantages in comparison to pure isolated for the degradation of synthetic dyes (Pal and Vimala, 2012). As different isolate may amend dye components over different positions or may expend the degraded products produced via other strain for further degradation (Forgacs et al., 2004). Documented literature proposes to use the microbes for remediating of dye and metal constituents from contaminated soil and water (Robinson et al., 2001). Thus, we need to explore the role of microbes for the restoration of our natural resources especially belonging to Ludhiana District of Punjab, the hub of textile sector, India.

The present study is a step towards the development of eco-friendly approach for the large-scale problem (effluent) prevailing due to the operation of textile dyeing units. The primary focus is to isolate the dye decolorizing bacteria from textile effluent

collected from Ludhiana district, Punjab, India and the assessment of these isolates to degrade the chosen commonly used dyes i.e. disperse dyes were analysed. The success of biotreatment was achieved when the treated water showed the effective result for germination of *Triticum aestivum,* which favour the usage of treated water in irrigation and life-supporting processes. Although, the microbes are omnipresent in nature, and continuous exposure of these toxic dyes evolves these microbes to detoxify and decolorize the chemical agents. The hypothesis of this study was that, the bacteria under constant exposure to the synthetic dyes alone, can be effectively used in remediating the textile mediated water pollution.

1.7 Scope of the current study

The conventional effluent treatment technologies in the form of physical and chemical treatment systems are not sufficient for the treatment of textile wastewater due to the presence of recalcitrant azo bonds and low biodegradability of textile dyes. Moreover, these methodologies are costly, inefficient and have less adaptability to diverse categories of dyes. In comparison, bioremediation is cost effective, safe and an economically feasible technique. Therefore, in the present study we focused on the use of naturally occuring for the degradation and decolorization of disperse dyes that are being used in the textile industries of Ludhiana city, Punjab, India.

1.8 Social impact of the research

- We are providing a biological technique for decolorization of various textile dyes.
- Use of adapted microorganism (bacteria) for the treatment of textile effluents containing dyes will serve dual purposes at the same time, one is low cost and another is eco-friendly approach for degradation of dyes.
- We are trying to improve the existing water quality for aquatic life and use of treated wastewater for irrigation purposes.

REVIEW OF LITERATURE

Chapter 2

REVIEW OF LITERATURE

2.1 Textile industries and water pollution

The textile industry has been one of the major polluters of surface and ground water resources because it uses as many as 8,000 chemicals and huge amount of water. Several reports suggest that an average sized textile industry consumes about 1.6 million litres of water per day for the production of about 8,000 kg of fabric (Khandare et al., 2015). According to the World Bank, 17% to 20% of textile industry water pollution originates by the steps used for dyeing and printing treatments that apply to the fabric (Holkar et al., 2016). The wastewater discharged from textile dyeing industry contains a total of 72 toxic chemicals out of which 30 chemicals cannot be removed by waste treatment processes (Chen and Burns 2006). The demand of chemical based textile dyes have increased significantly due to their cost-effectiveness and high stability of various parameters like temperature and light in contrast to natural dyes. This leads to discharge of highly polluted effluents (Couto, 2009). These textile dyes, which are readily soluble in water, have resulted in negative effects on the fragile ecosystems around the industries and represent serious environmental problems. Textile effluent due to its dark color usually blocks sunlight, which hinders the life of aquatic organisms (Ghaly et al., 2014). Currently, the dyes used in the textile mills are approximately ten thousand in number, out of which azo-dyes constitute the biggest and very recalcitrant category of dyes on a commercial scale (Chung and Chen, 2009). The release of these synthetic azo-dyes in the environment has detrimental effect on all forms of life (Singh and Arora, 2011). Textile printing and dyeing processes include pretreatment of fabric, dyeing with synthetic dyes, printing and finally finishing steps with each step leads to water contamination.

Organic and inorganic solid materials, acids and bases, coloring agents are the major contaminants present in the wastewater. Tannin and lignin are the coloring agents which assign color to the fabric (Singh et al., 2012). Dyeing basically includes application of dyes under appropriate conditions, to produce colored fabrics. In contrast, printing includes application of dyes to a restricted area on the fabric that is selected for applying the abstract of the design (Gausa and Abubakar, 2015). Finishing processes comprise

softening, cross-linking and water proofing but all these steps lead to water contamination (Babu et al., 2015). With these processing steps, different phases of production do exist, such as yarn and fabric formation, wet processing and textile fabrication. However, synthetic dyes pose rampant health hazards when they react with other chemicals and form non-degradable by-products. The presence of heavy metals (such as Cu, As, Pb, Cd, Ni and Co), soaps, detergents, sulphur and acetic acid have been reported by researchers to cause toxic effects. Organic materials such as formaldehyde based agents use to fix the dyes to fabric, chlorinated agents use to remove stain, softeners based on hydrocarbons, and certain other non-biodegradable synthetic chemical agents are carcinogenic. The effluent coming from textile dyeing mills is not only toxic, but it is also enriched by the presence of mutagenic, and teratogenic as well as carcinogenic chemicals (Novotny et al., 2006). For instance, the well-known carcinogen, benzidine, is the parent component of most of the azo dyes, which poses a threat to living organisms (De Aragao et al., 2005). The experimental studies on animal model conducted by Raj et al., (2015) showed that the main class of textile dyes, i.e. azo dyes is directly connected to bladder cancer in humans; to splenetic sarcomas; hepatocarcinomas which is the main cause of aberration of chromosomes in mammalian cells. Sudova et al., (2007) reported that malachite green being an important carcinogenic agent has been shown to cause serious effect on human reproductive system and on the immune system. Disperse blue 291 dye has been reported to cause nucleotide substitution in the DNA and addition or deletion of some base pairs which leads towards some errors in genetic code (De Aragao et al., 2005). Due to high thermal and photo stability of dyes, dyes can persist in the water environment for longer time period if left untreated. For instance, reactive Blue-19 in hydrolyzed form, having a half-life of about 46 years at 25°C and pH 7.0, can persist in polluted waters for years (Carmen and Daniela, 2012). Therefore, in current study an effort has been made to review the entire literature on cost effective and eco-friendly methods to degrade textile dyes present in textile effluents using biological approaches.

2.2 Textile Dyes

A total of about 10,000 different types of dyes currently being used in textile industry and more than 7 x 10^5 tons of synthetic dyes are produced worldwide annually (Anburaj

et al., 2011). Dyes are used for imparting color to the fabric because of the presence of chromophoric groups in their structure. The solubility of these dyes in water also differentiates them from one another. Dyes are predominantly classified on the basis of fibers on which it is to be imprinted and the chemical/physical nature of these dyes (Wesenberg et al., 2003). Chromophores mainly comprise a group of atoms which gives distinct color to the dye whereas the color enhancer named auxochromes is achieved by the electron substitution either by removing or donating. Chromophores contain functional groups such as –N═N–, –C═O, $–NO_2$ and $O=(C_6H_4)=O$ (quinoid assemblies) whereas the auxochromes of the functional groups such as $–NH_3$, –COOH, –OH and $–SO_3H$ (Srinivasan and Viraraghavan, 2010). Waqas et al., (2015) studied different auxochromes and classified into different categories of reactants, which could be acid or base, or astringic or disperse, pigmented, vat, anionic or in grain form, and also may be in dissolved or undissolved form. Dyes are also categorized on the basis of the reactive groups that are in association with -OH and -SH through the covalent bond formation which are then used on different fibers such as cotton, silk, nylon, and wool.

2.3 Textile Dyes: Impact on environment

The biggest threat to the environment is presence of untreated dyes in water bodies because many of these reactive dyes hinder the photosynthetic process of hydrophytes by blocking the light to penetrate, which further leads to the synthesis of toxic substances that are harmful against the marine organisms (Wang et al., 2009). The marine organisms are not the only one which is affected, but the flora and fauna of marine environment, soil fertility, and plant growth are also affected by toxic substances present in dyes. The colored discharge water from industries increases water pollution and are visually unpleasant (Damodhar and Reddy, 2013). Several studies have shown the unpleasant reality of these dyes which are initiator of cerebral carcinoma and skeletal abnormalities in the fetus. Murugesan and Kalaichelvan (2003) revealed that toxic aromatic amines are formed under the oxygen deficient environment in receiving media due to the discharge of untreated dye effluent. Some of the reports on azo dyes showed their linkage with many types of cancers of different organs such as bladder, spleen, liver and normal aberrations in model organisms and chromosomal deformities in mammalian cells (Murugesan and Kalaichelvan, 2003).

The major challenge associated with the treatment of wastewater containing dyes is related to degradation process. Different types of microbes have been employed under anaerobic environment for decolorizing the azo based dyes. Kirk and Farrel, (2017) studied the action mechanism of decolorization of azo bonded dyes under anaerobic condition using bacteria where electron transfer directly to the azo dyes resulting in bacterial catabolism and ATP generation. Nevertheless, Damodhar and Reddy, (2013) stated in their research that no bacteria catabolize the azo dyes for the synthesis of ATP as the final product instead, these azo dyes are reduced by Fe^{2+} or H_2S producing inorganic compounds under the anaerobic environment. Islam and Huda (2016) heightened the problems associated with discharge of effluents from textile industries causing hindrance in the photosynthesis process and affecting the marine bodies and phytoplankton negatively. The effluent discharged from these textile industries impart corrosive traits as well as additions to their physical traits such as unpleasant smell, decreased dissolved oxygen in water, and increase in insoluble materials in water. The colloidal substances present in the effluent when merge with fresh water streams alter water turbidity. Therefore, these dissolved impurities give rise in the salt level in the water, which makes it inappropriate for human consumption and irrigation. Cr and SO_3 are the toxic contaminants posing a serious threat to marine life and microorganism that are useful for the purification of the water streams. Sudden change in the amount of dissolved oxygen takes place due to the presence of nitrates, nitrites, starch and sulfides (Asamudo et al., 2005). Debris remains of organic materials such as starch cotton are degraded by bacteria which increases the demand for dissolved oxygen in water. Different chemicals used in textile processing cause the variation in pH in the water affecting the marine life (Singh et al., 2011). Solids which are dissolved in the effluent get settled on the surface of the sewers and cause corrosion to waste treatment pipes. Untreated textile industry discharge to water bodies poses a greater risk to ecosystems (Singh et al., 2011).

The effluent from textile industries affects negatively to soil fertility, plant growth and productivity of plants and make the plants susceptible to pathogens. This affects plant germination, seed survival, shoot and root length, etc. Chlorophyll content of the plants also decreases if textile industry effluent is used for irrigation. The decline in plant

protein, carbohydrates, and chlorophyll is a clear indication of toxic effects of textile dye effluents on plants (Jayanthy et al., 2014).

It has been found in the clinical trial of model organisms that azo dyes are responsible for the formation of liver nodules. Also, laborers working in the azo dye industries have shown susceptibility of developing the bladder cancer. A number of the azo dyes prompt the formation of nodes in liver in the experimental models (Puvaneswari et al., 2006). It is also suggested that laborers have high chances of bladder cancer as they are extremely exposed to large amounts of benzidine based azo bonded dyes (Golka et al., 2008). Puvaneswari et al., (2006) studied the consequences encountered on releasing benzidine and benzidine analogue to model organisms such as canines, hamsters, and mice, which provides the outcome indicating existence of probable aromatic amines and their associated N-acetylated derivative in their urine which can be tumorigenic. These studies clearly show that some of dye products used in the textile industry can cause cancer and other ailments in humans and other aquatic life.

2.4. Treatment Options for Dye Effluent

Nowadays, the concern is primarily focused on developing economical and effective methods to treat wastewater discharge from the textile industries to protect the aquatic life in water bodies. Therefore, the methods could be physico-chemical, biochemical or the combination of both which can provide effective technologies in removing contaminants from wastewater coming from the textile industries. Wesenberg et al., (2003) reported that physical and chemical methods can be cost-effective but they do not guarantee the complete elimination of the contaminants.

2.4.1 Physico-chemical methods

Although physico-chemical methods of wastewater treatment are easy to use, but these methods may not be always cost effective and environmental friendly (Gaehr et al., 1994). High electricity consumption is required with less output because of generations of the high number of byproducts and sludge which cannot be reused (Fajardo et al., 2016). These methods are not single step rather multistage treatment process with long retention time is the necessity. Generally, the chemical approaches are utilized for elimination of organic contaminants by employing coagulation as well as flocculation approaches (Ukiwe et al., 2014). Kim et al., (2002) highlighted efficacy of the coagulation treatment against the insoluble dye material on comparing with the soluble

dye material in the water. The chemicals which are used in coagulation processes, precipitation as pH modification are highly expensive. Major issues which limit the use of chemical method includes dewatering, pH modification, high levels of residues associated in the supernatant, cost towards precipitation, sludge generation and its disposes. The merits and demerits of various physio-chemical methods used for treatment of textile dyes are represented in Table 1. This table indicates that physio-chemical methods are available for treatment of textile industry effluents, but energy and environmental costs are high.

Adsorption, ion-exchange, oxidation process and irradiation are a few of the physical approaches that have been used for treating wastewater and have provided valuable results (Aplin et al., 2000). Significant absorption ability of the acid dye was shown by the absorbent chitin as it comprises amino nitrogen. Daassi et al., (2016) conveyed that waste materials contain an ample number of adsorbents which have the ability to decolorize the dye as well as colored organic material that provide color to the media even at low prices. Adegoke and Bello (2015) acknowledged about the activated carbon and its adsorption capability which has the potential to treat both acid and basic dyes. Abu-Saied et al., (2013) provided the insight about the inability of the adsorption method in treating the undissolved dye materials and finding the alternative desorption method. The irradiation technique has the potential in treating different colored water of small volume but requires high amount of dissolved oxygen. Whereas, the ion exchange methods manifest more deteriorating results in treating different dyes and show inadequate response in the presence of other additives in the same wastewater.

Table-1: Advantages and disadvantages of existing various physico-chemical methods available for treatment of textile dyes

S. No	Physico-chemical methods	Applications	Merits	Demerits	References
1	Carbon nanotubes	Point-of-use, heavily degradable contaminants (pharmaceuticals, antibiotics)	Highly accessible sorption sides, bactericidal, reusable	High production costs, possibly health risk	Qu et al., (2013)
2	Cucurbituril	A cyclic polymer with hydrophobic cavity and low solubility in aqueous solutions used for decolourization of dyes	Good sorption capacity for various dyes	High cost	Ramalho et al., (2002)
3	Electrochemical destruction	Environmental compatible, as its main reagent, the electron, is a clean reagent	Breakdown compounds are non-hazardous	High cost of electricity	El-Ashtoukhy et al., (2013)
4	Ion exchange	A ion-exchange resin captures charged ions formed due to breakdown of dyes	Regeneration: no adsorbent loss	Not effective for all kind of dyes	Raghu and Basha, (2007)
5	Irradiation	It generates radical species which reacts rapidly with textile dyes and leads to the destruction of its chemical structure.	Effective oxidation at lab scale	Requires a lot of dissolved O_2	Chen et al., (2006)
6	Ozonation	A powerful disinfection and a strong oxidant to remove colour and odour, eliminating trace toxic synthetic organic compounds and assisting in coagulation	Applied in gaseous state only	Short half-life (20 min)	Al-Kadsi et al., (2004)
7	Nanoparticles (Ag, ZnO, TiO_2, coated ceramic filters)	Large surface area, small diffusion resistance, high adsorption capacity, fast adsorption equilibrium	Offers an effective and economic way to environmental bioremediation protection	Leaching problem	Hosseinnia et al., (2010)

8	Nanoadsorbents	Point-of-use, removal of organics, heavy metals, bacteria	high specific surface, higher adsorption rates, small footprint	high production costs	Gehrke et al., (2015)
9	Nanometals and nanometal oxides	Removal of heavy metals (arsenic) and radionuclides, media filters, slurry reactors, powders, pellets	short intraparticle diffusion distance compressible, abrasion-resistant, magnetic photocatalytic (WO_3, TiO_2)	less reusable	Gehrke et al., (2015)
10	Membranes and membrane processes	All fields of water and wastewater treatment processes	reliable, largely automated process	Relative high energy demand, Concentrated sludge production	Gehrke et al., (2015)
11	Magnetic Nanoparticles	Wastewater Treatment	Provides stability, Protection from oxidation, provides surface to which contaminant specific ligands are attached.	In vitro cytotoxicity	Costa et al., (2011)
12	Peat	Peat's ability of cationic exchange is its exclusive property	Good adsorbent due to cellular structure	Specific surface areas for adsorption are lower than activated carbon	Wang et al., (2011)
13	Polymeric Nano adsorbents (dendrimers)	Removal of organics and heavy metals	Bifunctional (inner shell adsorbs organics, outer branches adsorb heavy metals), reusable	Complex multistage production process	Chou and Lien, (2011)
14	Photochemical with H_2O_2	The generation of highly reactive and non-selective hydroxyl radicals, which are able to degrade many organic pollutants	No sludge production	Formation of byproducts	Lodha et al., (2010)
15	Sodium Hypochlorite	The strong oxidizing potential of sodium hypochlorite towards the	Initiates and accelerates cleavage of azo bond	Release of aromatic amines	Wang et al., (2011)

		decolourization /degradation of dyes			
16	Silica gel	The high surface area, good sedimentation ability and great adsorption of SiO2 particles facilitates significant dye degradation	Effective for basic dye removal	Side reactions prevent	Zhao et al., (2008)
17	Wood chips	Wood chips had positive effects for degradation of the dyes from wastewater as they provide are natural environment for living microorganisms	Good sorption capacity for acid dyes	Requires long retention times	Turgay et al., (2011)
18	Zeolites	Disinfection processes	Controlled release of nanosilver, bactericidal	Reduced active surface through immobilization of nanosilver particles	Turgay et al., (2011)

2.4.2 Biological methods

Biological methods, i.e. degradation of dyes with the biological phenomenon such as bioremediation is an eco-friendly and economical technique with less operating time for removal of dyes from textile effluent (Ekambaram et al., 2016).

Biological based methods employed for degradation of the effluent from the textile industries have been successfully used. The biological degradation (i.e. bioremediation) is economically feasible, environmental-friendly and generates less volume of sludge when compared with other techniques. It causes the degradation of synthetic dyes to a comparatively less toxic inorganic compound because of breakdown of bond (i.e. chromophoric group) and finally helps in removal of color (Babu et al., 2015). The catabolism of the azo dyes takes place in two steps, firstly the dyes underwent through the breaking of the azo bonds forming the amines, and secondly the aromatic amines are further catabolized to small non-toxic molecules under aerobic environment (Chequer et al., 2011). The techniques are being developed to use the ability of bacteria to survive under the aerobic and anoxic conditions for the complete degradation of the azo bonds formed within the dyes.

Leena and Selva, (2008) gave the insight that biological process is efficient in reducing the COD and turbidity but inefficient in removing the color. Muda et al., (2013) reported success and benefit of the two-phase process in developing biological methods for decolorization in a future where the first phase involves anaerobic processes followed by aerobic process.

2.4.2.1 Fungal biodegradation of dyes

Different fungal species have been reported in the literature and several species have been tried for treating the wastewater discharge from textile dyeing industries (Aust, 1990). Combinations of aerobic and anaerobic treatment by different microbes have shown promising results for biological degradation of textile dyes (Lade et al., 2012). Several studies on fungal based dye decolorization have been conducted to replace present chemical and physical treatment methods (Placido et al., 2016). *Phanerochaete chrysosporium* emerges as an efficient remediation model for treating the effluent from textile, pulp and paper industries which contains polycyclic aromatic hydrocarbon (PAH). Senthilkumar et al., (2014) studied *Phanerocheate chrysosporium*, which produces extracellular enzymes such as, laccase and lignin peroxidase mediated decolorization of various dyes. Singh and Singh (2010) examined another fungus named as *Trichoderma harzianum* which has been employed for the cleaning the effluent from the textile industry. A fixed amount of Congo red and Bromophenol blue dyes were completely degraded by fungal mycelium by using semi-solid PDA medium. This study showed that the medium containing Congo red, Direct green, Bromophenol blue, Acid

red and Basic blue inhibited the growth of *Trichoderma harzianum* in comparison to the control treatment (growth in the medium without dye). Out of these dyes, Bromophenol blue was found to show the maximum growth inhibitory effect (43%). For bioremediation, diverse fungal cultures were used due to their characteristic features such as the fast growth, large amount of biomass and wide hyphal spectra which made fungus more effective as compared to the bacteria (Anastasi et al., 2013). Chairin et al., (2013) cited filamentous fungi as an effective degrader under certain niches because of their high surface-to-cell ratio. Singh et al. (2012) studied *Aspergillus* strain (MMF3) and optimized NaCl concentration for the effective degradation of textile dye orange 3R and reported efficiency about 96% on the 5^{th} day under ideal conditions. At 1.0% concentration of NaCl, the rate of decolorization of dye was 56%. With further increase in NaCl concentration up to 2.0%, the rate of dye decolorization decreases up to 42%. Maximum dye decolorization rate was observed on the 5th day at various NaCl concentrations. Due to high decolorization ability and ability to function in naïve conditions made this fungal strain of interest in biological treatment of the effluents of dyeing mills. Verma et al., (2012) reported the ability of marine derived fungus which can degrade the Reactive Blue-4 dye belonging to the category of anthraquinone dye and can also be utilized in bioremediation process. Degradation procedure works in two steps involving first the partial treatment of purified laccase and then with compounds (with low molecular weight) as the final enzymatic degradation process. The results confirmed 29% reduction in total carbon and a twofold reduction in toxicity. Corso et al., (2012) reported the paramorphogenic form of *Aspergillus oryzae*, which has been exploited for biosorption of azo dyes present in industrial wastewater. Chairin et al., (2013) studied decoloration and deprivation of bisphenol-A and other synthetic dye by using the laccase enzyme isolated from *Trametes polyzona* strain WR710-1. Anthraquinone dye (Reactive Blue 4) which showed decolorization of about 61% and reduced the COD by twofold in 12 hr as a result of laccase treatment. Characterization of metabolites synthesized during the whole process was done by mass spectrometry, UPLC, and UV-vis spectroscopy.

Biodegradation coupled with ozonation has been investigated recently for eliminating tannins along with dyes to minimize pollutant load in the effluent. Various fungal cultures, namely *Penicillium* sp. and *Aspergillus niger* have been extensively studied for the biodegradation of wattle extract and various synthetic dyes. This hybrid model of biodegradation and ozonation proved very successful for removal of tannins and dyes. The ozonation was better off than chemical methods when combined with biodegradation due to its better rate of degradation.

This combined model minimizes the pollution level of different physical parameters like BOD, COD, TOC, TSS and TDS (Kanagaraj and Mandal, 2012).

2.4.2.2 Bacterial degradation of dyes

Variety of microorganisms have been studied, but not many dye degradation bacteria have been identified. In the context of bioremediation, numerous assessments on bacteria having the ability to catabolize organic pollutants have been investigated (Ngulube et al., 2017).

The main advantage to work with bacteria is that they are easy to culture and can grow more quickly as compared to other microbes. The dye degradation ability of bacteria can easily be enhanced by the molecular genetic manipulation. Bacteria are able to catabolize chlorinated and aromatic hydrocarbon based organic pollutants, which can be decomposed by using them as an energy source (C source) (Yang et al., 2014) and have the ability to oxidize sulphur based textile dyes (Sulphur blue 15) to sulphuric acid (Nguyen et al., 2016). Many studies have shown favorable results in identifying bacteria which can degrade different azo based dyes at a faster rate. Different bacterial group under traditional aerobic, anaerobic and under extreme oxygen deficient conditions causes an azo dye reduction for decolorization. The chemical reaction during the reduction of the azo dyes starts with the breaking of azo bonds (-N=N-) under anaerobic environment by the azoreductase enzyme which results in a colorless solution of aromatic amines. Wang et al., (2014) reported that metabolites formed as a result of dye reduction can further be catabolized either by aerobic or anaerobic processes. The intermediate products synthesized during dye decolorization can also be reduced by other enzymes such as hydroxylase and oxygenase that are also produced by the bacteria (Elisangela et al., 2009). Several bacteria have been found to possess the ability to breakdown azo bond in textile dyes and resulted to produce colorless amines. Ali et al., (2011) studied the behavior of aerobic bacteria that were able to propagate in the presence of azo compounds. The intermediate sulfonated amines formed in this process may be aerobically degraded. The gram +ve bacteria such as *Clostridium* sp., *Bacillus* sp, *Brevibacillus* sp. *Paenibacillus azoreducens* and gram – ve bacteria such as *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas putida*, *Citrobacter* sp. and *Escherichia coli* also possess promising decolorizing efficacy on various dyes. This section shows that several studies have indicated on the possibility of identifying naturally occurring bacteria in the natural water and soil systems to biodegrade textile wastewater. Table 2 gives a list of various published studies on decolorization efficiency using different bacterial species for degradation of textile dyes in textile effluent. Future research needs to concentrate in this important area of microbial sciences to solve industry's environmental problems.

Table-2: Decolorization of various textile dyes by bacterial culture

S. No.	**Textile dyes**	**Bacteria**	**Reference**
1	Acid Red 128	*Bacillus endophyticus* VITABR13	Prasad and Rao, (2011)
2	Reactive Black-5, Direct Red-81, Acid Red 88	*Shewanella* sp. Strain IFN4	Imran et al., (2014)
3	Foron Yellow RD5GL, Foron Red RDRBLS, ForonRubine RDGFL, Foron Black RD3GRN, Foron Blue RDGLN Foron Turquoise SBLN	*Ganoderma lucidum* *Coriolus versicolor*	Sadaf et al., (2013)
4	Crystal Violet	*Bacillus subtilis*	Kochher and Kumar, (2011)
5	Reactive Blue 19; Dark Green Dye	*Enterococcus sp. and Klebsiella sp*	Gulati and Jha, (2014)
6	Crystal Violet	*Pseudomonas aeruginosa, Clostridium perfringens, Proteus vulgaris*	Ali and Akthar, (2014)
7	Light Red Dye	*Proteus* sp., *Klebsiella* sp.	Sethi et al., (2012)
8	Toludine Red	*Rhodococcus rhodochrous*	Shah, (2014)
9	Congo Red (Direct red 28), Direct blue 80	*Pseudomonas putida, Klebsiella ozaenae*	Shifanki et al., (2015)
10	Reactive red 4E8Y5	*Providencia* spp. RMG1, *Bacillus* spp. RMG2	Gudmalwar et al., (2012)
11	Crystal Violet	*Enterobacter* sp. Strain VP-64	Hemapriya and Vijayanad, (2014)
12	Basic Red 46	*Staphylococcus epidermidis* MTCC 10623	Pokharia and Ahluwalia, (2016)
13	C.I. Acid Blue 113 (AB-113) C.I. Basic Red 46 (BR- 46) C.I. Direct Blue 151 (DB-151)	*Pseudomonas aeruginosa, Pseudomonas putida*	Falavarjani et al., (2012)

	C.I. Direct Brown 2 (DB-2) Mixture of four dyes (Mix)		
14	Provisional Pink	*Bacillus* sp., *Aeromonas hydrophila* sp.	Celia and Suruthi, (2016)
15	Congo Red	*Bacillus pumillus*	Modi et al., (2015)
16	Indigo Dye	*Actinomyces* sp. (PK07), *Pseudomonas* sp. (PK18), *Stenotrophomonas* sp. (PK23), S*taphylococcus* sp. (PK28)	Rajendran et al., (2015)
17	Alizarin	*Burkholderia* sp.	Sharma and Sharma, (2015)
18	Drimarene Red	*Bacillus* sp. SG2	Oak et al., (2016)
19	Methyl red and Navy blue	*Bacillus* sp.	Ezhilarasu, (2016)
20	Remazol Blue	*Pseudomonas aeruginosa, Ochrobactrum* sp.	Kilic and Donmez, (2012)

Table-3: Decolorization of different dyes under anaerobic conditions by bacterial cultures

S. No.	**Textile dyes**	**Bacteria**	**Reference**
1	Congo Red and DB 38	*Escherichia coli*	Isik and Sponza, (2003)
2	Methyl-Orange	*Pseudomonas* sp.	Silveria et al., (2009)
3	Reactive Dye Red 22	*Escherichia coli*	Chang and Kuo, (2000)
4	Congo Red and Reactive Black 38	*Pseudomonas* sp.	Isik and Sponza, (2003)
5	Reactive Blue 13	*Pseudomonas* sp.	Lin et al., (2010)
6	Congo Red	*Mutant Bacillus* sp. ACT2	Gopinanth et al., (2009)
7	Napthol Green B	*Shewanella oneidensis* MR-1	Xiao et al., (2012)
8	Reactive Violet 5R	Bacterial mixed culture- SB4	Jain et al., (2012)
9	Remozol Orange	*Pseudomonas aeruginosa*	Sarayu et al., (2010)
10	Methyl Red	*Halomonas elongata, Escherichia coli*	Eslami et al., (2016)
11	Methyl Red	*Aeromonas jandaei* strain SCS5	Sharma et al., (2016)
12	Mono Azo Dye	*Clostridium perfringens*	Morrison and John, (2016)

2.4.2.3 Aerobic and anaerobic degradation of dyes

Panswad and Luangdilok (2000) have reported that reduction of azo bonds can further be catabolized under ideal aerobic environment as shown by Eq. (1) below:

$$Ar\text{-}N{=}N\text{-}A\acute{r} + 4e^- + 4H^+ \longrightarrow ArNH_2 + H_2NA\acute{r} \quad \text{.....Eq. (1).}$$

Van der Zee and Villaverde, 2005 conducted anaerobic study and found that organic carbon is needed as the energy source for catabolizing dyes into decolorized water under anaerobic environment. This shows the carbon source could be a simple source such as $C_6H_{12}O_6$, $C_2H_3O_2^-$ or a complex source such as tapioca or whey which is most extensively used for to complete anaerobic reaction for the decolorizing process. Table 3 emphasize list of various studies conducted by different researchers on the decolorization of azo bonded dyes under anoxic environments.

Recently, many strains of bacteria were isolated that have the ability of decolorizing the azo dyes under aerobic environments. Palanivelan et al., (2014) reported that different bacterial strains need organic carbon as a source of energy for their growth. But certain bacteria have also been reported who possess the ability to survive on the azo complex as it utilizes it as a source of carbon (Pandey et al., 2007). Bacteria contain enzymes that cause the splitting of –N=N– bonds lead to amine formation and use them as carbon and energy source for their growth. These types of bacterial isolates are highly substrate sensitive. *Xenophilus* is one of these kinds of bacterial strains. Four such bacterial strains have been isolated which utilizes methyl red as sole source of carbon. The two of such bacterial strains are *Vibrio logei* and *P. nitroreducens*. No existence of the amine product was assessed in the growth medium signifying degradation of the media (Adedayo et al., 2004). Table 4 highlight various studies conducted on the decolorization of wastewater by using different aerobic bacterial communities. These studies show that the possibility of isolating right bacterial strains for developing bioreactors for decontaminating textile wastewaters.

2.4.2.4 Degradation of textile dyes using algal species

Algae is generally found in fresh water as well as in saltwater and are being extensively studied as a bio-sorbent (Devi et al., 2015). Due to their large surface area and binding ability, algae have the highest bio-sorption potential and electrostatic force of attraction

for contaminants in the wastewater. Several studies have shown that metabolization of contaminant present in the wastewater is of has various forms such as -OH, $RCOO^{-}$, -NH_2, and PO_4^{3-} and get absorbed on the algae surface (Al-Fawwaz and Abdullah, 2016). Decolorization of the dye by algae involves three different mechanisms. First, algae utilize the chromophores to harvest the algal biomass, CO_2 and H_2O; second algae contribute to the conversion of the chromophore material to the non-chromophore material; and third, the resulting chromophore is adsorbed onto the algal biomass (Alvarez et al., 2015). Many studies have been reported showing effective use of the algae against azo dye degradation by producing the azoreductase enzyme for decolorization (Telke et al., 2008). It has also been reported that species such as *Chlorella pyrenoidosa* for methylene blue (Pathak et al., 2015) and *Oscillotoria* are effective in azo dye degradation into aromatic amine that are further catabolized into organic compounds. Some studies have reported that different algae species utilize azo dyes as C and N source for their growth (Waqas et al., 2015). Table 5 gives a list of various references of several studies that have been conducted on the use of different types of algae and consortium of algae cultures to remove textile dyes and other contaminants from textile industry effluent.

Table-4: Decolorization of different dyes under aerobic conditions by bacterial cultures

S. No.	**Textile Dyes**	**Bacteria**	**Reference**
1	Reactive Red 22	*Pseudomonas luteola*	Chang et al., (2001)
2	Acid Blue 25	*Klebsiella* sp.	Prasad, (2015)
3	Remazol Blue	*Bacillus megaterium, Micrococcus luteus, Bacillus pumilus*	Karatay et al., (2015)
4	Acid Scarlet 3R	*Scheffersomyces spartinae*TLHS-SF1	Tan et al., (2016)
5	Reactive Orange 4	*Lysinibacillus* sp	Saratale et al., (2015)
6	Reactive Black 5 (RB 5)	*Pseudomonas entomophila* BS1	Khan and Malik, (2015)
7	Reactive Blue 5	*Streptococcus thermophilus*	Zhang et al., (2015)
8	Congo Red	*Acinetobacter baumannii* YNWH 226	Li et al., (2015)
9	Methylene Blue	*Shewanella onediensis* MR-1	Liu et al., (2016)
10	Reactive Green-19Remazol Navy Blue Reactive Red-198	*Enterococcus durans* GM13	Das and Mishra, (2016)
11	Reactive Red M8B	*Bacillus subtilis*	Arulazhagan, (2016)
12	Reactive Blue 222, Reactive Black 5	*Pseudomonas* sp. D4, *Salmonella* sp. D7, *Aeromonas* sp. D11, *Klebisiella* sp. D22, *Bacillus* sp. D31	Mohamed, (2016)
13	Congo Red, Evans Blue and Eriochrome Black T	*Staphylococcus lentus*	Chaieb et al., (2016)

Table-5: Decolorization of different dyes by various algae

S. No.	Textile dyes	Name of Algae	Reference
1	Acid Red 247	*Spirogyra rhizopus*	Ozer et al., (2006)
2	Triphenylmethane Dye Malachite Green	*Cosmarium* sp	Daneshvar et al., (2006)
3	Reactive Red	*Synechocystis* sp.	Karacakaya et al., (2009)
4	Malachite Green	*Pithophora* sp.	Kumar et al., (2005)
5	Methylene Blue	*Cystoseira barbatala*	Caparkaya and Cavas, (2008)
6	Astrazon Blue, Astrazon Red, Methylene Blue	*Caulerpa lentillifera*	Marungrueng and Pavasant, 2007
7	RGB-Red Dye	*Nostoc muscorum* (blue green algae)	Sinha et al., (2015)
8	Methylene Blue	*Ulva lactuca, Sargassum*	Tahir et al., (2008)
9	Methylene Blue	*Caulerpa lentillifera*	Marungrueng and Pavasant, 2007
10	Methylene Blue	*Caulerpa racemosa* var. *cylindracea*	Cengiz and Cavas, (2008)
11	Acid Blue 9 (Triphenylmethane)	*Spirulina platensis*	Dotto et al., (2012)
12	Methylene Blue (MB), Malachite Green (MG)	*Desmodesmus* sp.	Al-Fawwaz and Abdullah, (2016)

2.4.2.5 Use of enzymes in the degradation of textile wastewater

Enzymes are the best molecules that facilitate in sequential breakdown and degradation of complex compounds efficiently. Several bacterial species have been discovered to comprise unspecific cytoplasmic enzymes that act like azoreductases (Dave et al., 2015). Table 6 features various studies conducted using different enzymes for removing contaminants from textile wastewater.

Table-6: Involvement of different enzymes for the treatment of textile dyes

S. No.	Textile dyes	Name of Enzyme	Biological Source	Reference
1	Acid Red	Azoreductase	*Acinetobacter radioresistens* (Bacteria)	Ramya et al., (2010)
2	Reactive Blue 19 Reactive Blue 4 Reactive Black 5	DyP-type peroxidases	*Thermobifida fusca* (Bacteria)	Van Bloois et al., (2010)
3	Amaranth dye	Lignin peroxidase; Laccases; Tyrosinase; NADH–DCIP reductase; Riboflavin reductase	*Acinetobacter calcoaceticus* NCIM 2890 (Bacteria)	Ghodake et al., (2011)
4	Reactive Red BL	Azoreductase	*Alcaligenes* sp. AA09 (Bacteria)	Pandey and Dubey, (2012)
5	Direct blue 14	Laccases; Mn Peroxidase	*Pleurotus florida,* (Bacteria) *P. ostreatus* (Bacteria) *P. citrinopileatus* (Bacteria) *P. eryngii* (Bacteria)	Vishwakarma, (2012)
6	Reactive Yellow 107 (RY107) Reactive Black 5 (RB5) Reactive Red 198 (RR198) Direct Blue 71 (DB71)	Tyrosinase	*Brevibacterium* sp. (Bacteria)	Franciscon, (2012)
7	Reactive Orange 13	Veratryl Alcohol Oxidase	*Alcaligenes faecalis* PMS-1(Bacteria)	Shah et al., (2012)

		Tyrosinase NADH–DCIP reductase		
8	Acid blue-25 (AB-25)	Horse raddish peroxidase	*horseradish roots* (Plant)	Gholami Borujeni et al., (2013)
9	Direct yellow 12	Horse radish peroxidase	*horseradish roots* (Plant)	Maddhinni et al., (2013)
10	Reactive Red 141	Azoreductases	*Bacillus lentus* BI377(Bacteria)	Oturkar et al., (2013)
11	Methyl Orange	Laccases	*Coprinopsis cineria*	Tian et al., (2014)
12	Methyl Orange	Soyabean peroxidase Luffa actangula peroxidase	Bio wastes of soyabean hulls (Plant) Luffa skin peels (Plant)	Chiong et al., (2016)
13	Navy Blue HE2R	lignin peroxidase Tyrosinase Laccase Riboflavin reductase	*Ipomoea hederifolia, Cladosporium cladosporioides* (Plant-Fungus consortium)	Patil et al., (2016)
14	Reactive Red 195A Reactive Blue 21 Reactive Yellow 145A	Manganese peroxidase	*Ganoderma lucidum* IBL-05 (Fungus)	Bilal et al., (2016)

Several researchers have reported the applications of bacterial cytoplasmic azoreductases in environmental biotechnology (Shah et al., 2013). Azoreductase enzyme can be classified on the basis of the primary amino acid level which is rarely found (Morrison et al., 2014). The phenoloxidases namely laccase have a large potential to degrade waste products of many aromatic compounds. These laccases enzymes result in the degradation of complex polyaromatic polymers, named lignins. Laccases (EC 1.10.3.2) belong to the class of oxidoreductases which are further the member of multicopper oxidases and have the ability of oxidizing phenols, polyphenols, and anilines by removing the one electron. Manganese peroxidase is an enzyme which is the member of class oxidoreductases. It attacks the phenolic compounds through the intermediary redox reaction with the help of Mn^{2+}/Mn^{3+} ions, whereas lignin peroxidase

attacks the non-phenolic methoxy substituted lignin subunits which behave as substrate (Hosseinzadeh et al., 2016).

Laccase enzyme employs the mechanism of free radical that is non-specific in nature for executing the degradation of the dyes of azo without forming toxic aromatic amines. 97% decolorization of Orange G azo dye was obtained by *Trametes versicolor* (Casas et al., 2007). Kalme et al., (2007) obtained effective results by the use the laccase enzyme (recovered from the *Pseudomonas desmolyticum* strain NCIM 2112) showing 100% degradation of dyes such as Direct blue-6 and Red HE7B. All above studies clearly show the role of different enzymes in removing various contaminants from industry discharged wastewaters. These studies were not conducted to determine the long-term effect on aquatic life in water bodies in case some enzymes are released into water bodies during the decontamination process. Therefore, more research is needed to investigate the effect of different wastewater treatment technologies on the aquatic life and environmental health of the ecosystem.

The aim of this study is to gather/fetch information or bring an impression on the new treatment options available for textile effluent containing chemical based dyes. Regardless of the actual fact that no uniform and constant standard is available currently, several countries have set stringent discharging standards. Now a-days many researchers are trying to find the way out and implementing some new treatment options, hybrid technology, a combination of physical and biological methods. Though lot much of the research has been conducted on the decolorization of textile dyes with existing treatment methodologies, but little research has been conducted on the use of cutting edge technologies. Extensive literature is available highlighting removal of textile dyes using various physico-chemical processes such as coagulation, photo oxidation and chemical based degradation. Hence, this research focuses on the development biological methods (based on micro-organisms such as bacteria) using wdye degrading enzymes.

OBJECTIVES

Objectives: On the basis of above research questions, following objectives were developed for this study:

- To determine physico-chemical and biological characteristics of textile dyes in the untreated textile industry wastewater at selected effluent discharge sites
- To isolate, identify, and characterize dye-degrading bacterial strains from textile industry wastewater
- To develop bacterial consortium for enhanced dye degrading capability in textile industry wastewater.
- To design and develop clean-up technology (biofilters) for in-situ bioremediation of textile industy effluents

Thesis Structure

This thesis is written in a chapter format. Chapter 1 gives introduction to the statement of the problem leading to the proposed study. It gives an overview and classification of textile dyes and treatment options for textile industry effluent. Chapter 2 comprises of literature review which describes the relevant studies conducted by various researchers on this topic and their research findings. Chapter 3 outlines the hypothesis which includes research questions that drives the direction of the study. Chapter 4 highlights various objectives of the study. The materials and methods used to achieve the goals of objectives are described in chapter 5. The results and discussions of this study are given in chapter 6. Finally, the summary and conclusions of this study are presented in chapter 7.

MATERIAL AND METHODS

Chapter 5

Material and Methods

5.1 Study area

Ludhiana is a cosmopolitan city of Punjab and a hub of historical heritage, situated at 30 56' N and 75 52' E, which is positioned about 10 km south of the Sutlej river. The wastewater drain, Buddha Nallah (old rivulet) runs through the city carrying majority of city's domestic and industrial sewage and merges into Sutlej river (Figure 5). This drain gets transformed into a flooding stream during the monsoon season and pollutes the Sutlej river. Figure 5(B) shows the dark colour of Sutlej river water where Buddha Nallah enters the Sutlej river.

(A) **(B)**

Figure-5: Buddha Nallah flows through the city Ludhiana. 5(A) Buddha Nallah carries industrial and domestic waste; 5(B) point of confluence where Buddha Nallah merges with river Sutlej

5.2 Chemicals

All the chemicals pertaining to present study were acquired from Loba Chemie Pvt. Ltd., Mumbai, India. The mineral Salt Medium (MSM) contained (g/l): Potassium dihydrogen phosphate KH_2PO_4 -3.0 g/l; Na_2HPO_4 - 6.0 g/l; NH_4Cl-5.0 g/l; NaCl-5.0 g/l; $C_6H_{12}O_6$- 8.0 g/l; $MgSO_4$- 0.1 g/l (Khalid et al., 2008).

5.3 Sampling and field investigation

In the first part of the study, two textile-dyeing industries designated as site 1 and site 2, were chosen for this study. These two industries are located on Tajpur road and Bahadur-Ke-road near the bank of Buddha Nallah (old rivulet) drain in the city of Ludhiana. Textile industry effluent and surface soil samples were collected monthly for a period of twelve months at two discharge points. The locations of two sampling stations are shown in Figure 6. The water

samples from textile industry discharge points were collected in the morning between 7 am and 9 am twice a month during the period of June 2014 to May 2015. Sterilized glass bottles labelled were used to collect water samples using a sample code and then were transported to the laboratory of School of Biosciences and Bioengineering, Lovely Professional University, Phagwara, Punjab, in an ice box and stored at 4˚C (Ogunlaja and Aemere, 2009). Total of 48 samples were collected form two selected sites of textile dyeing industries, viz. site 1 and site 2 located at Tajpur road and Bahadur-ke-road, Ludhiana. Various physico-chemical parameters like pH, total dissolved solids (TDS), total suspended solids (TSS), water temperature (T), biochemical oxygen demand (BOD) and chemical oxygen demand (COD) were tested (APHA, 2005). In addition, month wise rainfall data (mm) was collected from the "Climate Change and Agricultural Meteorology department", Punjab Agricultural University, Ludhiana.

5.3.1 Analysis of textile industry effluent samples for physico-chemical parameters

To analyse different physical and chemical parameters in the water samples collected from textile industry discharge points, standard methods of APHA, (2005) were followed (Figure 7). pH of the effluent samples was calculated by using electrometric method and by using digital desktop, pH Meter (Systronics-335). Water temperature of the wastewater recoderded by using glass thermometer. Likewise, TDS and TSS data were analyzed by using the gravimetric method, evaporating dishes, and whatmann filter paper no.1. BOD5 of the wastewater was measured by Winkler's method in 300 ml BOD bottles. Likewise, COD was determined by dichromate refluxe method.

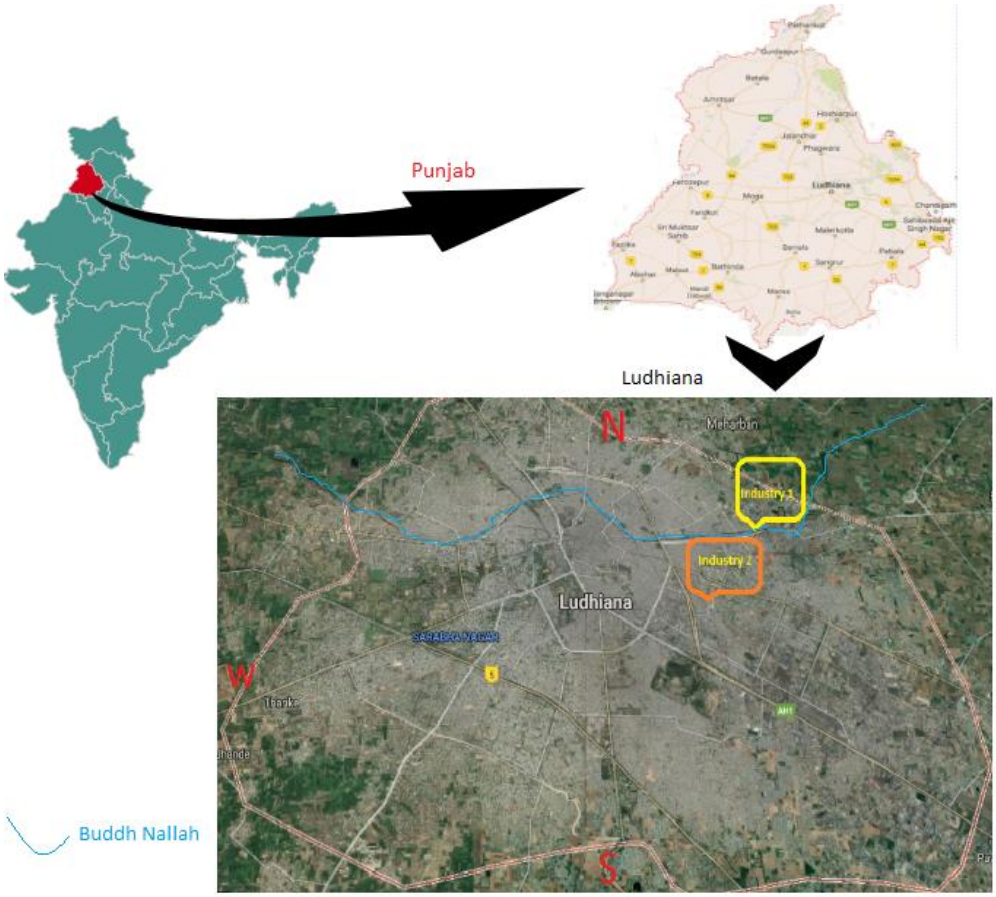


Figure-6: The map of Ludhiana showing the sampling sites (site-1 and site-2)

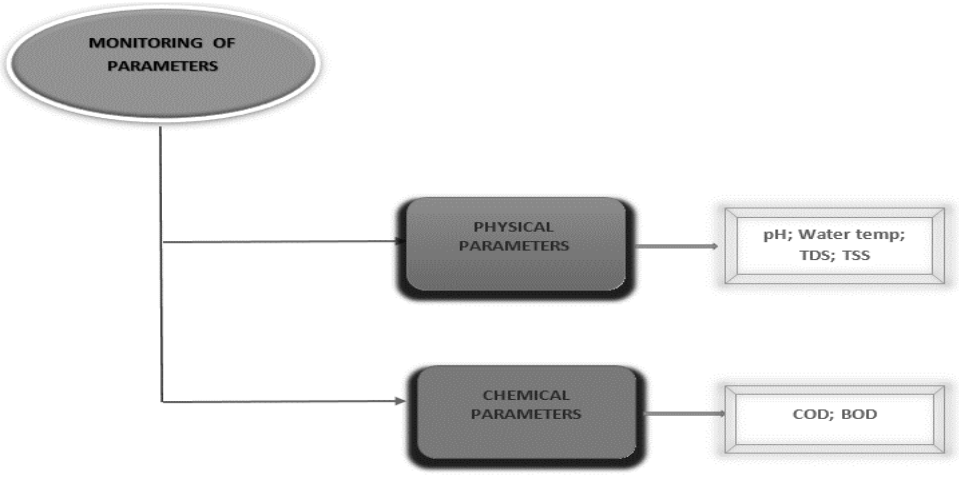


Figure-7: Grouping of parameters for monitoring of textile industry effluent

5.3.2 Data interpretation

To identify and interpret the relationship among different physico-chemical parameters, the data was collected for four climatic pattern seasons wise: i) rainy monsoon season (June 2014 to August 2014), ii) autumn season (September 2014 to November 2014), iii) winter season

(December 2014 to February 2015) and, iv) summer season (March 2015 to May 2015). An average of forty-eight data points of each sampling with respect to each selected parameter in a particular season was taken as one observed value. These data were analyzed statistically by using the JMP software and was represented in the form of Mean ± SEM. The student-t test was used to check the analogy and contrast in selected physicochemical attributes. Results in each experimental value were interpreted depending upon probabilities. If P-value was less than 0.05, results were found to be significant.

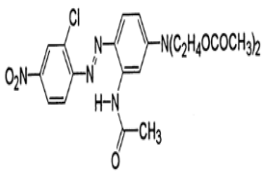
5.3.3 Correlation coefficients

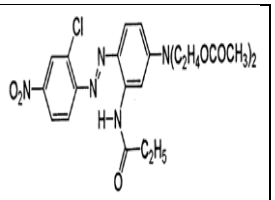
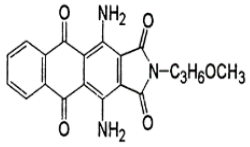
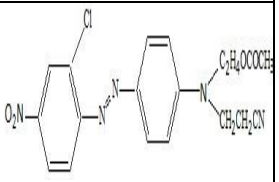
Correlation coefficients were calculated for the determination of relationships between various physio-chemical parameters for two different sites. The statistical analysis was done by using the JMP software, version 13.

5.4 Selection of dyes

The textile dyes disperse red 167.1 (rubine BL), disperse red 54 (scarlet RR), disperse red 167 (dark Red 2B) and disperse blue 60 (blue BG) were selected (generous gift from local textile industry) for the decolorization study. These dyes are used for both the seasons (winters and summers) to color polyester, acrylic and nylon fabrics in textile industries. Properties of dyes are given in table 8.

Table-8: Properties of selected textile dyes

Name of Dye	CI Name	CI Number	Molecular Weight	Molecular Formula	Structure
Rubine BL (azo disperse dye)	disperse red 167.1	11338:1	505.91	$C_{22}H_{24}ClN_5O_7$	

Dark Red 2B (azo disperse dye)	disperse red 167	11338	519.93	$C_{23}H_{26}ClN_5O_7$	
Blue BG (Anthraquinone disperse dye)	disperse Blue 60	61104	379.37	$C_{20}H_{17}N_3O_5$	
Scarlet RR (azo disperse dye)	disperse red 54	11131	415.83	$C_{19}H_{18}ClN_5O_4$	

5.4.1 Spectral analysis of selected azo-disperse dyes [to check absorption maxima (λ_{max}) of dyes]

The selected textile dyes were firstly studied for absorption spectrum by using UV-Vis double beam spectrophotometer (UV-1800, Shimadzu scientific works) in a range of 200nm to 900nm (Kalyani et al., 2008).

5.4.2 FTIR analysis of dyes

To check the presence of different functional groups, FTIR analysis of selected dyes was done in the mid IR region of 400-4000 cm^{-1} with 16 scan speed. The samples were prepared with spectroscopically pure KBr in the form of pellets (Lade et al., 2012).

5.4.3 Isolation and purification of dye decolorizing bacteria

Second part of this study was to identify and isolate those bacteria from textile industry wastewater that has the capability to decolorize the dye in wastewater stream. Bacterial strains were isolated from textile industry wastewater and soil samples on mineral salt medium (MSM) with serial dilutions from 10^{-1}-10^{-3} in physiological saline (Khalid et al., 2008) (Figure 8). Selected isolates were then purified by streaking it again on MSM pentagonal manner. The purification step was repeated thrice. After purification, the isolated cultures were preserved in the refrigerator at 4°C for successive decolorization and degradation study of selected dyes under investigation.

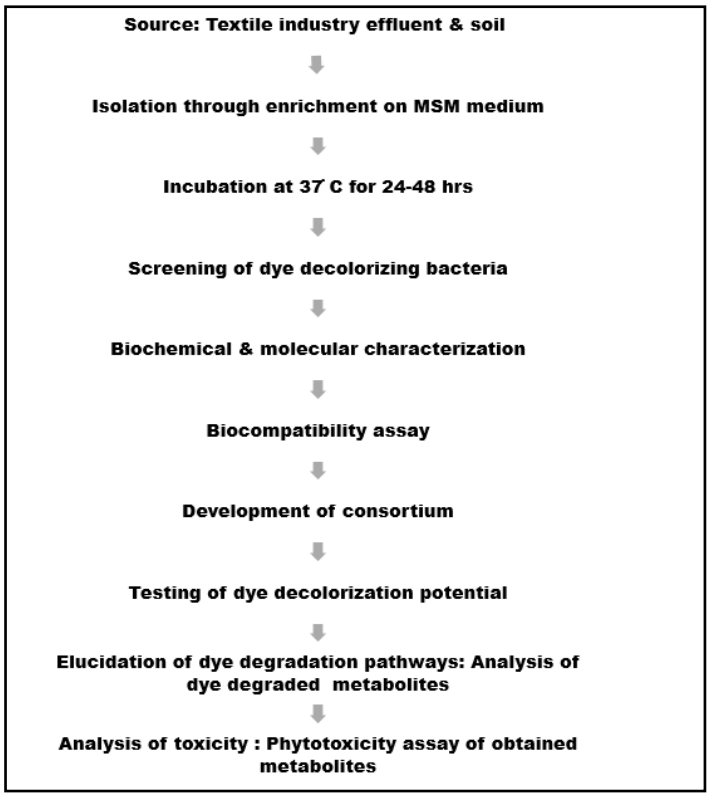


Figure-8: Key steps for isolation of efficient dye degrading bacteria and elucidation of dye degradation pathway

5.4.4 Dye decolorization assay at shaking condition

Fifteen isolates (IS-1 to IS-15) were cultured for 24 h in 250ml conical flasks having 100ml MSM broth and. Then, these isolates were modified separately using different concentration of each dye (50mg/L, 150 mg/L and 250 mg/L). The dye degradation competency of each isolates was examined under shaking conditions at 150 rpm with

temperature of 37°C in orbital shaking incubator (Khalid et al., 2008). After different interval of time, the 5 ml of culture media was taken and the sample was centrifuged for 10 min at 10,000 rpm for the separation of bacterial cell mass and supernatant. Thus, supernatant obtained was used for the analysis of decolorization ability. The test was conducted in triplicates. Abiotic controls were also included. Furthermore, dye decolorization assays with 15 isolated have been conducted out of which six isolates have been found to be effective in dye decolorization.

5.4.5 Biochemical, morphological and physiological characterization of bacterial isolates

The six selected bacterial isolates were further examined for their biochemical and physical characteristics including form, surface of colonies on petri plate, colony color and gram staining reaction. The biochemical and physiological properties of the selected bacterial isolates were also examined by catalase, indole, urease, oxidase test, Voges- proskauer and methyl red tests (Olutiola et al., 2000). The bacterial isolates obtained were then recognized by observing the characteristic features and comparative analysis of the standard description of bacterial strains in Bergey's Manual of Determinative Bacteriology.

5.4.6 Molecular characterization of bacterial isolates: DNA extraction PCR amplification of 16S rRNA gene

The 16S rRNA investigation was done to identify the bacterial strain which is effective in dye decolorizing. The isolate 1, 2 and 3 were sent to Samved Biotech Pvt. Ltd., Ahmedabad (Gujrat), India for identification of these bacterial strain. The PCR was performed on the isolated DNA to amplify the fragment of 16S rDNA gene. The amplified DNA of 1500bp size was resolved on the agarose gel and further analyzed for undue contaminants. For the amplification, the primers 27F (Forward) and 1492R (Reverse) were used with BDT v3.1 cycle sequencing kit on ABI 3730xl Genetic Analyzer. The BLAST analysis was done on the obtained sequences by comparing it with the nucleotide database of GenBank database. On the basis of the highest identity score, the 10 sequences were chosen and aligned with ClustalW. RDP database was employed for generating Distance matrix with MEGA 4 was employed for constructing the phylogenetic tree. For assessing the evolutionary history, the NJ method was employed (Saitou and Nei, 1987). The optimum tree was found to be of branch length = 0.00631033. The evolutionary distances were calculated with the help of the Kimura 2-parameter method (Kimura, 1980) and units of the number of base substitutions per site. The study comprises of 11 nucleotide sequences.

The isolate 6, 11 and 15 were sent to Yaazh Xenomics, East Chennai, Tamilnadu for molecular characterization. Genomic DNA of the bacteria was extracted using the InstaGeneTM matrix genomic DNA isolation kit. The protocol involves the following steps: firstly, colony of the isolated bacterial was collected and suspended in 1ml of sterile water in a microfuge tube. After that the sample is centrifuged for 1 minute at 10,000 rpm, the supernatant obtained is then removed. 200 µl of Insta Gene matrix was added to pellet and the microfuge was incubated for 15 minutes at 56°C. Sample is then vortexed at high speed for 10 seconds and after that it is placed for 8 minutes at 100 °C in water bath in order to give the heat shock. Again, the content obtained was vortexed at high speed for 10 seconds and centrifuged for 2 minutes at 10,000

rpm. For the PCR reaction, 20µl of the supernatant was used to prepare the 50 µl PCR reaction mixture. Then, 16S rRNA region was amplified with the help of MJ Research Peltier Thermal Cycler. The Primer 518F/800R were used for the sequencing of the PCR product. Sequencing was done with ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems). The 16s rRNA sequence obtained was analyzed with the help of blast tool by assessing the similarity score of the nucleotide which is already present in the NCBI Database. The phylogeny analysis of the closely related sequence obtained on performing blast was evaluated with multiple sequence alignment tool, MUSCLE ver. 3.7 was used (Edgar, 2004). The result obtained by aligning sequences were amended using the program Gblocks ver. 0.91b. Finally, for the phylogeny analysis, PhyML ver. 3.0 aLRT program was used and HKY85 was used as the Substitution model. For rendering the phylogenetic tree, program tree Dyn ver. 198.3 was employed (Dereeper et al., 2008).

5.5 Biocompatibility assay of efficient dye degrading bacteria for dyes

To analyze the synergistic activity among isolated bacterial species for the development of consortium, biocompatibility assay was performed by following the method described by Nikam et al., (2007). The selected bacterial isolates were spreaded on the growth media and allowed to grow for 24 h at 37°C. The sterilized discs of 5 mm (whatmann paper no-1) impregnated with bacterial suspension of individual isolates were placed at the distance of 5 mm from the periphery of petriplates already having growth of cultures inoculated in different pattern to check the antagonistic/syneristic effect of bacterial cultures on each other.

5.6 Development of consortium

Initially, a loop full of individual bacterial pure cultures were inoculated separately in minimal media and incubated for 24 h at 30ºC. For the development of bacterial consortium, 6 h old cultures were then transferred aseptically into nutrient medium followed by 24 h incubation at 30ºC. The consortium thus obtained was used as the source of inoculum for further studies (Lade et al., 2012; Das and Mishra, 2017)

5.7 Analytical methods: dye decolorization assay

The Absorbance of supernatants withdrawn at different intervals of time were determined at their maximum absorption wavelength for particular dye which is present in the visible region by UV-Vis double beam spectrophotometer (UV-1800, Shimadzu scientific works). The decolorization percentage was assessed by the following formula.

$$\text{Decolorization percent (\%)} = \frac{(I.A) - (O.B)}{} \times 100$$

(I.A)

Where, I. A= Initial Absorbance and O. B = Observed Absorbance

5.7.1 Analysis of degradation products: GC-MS analysis and FTIR

After decolorization assay, the supernatants, which contains the degraded or biotransformed products of dye were extracted by centrifugation (10,000 rpm) for 20 min at 4°C. The resultant supernatant was further added to equal volume of ethyl acetate and mixed strongly to dissolve metabolites and dried over anhydrous Na_2SO_4. Further, solvent layer was air-evaporated in rotary vacuum evaporator. The remaining fractions were scrapped, collected, and dissolved in 2-3 mL of HPLC grade methanol. Finally, the samples were filtered through 0.22 μm syringe filter (Millipore Millex with PES membrane) and then subjected to FTIR and GC-MS analysis to confirm biodegradation of dyes (Lade et al., 2012). The samples for FTIR analysis were prepared by mixing it with spectroscopically pure KBr and analyzed by using the methods described by Lade et al., (2012). The GC-MS analysis of the fractions was performed by using a Perkin Elmer MS Engine, equipped with integrated gas chromatograph with a HP1 column (30 m long, 0.25 mm id, nonpolar). The flow rate of carrier gas (Helium gas) was kept at 1 ml per minute. The injector temperature was maintained at 280°C with alterations in oven conditions as follows: Initially maintained at 80°C kept constant for 2 min and increased up to 200°C with 10°C min^{-1} and finally, raising the temperature up to 280 °C with an increment of 20°C min^{-1} rate (Jadhav et al., 2009; Kurade et al., 2012). The compounds obtained as a result were then identified on the basis of mass spectra and using the standard NIST library.

5.8 Toxicology study of dyes: Phytotoxicity assay

5.8.1 Phytotoxicity assay

The phytotoxicity assay was performed to assess the toxicity of selected dyes and the degraded metabolites with respect to wheat variety PBW 550 (*Triticum aestivum*) collected from Punjab Agricultural University, Ludhiana, commonly used in Indian agriculture. Toxicity study was conducted by following the method of Parshetti et al., (2006).

5.9 Screening of bacterial isolates for the activity of dye degrading enzymes

5.9.1 Preparation of cell free extract

The bacterial cells were subjected to centrifugation (10,000 rpm for 15-20 min at 4°C) and the resulted supernatant was used for screening of enzyme activities. The separated biomass of bacterial cells was suspended in 50 mM potassium phosphate buffer (pH 7.4), mildly

homogenized and further sonicated by giving 12 strokes for 30sec with one min interval based on 60 amplitude keeping sonifier output below 4 °C. The subsequent extracts were then used as enzyme source (Telke et al., 2008; More et al., 2011).

5.9.2 Screening for enzymatic activities

Azo reductase activity was assayed by following the protocol described by Telke et al., (2008). The reaction mixture (2 ml) contains 4.45 μM of azo-disperse dye, 50 μM NADH, 1.2 ml of potassium phosphate buffer (20 mM, pH 7.5). The addition of NADH was done after 4 min pre-incubation of reaction assay mixture (37 ℃) and monitored for the decrease in absorbance (430nm) in UV-VIS spectrophotometer. The reaction was initiated by the addition of 0.2 ml of the enzyme solution. Azo-disperse dye reduction was calculated by using its molar extinction coefficient of 0.023 μM^{-1} cm^{-1}. One unit of azoreductase enzyme activity was defined as quantity of enzyme required to catalyze 1 μM of substrate min^{-1} mg of $protein^{-1}$. All the enzyme assays were run in triplicates.

The activity of laccase enzyme was monitored by the oxidation of ABTS using the protocol of More et al., (2011). The assay mixture contained 2.8 mL of 0.5 mM ABTS in 200 mM sodium acetate buffer of pH 5.5 and 0.2 mL of enzyme and incubated for 5 min at 35°C. The oxidation of ABTS was determined by observing the increase in spectrophotometer readings (420 nm, $3.6 \times 104\ M^{-1}\ cm^{-1}$) resulting in an intense bluish-green color read at 420 nm against a suitable blank. One unit of laccase enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of ABTS substrate min^{-1} and expressed as units per g of dry substrate (U gds^{-1}).

5.10 Development of bio-filter: continuous column studies

5.10.1 Preparation and characterization of bio-adsorbent

Final part of this research was the development of bio-filter. To accomplish this part of research, corn cobs were collected from the local market in Phagwara, Punjab and washed with distilled water. The dried corn cobs were then stored in desiccators to avoid moisture absorption. After this, the dried corn cobs were grounded and sieved to obtain fraction < 1mm and were then autoclaved (Miyah et al., 2016; Paliwal et al., 2015).

5.10.2 Analysis of surface characterization and morphology of corncobs

5.10.2.1 Electrical conductivity (EC) and pH

The electrical conductivity and pH of the powdered corncob was measured by preparing a suspension of 1% (wt./wt.) corncob with distilled water then heated up to 90°C in incubator

and stirred for 20 minutes. This process led to the mixing and dissolution of the soluble components. After cooling it to room temperature, pH was measured by using digital pH meter and conductivity by conductivity meter (Zhu et al., 2014).

5.10.2.2 Bulk density of corncob

Bulk density of corn cob particles was calculated by following the protocol described by Ahmedna et al., (1997). The oven dried corncob was weighed and filled into the graduated measuring cylinder (50ml). The bulk density was calculated by using formula:

Bulk density = Mass of sample/Volume of cylinder

5.10.2.3 Particle density of corncob

Corncob particles was crushed into small particles and then added again into a graduated measuring cylinder. Volume of water displaced in cylinder was measured after measuring the height of the corncob particles filled in the cylinder (Ahmedna et al., 1997).

Following formula was used to calculate particle density:

Particle density = Mass of sample/Volume of water displaced by corncob in cylinder

=A /V

Where, A= Mass of sample in gram; V= volume of the sample in cubic centimeters.

5.10.2.4 Scanning electron microscope analysis

The surface morphology of corncob particles was studied by JEOL USA Scanning electron microscope (SEM) [Model-JSM 6510LV]. The samples were prepared in powdered form and vacuum dried for 5-6 h. The dried samples are then gold sputtered for 30 seconds using DC magnetron sputtering machine and thereafter the coated samples are viewed under SEM with 10 KV accelerating voltage.

5.10.2.5 XRD analysis

The X-Ray diffraction technique was used for determination of the crystallinity behaviour of corncob biomass. In order to perform the XRD experiment, an analytical XRD diffractometer (Model: -X'Pert PRO) * was used. The X-ray diffractometer used a voltage of 30 kV with a current density of 15 mA during the experiment. The scanning range varied from 2θ value 10^{o} to 70^{o} and the scan speed was 0.050/s. CuKα X-rays with the wavelength, λ= 1.54056 Å were used for the XRD study in this work.

5.10.2.6 FTIR characterization

FTIR (Fourier transforms infrared spectroscopy) at room temperature in the range of wavelength (400-4000 cm^{-1}) was performed to analyze the surface properties and the presence of different functional groups on corncob (Leng et al., 2015).

5.11 Immobilization of bacterial consortium on corncob

The immobilization of bacterial consortium on the surface of corncob was done by the method described by He et al., (2017). The bacterial consortium culture was cultivated in minimal salt medium for 72 h at 30℃. Cells were harvested at the mid-log phase of the growth curve, as shown by an optical density of 0.3–0.5 at 600 nm (OD_{600}), by centrifugation (5000 rpm) at 4℃ for 15 min. The collected bacteria pellet was washed five times with distilled water and then re-suspended in distilled water. Then, 1 gram of corncob (grounded) was mixed with 1 gram of cell (wet weight) in 500 mL mineral salt culture medium. To achieve immobilization, the flask was placed in the orbital shaker for 2 h at 30 ℃ until cells adsorbed on the surface of corncob particles. The immobilized cells were then washed gently two times with the distilled water to remove free cells. Then immobilized corncobs were further used for the column studies.

5.12 Continuous column studies: experimental set up

For experimental set up, the fixed bed glass column of 2.5 cm inner diameter and 35 cm height was used for this study. At the bottom and top of the glass column, glass wool of 2 cm thickness was filled (Kundu and Gupta, 2005). A known amount of immobilized corncob (after weighing) was packed in the glass column to get the desired bed height (5 cm and 10 cm, respectively). The column was fed continuously with dye solution (with concentrations of 50 mg/L, 150 mg/L and 250 mg/L) by maintaining a flow rate of 7 mL/min with the help of standard I.V. Infusion set, against the gravity (from downward to upward direction) (Figure 9). The column studies were conducted to investigate the effect of various parameters such as dye concentration and the bed height on the effectiveness of biofilter (on dye adsoption to corncob particles immoblised with bacterial consortium). Further, sample of dye solution passing through the biofilter was collected at different intervals from the exit point of the column and dye concentrations were analyzed by using the double beam UV-VIS spectrophotometer (Shimadzu, Japan) at absorption maxima (λ_{max}) of respective dye. The functioning parameters of the glass column experimental set up are shown in Table 9. Standard calibration curves were also plotted between absorbance and concentration of the dye solution (20-400 ppm).

Table-9: Functioning parameters of the glass column experimental set up

Flow rate (mL/min)	7
Height of glass column (cm)	35
Internal diameter of column (cm)	2.5
Area of glass column (cm^2)	284.71
Material used for column	Borosilicate glass
Bed height (cm)	10 and 15
Bed volume (cm^3)	49.08 and 73.63
Mass of adsorbent (g)	25 and 35
Inlet concentration of dye (mg/L)	50, 150 and 250
Mode of flow	Continuous Up flow (against the gravitation)

5.12.1 Analysis and breakthrough modelling of column data

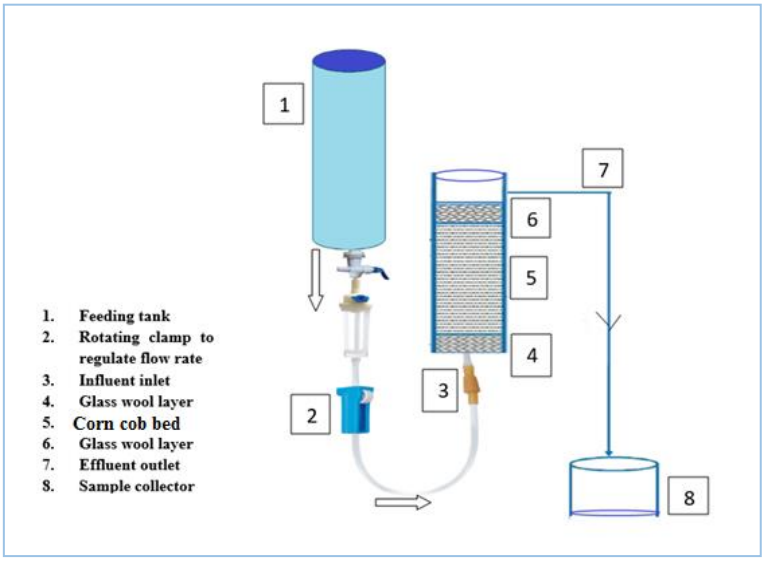


Figure-9: Experimental setup of continuous column

The breakthrough curves of dye concentrations were obtained by plotting C_t/C_o as a function of time. Relative concentration is defined as the ratio of dye concentration in effluent to dye

concentration in an influent (Ct/Co), as a function of time (t in min). The effluent volume can be calculated as:

$$V_{eff} = Qt_{total} \quad (1)$$

Where, t_{total} and Q are the total flow time (min) and volumetric flow rate (mL/min). The total adsorbed dye (q_t) in the column for a given solute concentration and flow rate is calculated from the following equation:

$$q_{total} = \frac{Q}{1000} \int_{t_0}^{t_{total}} C_{ad}\, \mathrm{dt} \quad (2)$$

where Q (mL/min) is the flow rate and C_{ad} (mg/L) is the adsorbed dye concentration [(inlet concentration (C_o) and outlet concentration (C_t)], and t_{total} is the total flow time (min). Equilibrium dye uptake in the column or maximum capacity of the column (q_{eq}) was defined by Eq. (3) as the total amount of dye adsorbed (q_{total}) per g of the adsorbent (w) at the end of the total flow time.

$$q_{eq} = \frac{q_{total}}{w} \quad (3)$$

5.12.1.1 Adams-bohart model

Bohart-Adams (Hutchins, 1973) model was used to predict the initial part of the breakthrough curve. The mathematical equation of the model can be written as:

$$ln \frac{C_t}{C_0} = k_{AB} C_0 t - k_{AB} N_0 \frac{Z}{F} \quad (4)$$

where C_o (mg/L) is the inlet concentration and C_t (mg/L) is the effluent concentration. k_{AB} (L/mgmin) is the Adams Bohart kinetic constant, F (cm/min) is the linear velocity which can be calculated by dividing the flow rate in the column section area, Z (cm) is the bed depth of column and N_o (mg/L) is the saturation concentration. A linear plot of ln Ct/Co against time (t) was determined by the values of k_{AB} and N_o from the intercepts and slopes of the plot.

5.12.1.2 Thomas model

The expression developed by Thomas (Thomas, 1944) calculates the maximum solid phase concentration of the solute on the adsorbent and the adsorption rate constant for a continuous adsorption process in column. The linearized form of the model is expressed as:

$$ln\left(\frac{C_0}{C_t} - 1\right) = \frac{k_{TH} q_0 m}{Q} - k_{TH} C_0\, t \qquad (5)$$

where k_{Th} (mL/min mg) is the Thomas rate constant, q_o (mg/g) is the equilibrium effluent uptake per g of the adsorbent, C_o (mg/L) and C_t (mg/L) are the inlet effluent concentration and outlet concentration at time t; m (g) the mass of adsorbent, Q (mL/min) the feed flow rate, and t_{total} (min) stands for flow time. The value of C_t/C_o is the ratio of outlet and inlet effluent concentrations. A linear plot of $\ln[(C_o/C_t)-1]$ against time (t) was used to determine values of k_{Th} and q_o from the intercepts and slopes of the plot.

RESULTS AND DISCUSSIONS

Chapter 6

Results and Discussions

6.1 Analysis of textile industry effluent samples for physio-chemical parameters

Discharge of treated and untreated effluents by the textile industries disturbs the environmental integrity of the region. Two textile dyeing industries identified as site 1 and site 2, were selected for this study. These two industries are located in the vicinity of Buddha Nallah drain on Tajpur road and on Bahadur-Ke-road. The results of the physicochemical properties of the effluent samples collected from these two selected sites are given in Table 10. The mean and standard deviation in seasonal variation of physicochemical parameters such as pH, temperature, total dissolved solids (TDS), total suspended solids (TSS), biochemical oxygen demand (BOD) and chemical oxygen demand (COD) were calculated from the collected data for the year 2014-15 and are shown in Tables 11 and 12.

The pH of collected textile effluent samples were analyzed and found to be between 6.1-10.7, respectively, for two sites against the WHO standards between pH range of 6.5-8.5. Figure 2 shows the mean seasonal variation of pH at two sites along with the mean seasonal rainfall. For Site 1 for the summer season, the value of pH was in the alkaline range of 8.78±0.470 ($p < 0.05$) which is highly significant with similar trends for Site 2, in the range of 8.51±0.411. The values of pH for the rainy season for site 1, were found to be in range of 7.38±0.583 as compared to Site 2 of 7.11±0.598 which shows non-significant difference. Effluent samples from Site 1, in autumn and winter season showed alkaline values of pH in the range of 8.58±1.40 and 8.33±0.970 (Figure 12A), respectively. Similar results were obtained for Site 2. The reason for alkaline range pH in effluent water samples is possibly due to the presence of certain chemicals like NaOCl, sodium hydroxide, various surfactants and sodium phosphate used in mercerizing the fabric and yarn (Pual et al., 2012). Similar results were obtained by Ramamurthy et al., (2011) and pH values higher than the observed range have also been reported by Desai and Kore, (2011), Tiwari and Chauhan, (2006) and Aher et al., (2007). The low pH values observed in the rainy season were possibly due to the accumulation of free carbon dioxide and increased rate of respiration by the organisms.

An opposite trend between pH and CO_2 has also been reported by Jindal and Rumana, (2000) for Yamuna River. Indian rivers polluted with industrial effluents have been found to have alkaline or acidic pH (Gurumayum et al., 2002). The important parameter which affects many chemical and biological processes in wastewater is temperature. In the present study, variation in the values of water temperature was observed. The maximum mean temperature was recorded in summer i.e. 41.16°C ±4.99 and lowest mean temperature in winter i.e. 39.25°C ±2.25 at site 2 and the maximum mean temperature in the rainy season was i.e. 40.83°C±2.42 and the minimum temperature was in autumn of 37.66°C±2.58 respectively. Figure 11(B) shows the mean seasonal variation in water temperature at two sites along with the mean seasonal rainfall. During the rainy monsoon season, the highest temperature of 45.1°C was observed. If the temperature of effluents being discharged from industries is high, then it will ultimately affect the properties of the receiving water body. Temperature may impact the aquatic life in receiving waters. With increase in temperature, the rate of biological activity will get altered, likewise solubility of oxygen and other gases will decrease which is harmful to aquatic community. In our research work, with increasing water temperature, the concentration of dissolved oxygen decreases and similar findings were also reported by Maurya et al., (2012). Total dissolved solids (TDS) signify the presence of inorganic ($Ca^{2+,}$ $Mg^{2+,}$ K^{+}, Na^{+}, $HCO^{3-,}$ Cl^{-}, SO_4^{2-}) and organic matter present in the form of suspension in water. The mean value of TDS was highest in autumn 2783mg/L±321.26 ($p<0.05$) and lowest in winter 1456±153.06 for Site 1. Similar trends were observed for the effluent of Site 2, the maximum value of TDS in autumn i.e. 2678mg/L±346.68 which is highly significant ($p<0.05$) and minimum in winter season i.e. 1463.8mg/L±164.22; 1618.5mg/L±180.03 in summer and 1783.1±290.37 in monsoon season (Figure 11C). Overall, the TDS values were higher in autumn and monsoon, while within the desirable limits of WHO standards in winter. Similar study was done by Paul et al., (2012) and and they reported higher values of TDS in the range of (2264mg/L-7072mg/L) in the textile wastewater. Higher TDS affect water quality and is unsuitable for the portability and industrial applications (Dattaray, 2015). According to the standards of WHO, the limit for TDS is 500mg/L and maximum limit is 2000mg/L (Paul et al., 2012).

In our study, TDS values were higher in autumn and Monsoon, while within the desirable limits of WHO in winter. Similar study has been conducted for Godavari River flowing little downstream from Nanded, Maharastra, by Srinivasrao et al., (2007). They found similar trends for TDS which causes changes into ionic concentration and nature of the deposits present at bottom of the river. The low TDS content in the groundwater of the study area could be either a result of short residence time or because of slow weathering of granitic or granitic gneiss terrain. Excessive concentration of TDS in ground water affect the human health, induces unfavorable physiological, aesthetically unsatisfactory for bathing, washing and increases the boiling point (Jindal and Rumana, 2000). High TDS in groundwater is due to discharge of wastewater into pit ponds and the lagoons enabling the waste percolate down to the water table (Dattaray, 2015). The higher TDS values leading to higher ionic concentration also causes gastro intestinal complications.

Total suspended solids (TSS) is a parameter which is used to assess the quality of waste water after treatment in waste water treatment Plant. High values of suspended solids correspond to the use of different kind of synthetic dyes (Mohabansi et al., 2011). The sediments of size > 0.7 µm - <63 µm and detrital organic matter > 0.7 µm compounds to suspended solids. In the present investigation, the value of total suspended solids was recorded in autumn for two sites i.e. 143.5mg/L±75.01 and 139.66mg/L±71.87, respectively which is higher than the permissible limit given by WHO and minimum value of TDS was recorded the in rainy season of 86.50mg/L±15.10 for Site 1 and 84g/l ±27.59 for Site 2 in summer. The excessive levels of TSS in water bodies can have significant detrimental impacts on the physical, chemical and biological properties of the water body (Bilotta and Brazier, 2008). If this type of effluent is used for irrigation purposes, it will result in clogging of pores of soil and loss in productivity of soil. Biochemical oxygen demand (BOD) is the amount of dissolved oxygen required for the biochemical decomposition of organic compounds and the oxidation of certain inorganic materials (e.g., iron, sulfites) Garg et al., (2010). Biochemical oxygen demand indicates the presence of organic impurities in the effluent. Figure 11(E) clearly shows us that the values for BOD of the effluent at two sites are more than the permissible limits set by WHO. In the present

study, the highest value for BOD was observed in autumn i.e. 361.67mg/L±130.44 for Site 1 and 341.6mg/L±123.84 for Site 2 which is highly significant ($p < 0.05$). Nirgude et al., (2013) reported similar findings for BOD values of textile industry effluent from vapi industrial area in Gujrat in the range of 90-678mg/L. The WHO has set the limit of 250 mg/L for chemical oxygen demand (COD). Our study shows that for both the sites; COD value is greater than of permissible limit set by WHO. The seasonal variation of COD can be clearly seen in the Figure 11(F). The maximum value for COD was observed in winter i.e. 810.67mg/L±126.31 for Site 1 and 809.16mg/L±86.39 for Site 2 which is highly significant ($p < 0.05$).

The textile industry effluents in the rainy season show COD value as high as 900 mg/L, which may be due to higher level of pollution in surface runoff generated by monsoon. Singh et al., (2013) conducted a similar study on collecting textile industry effluents from 7 different dyeing mills and reported high values of BOD and COD of 108-790mg/L and 195-3050 mg/L, respectively. High level of chemical oxygen demands indicates noxious conditions and presence of resistant organic impurities (Mohabansi et al., 2011).

6.1.1 Correlation between various physio-chemical parameters at two different sites during four seasons

Correlation is used to evaluate the degree of interdependence and gives a relationship between two or more experimental variables. The systematic study of correlation between various physio-chemical parameters of water, not only helps us to determine quality of water but also quantifies the concentration of pollutants in water. It expresses the extent to which two variables vary together in either same or opposite directions (Khambete and Christian, 2014). Therefore, it provides a relative information for implementation of water quality treatment systems (Verandani and Vardhan, 2012). The correlation between variables is characterized as strong, in the range of +0.8 to 1 and -0.8 to -1, moderate in the range of +0.5 to 0.8 and -0.5 to -0.8, and weak in the range of +0.0 to 0.5 and -0.0 to -0.5. Correlation coefficients among various water quality parameters at two different sites for different seasons are represented in Figure 10. The results obtained in this study clearly

indicated a strong positive correlation between pH and water temperature, TDS, TSS, BOD and COD for both sites in autumn season [Figure 10(A) and 10(B)]. Decrease in disoolved oxygen will lead to increase in biochemical oxygen demand (BOD). However, BOD does not have direct relationship with TSS. But generally, more dissolved solids in effluent leads to more organic impurity that is BOD (Srinivasarao et al., 2007). In contrast, for Site 1, in rainy monsoon season, the strong positive correlation was observed between pH, BOD, COD and water temperature. TDS showed positive correlation between TSS, BOD and COD. As DO and BOD have inverse relationship, reduction in DO will lead to increased BOD in due course of time (Verandani and Vardhan, 2012). Water temperature exhibited negative correlation with other parameters [Figure 10(C and D)]. For site 2, in rainy monsoon season, water temperature and TSS exhibited negative correlation with other parameters. BOD showed positive correlation with TDS and COD for both sites in rainy monsoon season. In summer season, for both sites, moderate positive correlation was found between pH, water temperature and TDS. BOD has a moderate positive correlation with COD and TSS. Abdalla and Hammam, (2014) studied the correlation between BOD and COD for wastewater treatment plants in Egypt to check the biodegradability indicies to evaluate the treatment process and improve the performances. Our results for the correlation between BOD and COD for four different seasons are in accordance with their study. They reported if the ratio of BOD/COD is greater than 0.6, then the wastewater is biodegradable. If the BOD/COD ratio is between 0.3-0.6, then biodegrdation process will be very slow. In comparison, if the ratio of BOD/COD is less than 0.3, then the wastewater can be treated biologically. In our study, water temperature showed positive correlation with TDS [Figure 10(E and F)]. In contrast, TDS has a weak positive correlation with COD but exhibited strong correlation with BOD for both sites in winter season. Our results are in accordance with the previous study conducted by Hill et al., (2005). Hill et al., (2005) determined the wastewater quality index and studied statistical interrelationships amongst different parameters by correlation cofficients and regression analysis. They found positive correlation between TDS-TSS, TDS-BOD, TDS-COD, TDS-pH, and BOD-COD.

Table-10: Physicochemical parameters analysis of textile industry effluents at two selected sites in Ludhiana (2014-15)

Month	Date	Season	Rain fall (mm)	pH		Water Temperature (°C)		TSS (mg/L)		TDS (mg/L)		BOD (mg/L)		COD (mg/L)	
				Site 1	Site 2	Site 1	Site 2	Site 1	Site 2	Site 1	Site 2	Site 1	Site 2	Site 1	Site 2
June (2014)	02/06/2014	Rainy Monsoon	19.8	7.8	7.4	44.5	44.5	75	79	1323	1333	122	121	280	265
	16/06/2014			7.5	7.0	43	45.5	87	85	1552	1542	124	123	260	240
July	1/07/2014	Rainy Monsoon	90.8	6.5	6.1	40	35	114	110	1856	1826	170	175	320	300
	15/07/2014			7.2	6.9	38	38	90	86	1950	1930	185	180	280	290
August	1/08/2014	Rainy Monsoon	32.2	7.5	7.8	40	38	81	79	1978	1958	320	310	416	390
	16/08/2014			7.8	7.5	39.5	35.5	72	69	2120	2110	380	340	350	330
September	1/09/2014	Autumn	78.8	7.43	7.2	36.5	35	71	72	2360	2160	580	520	282	272
	15/09/2014			7.5	7.6	35	32	70	69	2550	2430	450	470	310	290
October	1/10/2014	Autumn	5.0	10.78	10.2	40	38	265	255	3050	2950	350	320	784	786
	15/10/2014			9.9	10	41.5	37.5	190	187	3220	3113	250	270	640	643
November	1/11/2014	Autumn	0.0	8.02	7.9	35.5	32	145	142	2860	2763	280	240	576	546
	15/11/2014			7.89	7.7	37.5	35	120	113	2662	2652	260	230	680	590
December	1/12/2014	Winter	14.6	7.33	7.2	38	35.5	84	82	1669	1769	385	345	650	790
	15/12/2014			7.5	7.6	40	38	127	117	1624	1526	290	280	990	895
January (2015)	1/01/2015	Winter	24.6	8.3	8.1	41	40	154	144	1336	1326	145	125	840	855
	15/01/2015			8.0	7.9	38	39.5	145	135	1418	1405	190	175	900	895
February	2/02/2015	Winter	38.6	9.9	9.6	40	42	55	52	1386	1358	185	195	700	695
	16/02/2015			9.0	8.9	42.5	40.5	82	79	1305	1399	250	230	784	725
March	2/03/2015	Summer	84.6	8.5	8.1	41	40	92	87	1450	1423	220	210	568	548
	16/03/2015			9.0	8.7	38	42	78	72	1478	1458	190	170	560	555
April	1/04/2015	Summer	17.5	8.5	8.3	35	32	122	125	1643	1645	152	134	440	435
	15/04/2015			8.2	8.1	40	45	114	101	1550	1544	178	158	420	410
May	1/05/2015	Summer	27.2	9.0	8.8	42	42	56	58	1896	1892	130	120	432	420
	15/05/15			9.5	9.1	45	46	59	61	1750	1749	155	125	380	375

Table-11: Physicochemical parameters analysis at site-1 in Ludhiana (2014-15)

Parameters	WHO standard	Summer (Mean± SD)		Rainy Monsoon (Mean± SD)		Autumn (Mean± SD)		Winter (Mean± SD)	
		Site 1	p value (two tailed)	Site 1	p value (two tailed)	Site 1	p value (two tailed)	Site 1	p value (two tailed)
pH	6.5-8.5	8.78±0.470	0.024*	7.38±0.487	0.81	8.58±1.40	0.165	8.33±0.970	0.190
Total Suspended Solids (mg/L)	100 mg/L	86.83±27.58	0.026*	86.50±15.10	0.053	143.5±75.01	0.185	107.8±39.76	0.63
Total dissolved solids (mg/L)	2000 mg/L	1627.8±171.58	0.000*	1796.5±299.55	0.12	2783.7±321.26	0.00*	1456.3±153.06	5.60E0-6*
Chemical oxygen demand (mg/L)	250 mg/L	466.67±78.20	0.00004*	317.67±58.10	0.01*	545.33±204.80	0.00*	810.67±126.31	7.35E-07*
Biochemical oxygen demand (mg/L)	30 mg/L	170.83±96	7.87E-07*	216.83±107.78	0.000*	361.67±130.44	9.78E-05*	240.83±87.37	0.000*
Water temperature (°C)	Not exceed 40°C	40.16±3.43	0.907	40.83±2.42	0.419	37.66±2.58	0.051	39.91±1.74	0.901

Table-12: Physicochemical parameters analysis at site-1 in Ludhiana (2014-15)

Parameters	WHO standard	Summer (Mean± SD)		Rainy Monsoon (Mean± SD)		Autumn (Mean± SD)		Winter (Mean± SD)	
		Site 2	p value (two tailed)	Site 2	p value (two tailed)	Site 2	p value (two tailed)	Site 2	p value (two tailed)
pH	6.5-8.5	8.51±0.411	0.059	7.11±0.598	0.469	8.43±1.31	0.210	8.21±0.88	0.240
Total Suspended Solids (mg/L)	100 mg/L	84.00±25.79	0.159	84.66±13.80	0.021*	139.66±71.87	0.206	101.5±36.07	0.920
Total dissolved solids (mg/L)	2000 mg/L	1618.5±180.03	0.000*	1783.1±290.37	0.097	2678±346.68	0.000*	1463.8±164.22	1.18137E-05*
Chemical oxygen demand (mg/L)	250 mg/L	457.16±75.72	5.35578E-05*	302.5±52.70	0.034*	521.16±202.90	0.008*	809.16±86.39	2.05E-08*
Biochemical oxygen demand (mg/L)	30 mg/L	152.83±34.04	4.88E-06*	208.16±94.33	0.000*	341.66±123.84	0.000*	225±78.54	0.000*
Water temperature (°C)	Not exceed 40°C	41.16±4.99	0.579	39.41±4.51	0.751	34.91±2.57	0.000*	39.25±2.25	0.909

***P< 0.05 (Highly significant)**

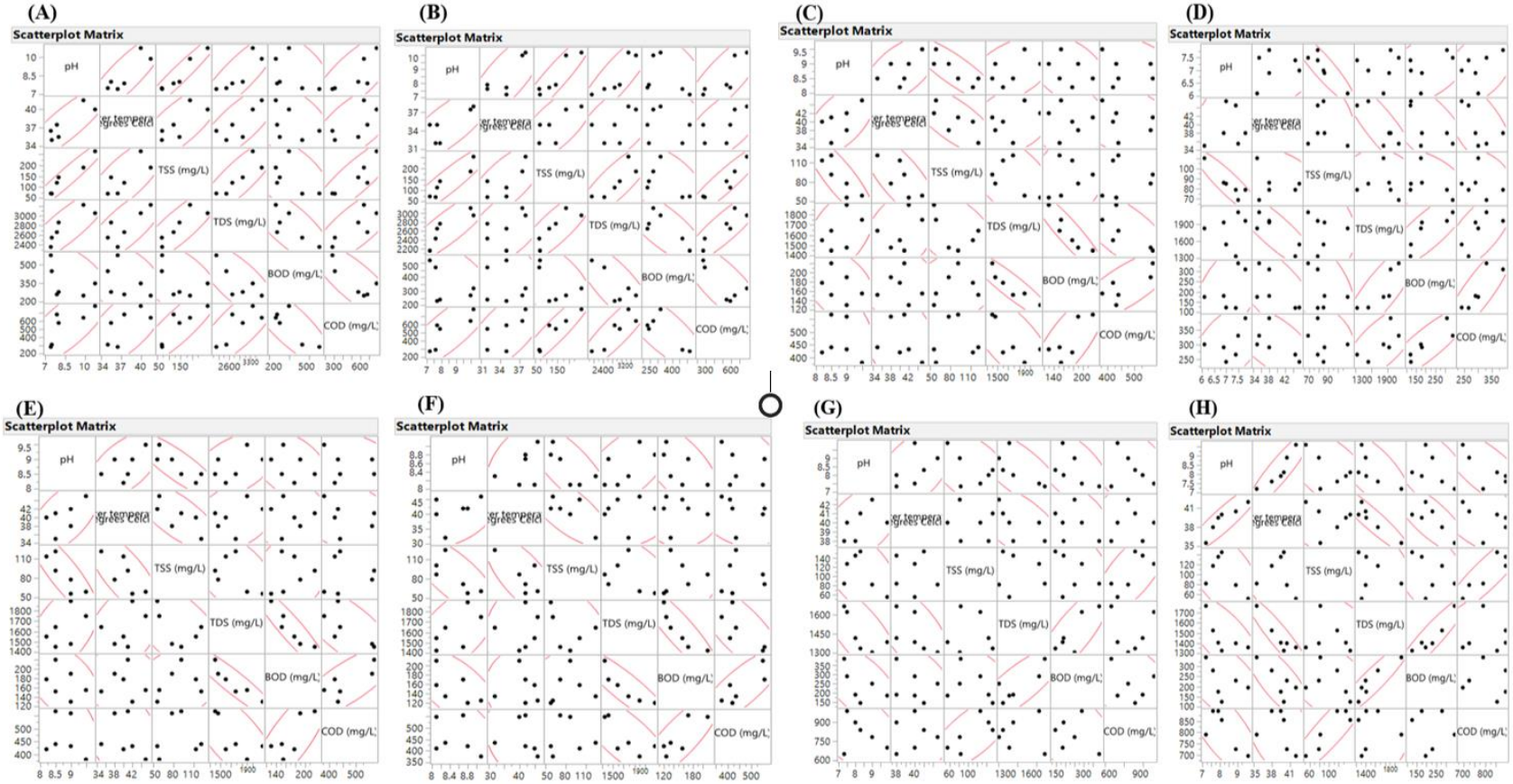
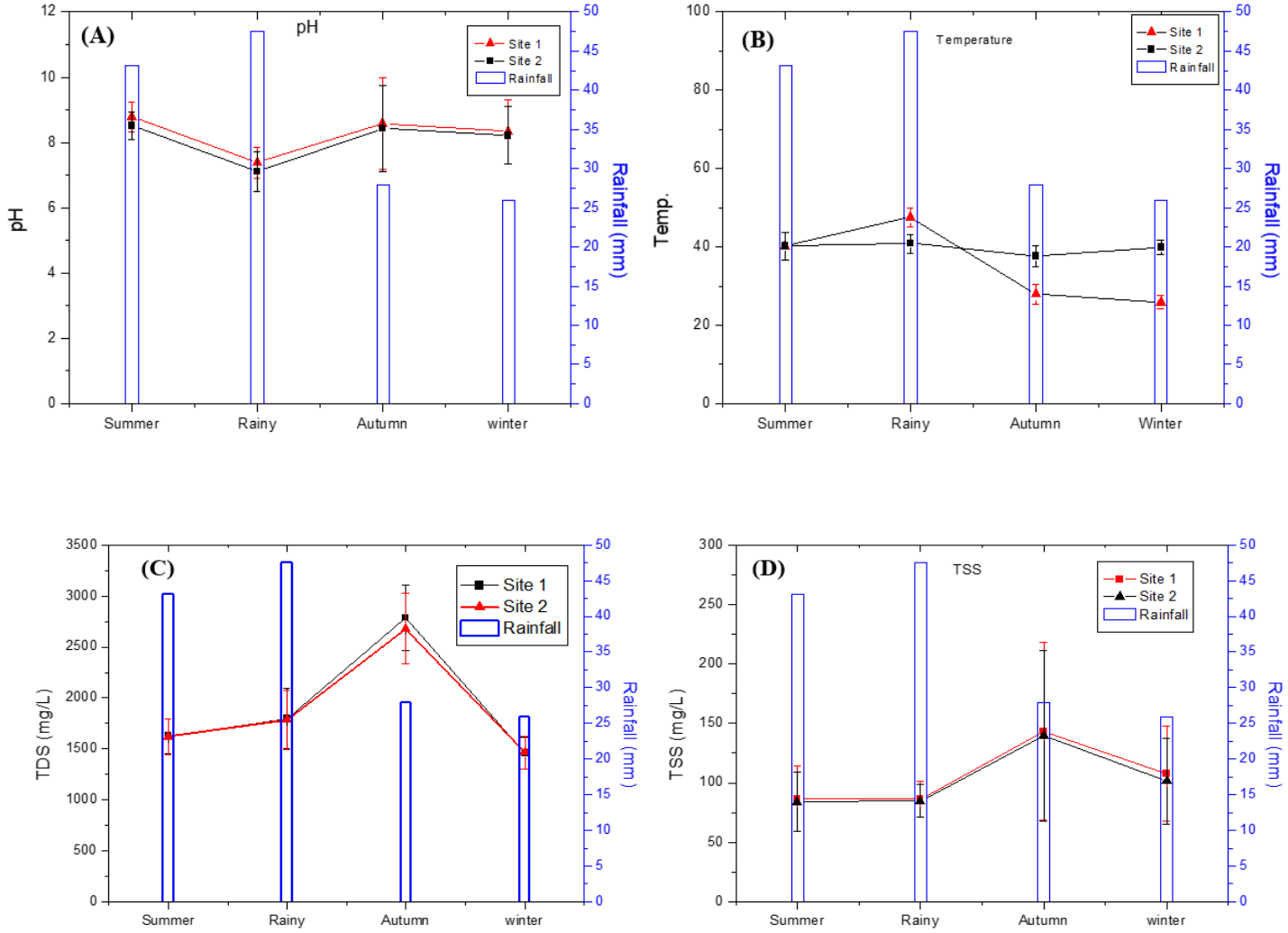


Figure-10: The correlation matrix for the physicochemical parameters of Site 1 and Site 2 in four seasons. 10(A) autumn season (Site 1); 10(B) autumn season (Site 2); 10(C) rainy monsoon season (Site 1); 10(D) rainy monsoon season (Site 2); 10(E) summer season (Site 1); 10(F) summer season (Site 2); 10(G) winter season (Site 1); 10(H) winter season (Site


(A)
pH
Site 1
Site 2
Rainfall
pH
Rainfall (mm)
Summer
Rainy
Autumn
winter
(B)
Temperature
Site 1
Site 2
Rainfall
Temp.
Rainfall (mm)
Summer
Rainy
Autumn
Winter
(C)
Site 1
Site 2
Rainfall
TDS (mg/L)
Rainfall (mm)
Summer
Rainy
Autumn
winter
(D)
TSS
Site 1
Site 2
Rainfall
TSS (mg/L)
Rainfall (mm)
Summer
Rainy
Autumn
winter

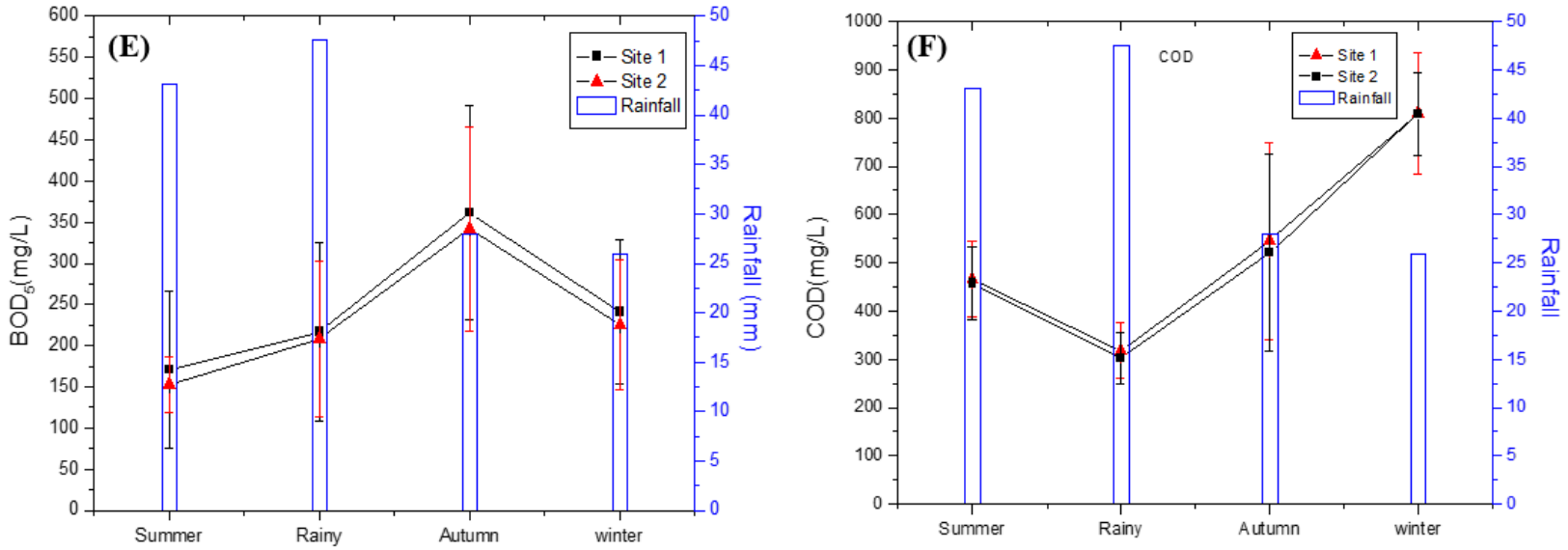


Figure-11: Graphs showing average seasonal variations of various selected physicochemical parameters of different seasons of Site 1 and Site 2 Ludhiana, Punjab. (June 2014 - 15). 11(A) seasonal variations of pH; 11(B) seasonal variations of water temperature; 11(C) seasonal variations of total dissolved solids (TDS); 11(D) seasonal variations of total suspended solids (TSS); 11(E) seasonal variations of biochemical oxygen demand (BOD); .11(F) seasonal variations of chemical oxygen demand (COD)

6.2 Selection and properties of textile dyes:

6.2.1 Spectrum study: Spectral analysis of dyes [to check absorption maxima (λ_{max}) of dyes]

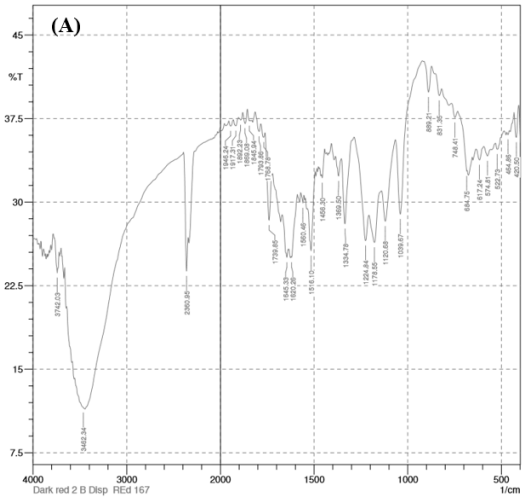
The selected textile dyes were firstly studied for absorption spectrum by UV-Vis double beam spectrophotometer (UV-1800, Shimadzu scientific works) in a range of 200nm to 900nm (Jadhav et al., 2009). The absorption maxima are presented in Table 13.

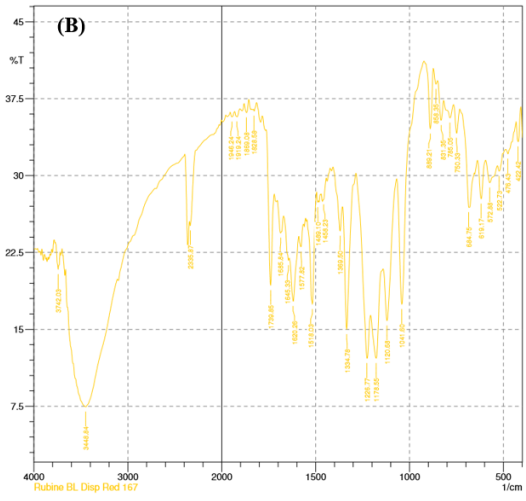
Table-13: Spectral analysis of dyes [to check absorption maxima (λ_{max}) of dyes]

Name of Dye	Color Index Name	λ max (nm)
Dark red 2B	Disperse red 167	471
Rubine BL	Disperse red 167.1	469
Blue BG	Disperse blue 60	782
Scarlet RR	Disperse red 54	531

6.2.2 FTIR analysis of dyes

To check the presence of different functional groups, FTIR analysis of selected dyes was done in the mid IR region of 400-4000 cm^{-1} with 16 scan speed. The samples were prepared with spectroscopically pure KBr in the form of pellets (Lade et al., 2012).





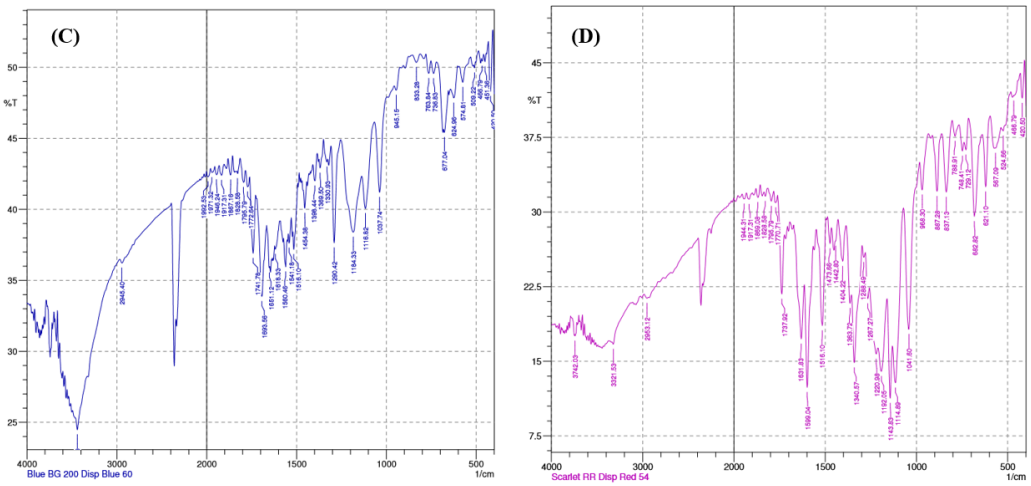


Figure-12: FTIR spectra of selected control dyes. 12(A) FTIR spectra of disperse red 167; 12(B) FTIR spectra of disperse red 167.1; 12(C) FTIR spectra of disperse blue 60; 12(D) FTIR spectra of disperse red 54

The FTIR spectrum of control disperse red 167 is displayed in Figure 12(A). In control dye, the spectra revealed the presence of peaks at 1575.46 cm^{-1} for H-N-H bending. The stretching of (CO)-H was reported at 2852.81 cm^{-1} and the peak at 1609.65 cm^{-1} that showed carbonyl stretching vibration. In control, the peaks at 1560.46 cm^{-1} and 1621.22 cm^{-1} is due to the presence of azo groups –N=N– stretching whereas the spectrum of control disperse red 167.1 is presented in Figure 12(B). The peak in the range of 1595-1600 cm^{-1} corresponds to the presence of azo bond (-N=N-), peak at 831 cm^{-1} for C-H deformation in alkanes and N-O stretching in nitrites, peak at 734-750 cm^{-1} for the presence of benzene ring. Furthermore, the peak at 619.17 cm^{-1} corresponds to the C–N stretching in acyclic compounds. The C-N vibrations were found at 1120.68 cm^{-1}, -OH stretching vibration at 3500–3400 cm^{-1}. Similar functional groups were found on Rubine GFL by Waghmode et al., (2012) and Pan et al., (2017). The FTIR spectra of parent disperse blue 60 is shown in Figure 12(C). The parent dye (DB 60) displaying various peaks in the finger print region for para-disubstituted and meta-disubstituted benzene rings. The peak at 1741.38 cm^{-1} corresponds to C=0 stretch and peak at 2945.22 cm^{-1} for asymmetric CH_3 stretch, peak at 1454.38 cm^{-1} designated C-C stretch in aromatics, a peak at 945.15 cm^{-1} for O-H bend of carboxylic acid and presence of peaks at 677.04 cm^{-1}, 736.83 cm^{-1},763.84 cm^{-1} indicates aromaticity or the presence of benzene ring. A peak at 3439.19 cm^{-1} for –OH stretching vibration and -NH stretching

vibration, a peak at 1651 cm^{-1} shows -C=C- stretching. Other peaks at 1516.1 and 1560.46 cm^{-1} are characteristic of >C=C< (anthraquinone); while peaks at 1651 cm^{-1} to >C=O (anthraquinone) (Ortiz et al., 2017; Pan et al. 2017). The FTIR spectra of control disperse red 54 dye is represented in fig 12(D) which confirms the structure of parent dye. The peaks at 3321.53 cm^{-1} corresponds to -N-H stretching as in amines, 1599.04 cm cm^{-1} for N=N stretching as in azo groups and -C-N stretching as in amides, 1114.89 cm^{-1} for C–O-H stretching as in alcohols, 1041.6 cm^{-1} for S–O stretching which displayed the sulfoxide nature of dye, and 682.82 cm^{-1} for C–Cl stretching, 2953.13 cm^{-1} for –C-H stretching, 1363.72 cm^{-1} for CH_3 vibration, 1442.8 and 1473.66 cm^{-1} corresponds to CH_2 bends and 1737.92 cm^{-1} for –C=O (ketonic) vibration (Jadhav et al., 2009; Kurade et al., 2012)

6.3 Isolation and screening of bacterial strains from textile industry effluent and soil

For second part of this study, water samples and soil samples from textile industry were collected for the isolation of 15 morphologically different bacterial isolates. The pure cultures were preserved on nutrient agar medium at 4^oC. All 15 isolates were tested individually for their ability to decolorize four selected textile dyes (disperse red 167, disperse red 167.1, disperse blue 60 and disperse red 54) separately at the concentration of 50 mg/L each dye. Dye decolorization efficiency of 15 isolates for decolorization of each dye is shown in Table 14. All 15 isolates decolorize the dyes with different capacity ranging from lowest 50.76 ± 0.25 to highest 84.80 ± 0.35 in case of disperse red 167; 55.30 ± 0.51 to maximum 78.13 ± 0.37 for disperse red 167.1; 54.43 ± 0.55 to 76.29 ± 0.51 for disperse blue 60 and 53.73 ± 0.46 to 84.17 ± 0.16 in case of disperse red 54. A number of researchers considered textile industry effluent as an efficient source of potent dye decolorizing bacteria due to the natural adaptation to the higher dye concentration and its toxic effect (Khehra et al., 2005; Ali et al., 2009; Prasad and Rao, 2010 and Pokharia and Ahluwalia, 2013).

Table-14: Percent decolorization of four selected dyes by 15 bacterial isolates (isolate 1 to isolate 15; 50mg/L in 24 h)

Isolate	Disperse red 167	Disperse red 167.1	Disperse blue 60	Disperse red 54
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Isolate 1	**74.93 ± 0.91**	**73.51 ± 0.46**	**76.29 ± 0.51**	**84.17 ± 0.16**
Isolate 2	**70.23 ± 0.25**	**73.15 ± 0.44**	**73.80 ± 0.30**	**71.93±0.11**
Isolate 3	**76.06 ± 0.56**	**72.80 ± 0.32**	**73.20 ± 0.43**	**76.50 ± 0.42**
Isolate 4	56.86 ± 0.80	67.10 ± 0.65	63.13 ± 0.70	59.63 ± 0.47
Isolate 5	52.10 ± 0.65	60.57 ± 0.80	64.60 ± 0.52	58.30 ± 0.51
Isolate 6	**84.80 ± 0.35**	**72.16 ± 0.28**	**65.22 ± 0.68**	**61.50 ± 0.49**
Isolate 7	52.93 ± 0.90	66.26 ± 0.45	60.64 ± 0.55	58.73 ± 0.58
Isolate 8	53.93 ± 0.83	64.55 ± 0.50	64.06 ± 0.70	60.73 ± 0.64
Isolate 9	50.76 ± 0.25	69.00 ± 0.50	62.23 ± 0.49	57.43 ± 0.45
Isolate 10	54.63 ± 0.55	63.83 ± 0.50	57.56 ± 0.51	60.16 ± 0.76
Isolate 11	**70.16 ± 0.21**	**71.13 ± 0.32**	**73.55 ± 0.40**	**78.61 ± 0.53**
Isolate 12	57.56 ± 0.40	62.47 ± 0.41	54.99 ± 0.95	59.36 ± 0.55
Isolate 13	56.13 ± 0.41	65.83 ± 0.76	54.43 ± 0.55	53.73 ± 0.46
Isolate 14	51.16 ± 0.29	55.30 ± 0.51	70.52 ± 0.50	74.21 ± 0.37
Isolate 15	**58.83 ± 0.76**	**78.13 ± 0.37**	**65.90 ± 0.17**	**78.10 ± 0.36**

Out of these fifteen strains, six strains showing the highest decolorization potential and tolerance to dye mixture were selected and were designated as isolate 1, isolate 2, isolate 3, isolate 6, isolate 11 and isolate 15. These six bacterial isolates were purified and used for further studies (Figure 13). The concentration of dye in textile effluent from textile dyeing industry is approximately in the range of 50 to 200 mg/L. This value is typical of those used in studies on treatment for azo dye containing effluent (Zhao and Hardin, 2007). However, change in operating processes may lead to still high concentration of dye in effluent. Keeping in mind the above fact, we used 50-250 mg/L dye concentrations to check their ability to decolorize different dyes. The difference in decolorization pattern is due to the dissimilarity in specificities, structure and complexity, particularly on the nature and position of substituent in the aromatic rings and the interaction with azo bond with different dyes as reported by many authors (Chen et al., 2003; Prasad and Rao, 2010) moreover due to the loss of ecological interaction, which they might be sharing with each other under natural conditions (Wang et al., 2009). The isolation of different microorganisms from the sample indicates the natural

adaptation of microorganisms to survive in the presence of toxic dyes. Several studies have been carried out to isolate indigenous bacteria from the textile dye effluent (Ponraj et al., 2011, Kaushik and Malik, 2009, Prasad and Rao, 2010), activated sludge (Wang et al., 2009) and soil contaminated with dyes (lake-mud and wastewater treatment plant) which indicated the natural adaptation of these isolates to high dye concentration (Leena and Selva, 2008; Telke et al., 2008) and their survival in the presence of toxic dyes.

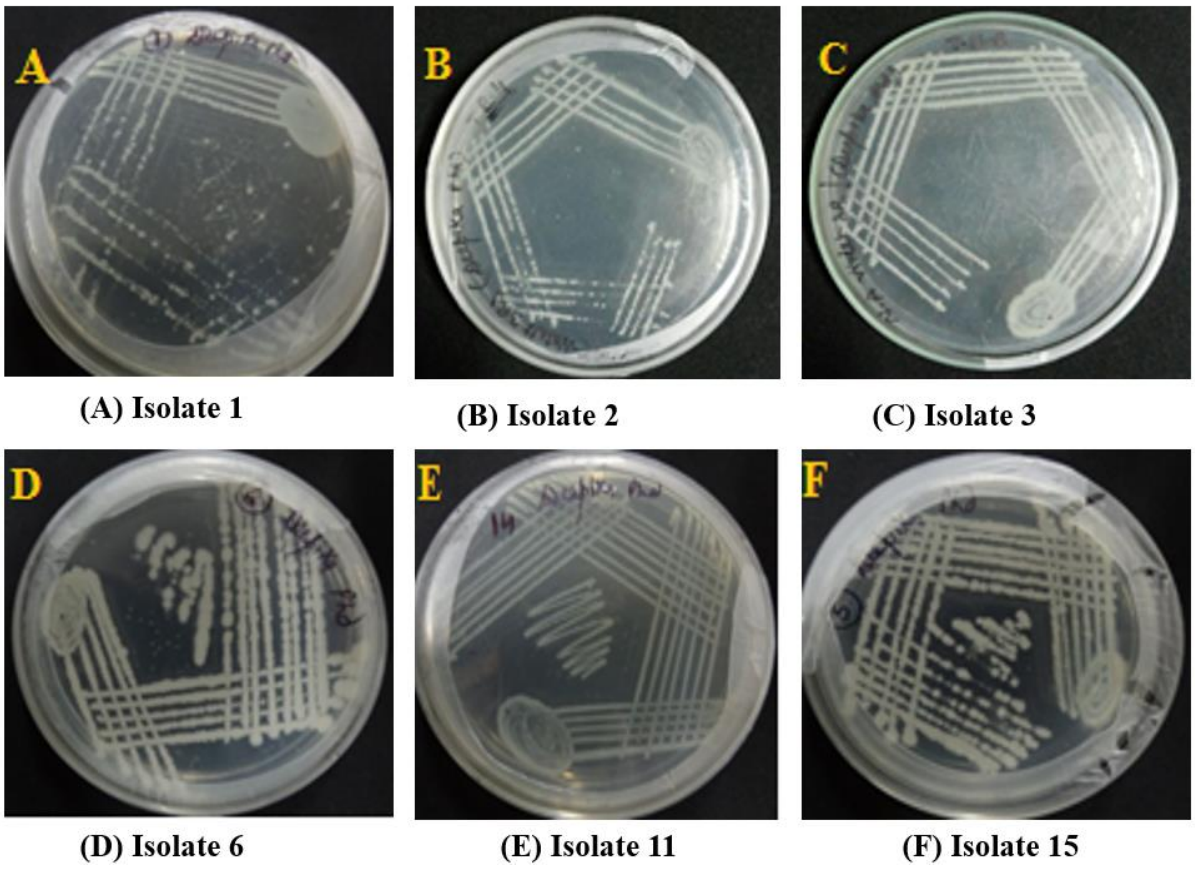


Figure-13: Six potent dye decolorizing purified bacterial isolates. 13(A) Isolate 1: 13(B) Isolate 2: 13(C) Isolate 3; 13(D) Isolate 6; 13(E) Isolate 11 and 13(F) Isolate 15

6.3.1 Biochemical, morphological and physiological characterization of bacterial isolates

Six efficient bacterial isolates were considered for their characterization, based on cell morphology, surface, colony characteristics (color) and gram's reaction. The Gram stain characteristics of the all bacterial strains indicated that they were Gram positive rod shaped (Figure-14). Table 19 shows the results of various characteristic properties

of six bacterial isolates. The isolates were examined for the biochemical texts as well (catalase test, indole, urease, oxidase, methyl red and voges proskauer tests). The isolated bacterial strains appeared white to milky off-white colonies having dry, mucoid, smooth and ellipsoidal surface with variable size and margins. All bacterial isolates showed positive tests for catalase and negative for indole, methyl red and voges proskauer test. Isolate 1 and isolate 2 showed positive results for urease in comparison to rest of the three isolates which showed negative results for urease test. Besides for oxidase test, except isolate 15 (displayed negative results), remained five bacterial isolates were showing positive results. The six most efficient bacterial species were found to share the main phenotypic features of the genus *Bacillus* (Table 15). After 16S rRNA gene sequence analysis all bacterial isolates namely, isolate 1, isolate 2, isolate 3, isolate 6, isolate 11 and isolate 15 were identified by BLAST search and submitted to NCBI for obtaining accession numbers. Figure 15 shows the phylogenetic relationship between the isolated bacterial strains and other related bacteria found in the GenBank database. The homology indicated that the isolate 1 identified as *Bacillus cereus* with accession number KX034566, isolate 2 identified as *Bacillus sphaericus* (KX034564), isolate 3 identified as *Paenibacillus pocheonensis* (KX034565), isolate 6 identified as *Paenochrobactrum glacei* (KX034561), isolate 11 identified as *Bacillus subtilis* (KX034562) and isolate 15 identified as *Brevibacillus panacihumi* (KX034559).

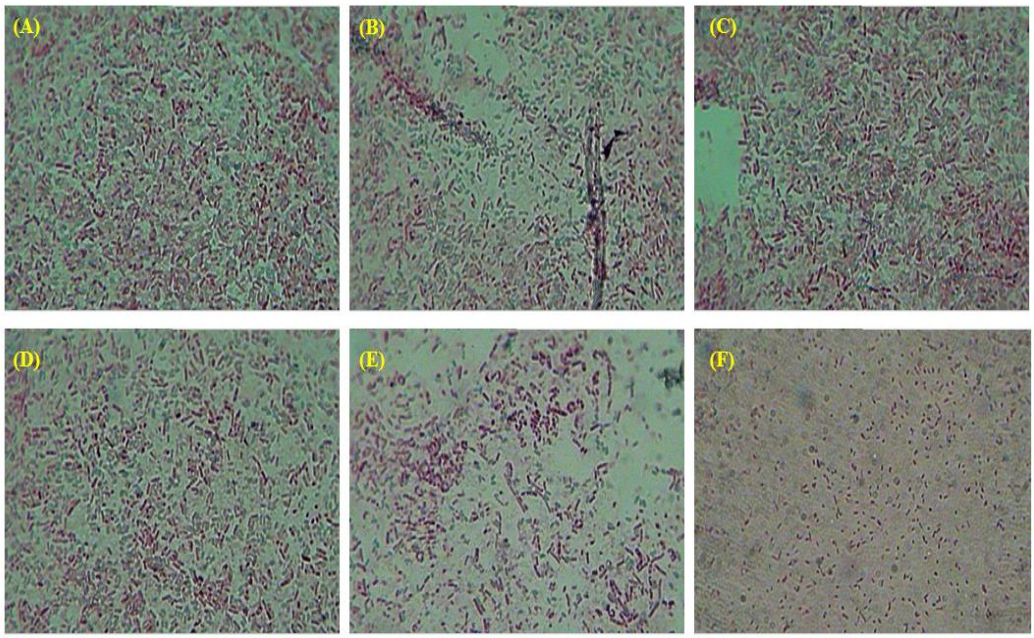
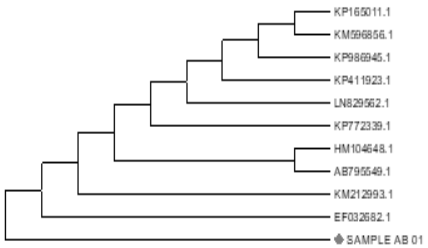
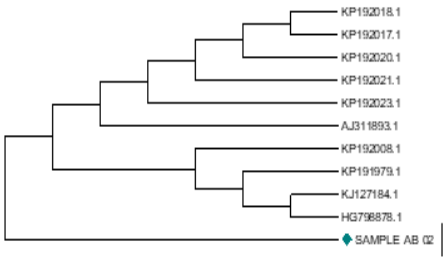


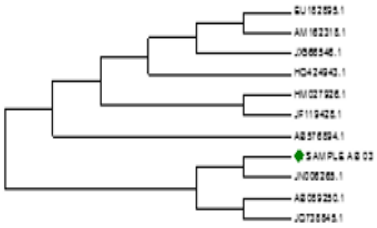
Figure-14: Gram staining of bacterial isolates. 14(A) Isolate 1; 14(B) Isolate 2; 14(C) Isolate 3; 14(D) Isolate 6; 14(E) Isolate 11 and 14(F) Isolate 15



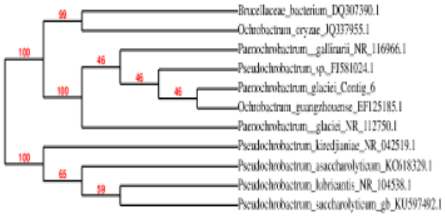
(A) Isolate 1-*Bacillus cereus* ***AU50*** (KX034566)



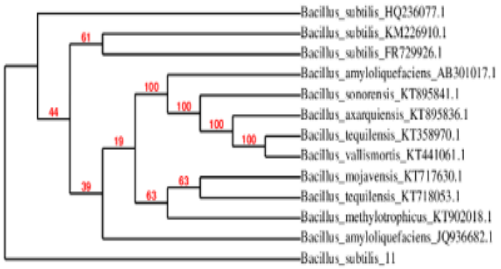
(B) Isolate 2-*Bacillus sphaericus* (KX034564)



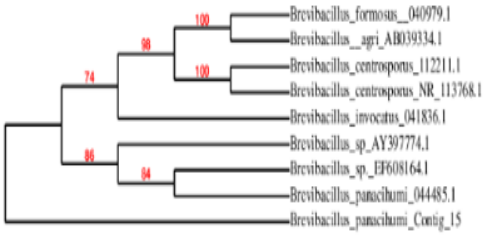
(C) Isolate 3-*Paenibacillus sp. C-2* (KX034565)



(D) Isolate 6- *Paenochrobacterum glacei* (KX034561)



(E) Isolate 11- *Bacillus subtilis* (KX034562)



(F)Isolate 15- *Brevibacillus panacihumi* (KX034559)

Figure-15: Phylogenetic tree of isolated bacterial species (1 to 7) based on their 16s rRNA gene sequence analysis. 15(A) identified as *Bacillus cereus* AU50; 15(B) identified as *Bacillus sphaericus*; 15(C) identified as *Paenibacillus* sp. C-2); 15(D) identified as *Paenochrobacterum glacei*; 15(E) identified as *Bacillus subtilis*; 15(F) identified as *Brevibacillus panacihumi*

Table-15: Biochemical, morphological and physiological characterization of bacterial isolates

Bacterial Isolates	**Isolate 1**	**Isolate 2**	**Isolate 3**	**Isolate 6**	**Isolate 11**	**Isolate 15**
Morphology	Irregular	Round	Irregular	Irregular	Irregular	Circular
Surface	Smooth	Smooth; Flat	Undulate; Concentric	Smooth	Dry; Flat	Ellipsoidal
Colony color	White	Off-White	Off-White	White	White	Cream-White
Gram reaction	Positive	Positive	Positive	Positive	Positive	Positive
Catalase Test	Positive	Positive	Positive	Positive	Positive	Positive
Indole Test	Negative	Negative	Negative	Negative	Negative	Negative
Urease Test	Positive	Positive	Negative	Negative	Negative	Negative
Oxidase Test	Positive	Positive	Positive	Positive	Positive	Negative
Voges Proskauer Test	Negative	Negative	Negative	--	Negative	Negative
Methyl Red Test	Negative	Negative	Negative	--	Negative	Negative
Molecular Identification	*Bacillus cereus* AU 50	*Bacillus sphaericus*	*Paenibacillus pocheonensis*	*Paenochrobactrum glacei*	*Bacillus subtilis*	*Brevibacillus panacihumi*
NCBI Accession No.	KX034566	KX034564	KX034565	KX034561	KX034562	KX034559

6.4 Biocompatibility assay of efficient dye degrading bacteria for dyes

To analyze the synergistic activity among isolated bacterial species for the development of consortium, biocompatibility assay was performed by following the method described by Nikam et al., (2007) with slight modifications. The selected bacterial isolates were spreaded on the growth media and allowed to grow for 24 h at 37°C. 5 mm size sterilized (whatmann paper no 1) discs impregnated with bacterial suspension of individual isolates were placed at the distance of 5 mm from the periphery of petriplates already having growth of cultures inoculated in different pattern to check the antagonistic/syneristic effect of bacterial cultures on each other.

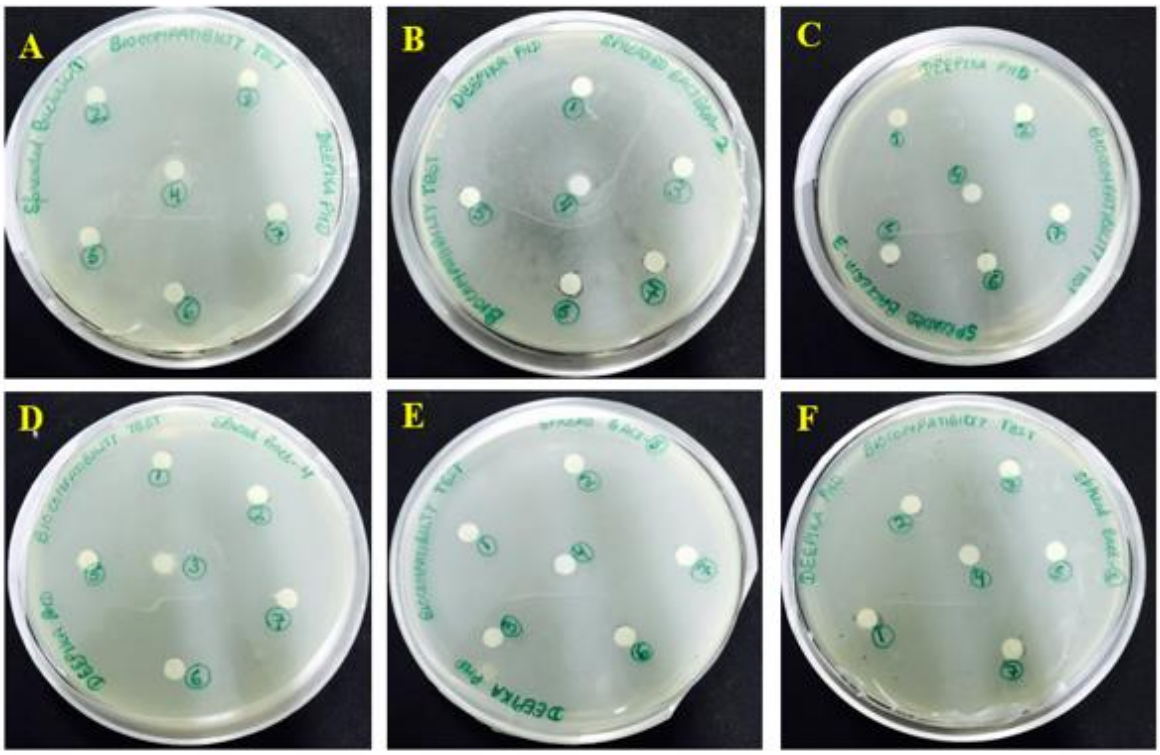


Figure-16: Test of biocompatibility between six potent identified dye decolorizing strains

After 24 h the growth was observed which was showing no zone of inhibition in any of the bacterial culture; hence all the bacterial cultures are compatible to each other and can be proved as a good consortium (Figure 16A to 16F). Our results were similar with the findings of Anandaraj et al., (2010) in which the species of *Rhizobium sp., Bacillus megaterium* and *P. fluorescens* was compatible with each other in cross streak plate assay. Thus, treatment of seeds with effective strains of *Rhizobium, Bacillus* alone or

in combination with other beneficial microorganisms may be preferred, because of their multiple potentials to control disease, fix nitrogen, increase crop productivity and improve of soil fertility besides reducing the negative environmental impact associated with chemical use (Huang and Erickson, 2007). In a similar study to evaluate the antimicrobial potential of axenic cultures of *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* was done by cross streak perpendicular assay (Singh et al., 2016). The above-mentioned isolates were screened for antagonistic or synergistic activity among each other. The interactions between microbes were observed by measuring the zone of inhibition (mm).

6.5 Development of consortium

The decolorization of textile dyes by bacteria was found to be quick and more efficient than any other microbial culture, but individual bacterial isolates may not be able to degrade the azo-bonded dyes completely. The intermediates may be carcinogenic amines, which should be degraded further for complete removal of textile dyes from wastewater effluents (Jadhav et al., 2008). Therefore, the treatment systems should comprise of mixed microbial cultures which are able to possess maximum biodegradation of dyes due to synergistic activities and is more advantageous in comparison to pure individual bacterial cultures (Khehra et al., 2005). Therefore, in this study, an attempt was made to develop the bacterial consortium containing six efficient dye degrading bacteria after investigating their compatibility and synergistic activities among each other (as discussed in previous section under biocompatibility assay). Initially a loop full of individual bacterial pure cultures were inoculated separately in minimal media and incubated for 24 h at 30ºC. For the development of bacterial consortium, 6 h old cultures were then transferred aseptically into nutrient medium followed by 24 h incubation at 30ºC. The consortium thus obtained was used as the source of inoculum for further studies (Lade et al., 2012; Das and Mishra, 2017). Similar study was conducted by Waghmode et al., (2012) for the development of consortium containing microbial culture of *G. geotrichum* and *B. laterosporus* in 100 ml nutrient medium (malt extract). Further this consortium was analyzed for the degradation of Rubine GFL. Their results indicated that the consortium was showing better rate of biodecolorization and biodegradation of Rubine GFL in comparison to the

individual organisms. The consortium lead to 100% decolorization of Rubine GFL (50 mg/L) in 30 h with significant reduction in chemical oxygen demand (COD) (79%) and total organic carbon (TOC) (68%). Our results are in accordance, the consortium (comprises six bacterial isolates) was showing maximum rate of decolorization (82.76± 0.255%) for disperse red 167.1 (rubine BL) at three different concentrations within 24 h, with increase in enzymatic activities. Our bacterial consortium was also showing better results for the decolorization (83.46± 0.26%) of disperse red 54 (scarlet RR) in contrast to individual bacterial isolates. Likewise, the rate of decolorization for disperse blue 60 was (76.12±0.97%) shown by consortium in contrast to individual bacterial isolates within 24 h The reason for better decolorization by consortium may be, the metabolites or by-products produced by individual strains, utilized by the co-existing strains for further degradation of dye in the form of consortium. Furthermore, this may lead to the enhancement for enzymatic activity also (Saratale et al., 2009). Our results are in agreement with the previous findings of Lade et al., (2012). The outcome of their study indicated that the microbial consortium containing (*P. rettgeri* strain HSL1 and *Pseudomonas sp*. SUK1) was showing 99% decolorization for the selected azo bonded textile dyes viz., reactive black 5, disperse red 78, direct red 81 and reactive orange 16 at concentration of 100mg/L in 30 h Moreover the previous results obtained by Saratale et al., (2009) for the development of microbial consortium containing (*Proteus vulgaris* NCIM-2027 and *Micrococcus glutamicus* NCIM-2168), was found to perform better rather than individual cultures. The consortium developed by this study was showing complete degradation of scarlet RR with 90% reduction of total organic carbon and chemical oxygen demand) in 3 h incubation time. The induction of dye degradaing enzymes such as riboflavin reductase and NADH reductase was observed in the consortium, which witnessed the involvement of these enzymes during quick bio-decolorization process of scarlet RR. The earlier study conducted by Kurade et al., (2012) for the decolorization and degradation of scarlet RR, the consortium of bacteria and yeast BLGG was showing 98% removal of color in 18 h, 68% decrease in biochemical oxygen demand and 74% decrease in chemical oxygen demand at 50mg/L in 48 h.

6.6 Dye decolorization assay with six efficient dye degrading bacteria (individually) and with consortium at different concentrations of dye (50 mg/L; 150 mg/L and 250 mg/L)

In the present investigation, decolorization of four selected dyes was investigated by using six selected bacterial species individually and with the developed consortium at three different concentration (50mg/L, 150mg/L, 250 mg/L). The rate of decolorization at 50 mg/L with time for the respective dye by bacterial isolates and with consortium is depicted in Figure 17; at 150 mg/L in Figure 19 and at 250 mg/L in Figure 20. The image analysis of dye decolorization (50 mg/L) obtained by bacterial isolates and consortium after 24 h against four selected dyes is displayed in Figure 18. Literature search revealed that no research has been conducted on the decolorization of disperse red 167 and 167.1 using a bacreial consortium. To the best of my knowledge no literature is available for the biodecolorization and biodegradation by microbial system for azo-bonded disperse red 167 (dark red 2b) till date. Only photocatalytic based decolorization of coralene dark red 2B is available which is based on the use of calcium zincate nanoparticles in the presence of sunlight (Madhusudhana et al., 2011). The results obtained by their study indicated that, the calcium zincate was found to be suitable for complete decolorization of dark red 2B under solar rays. Our study will be first finding for the bio-decolorization of disperse red 167 (dark red 2B). Disperse red 167.1 (rubine BL) and disperse red 167 (dark red 2B), both are single azo dyes as a result there is structure similarity with only difference in side chain group. The maximum decolorizing efficiency was exhibited by *Paenochrobactrum glacei* up to 84.80±0.34% for disperse red 167 at 50 mg/L [Figure (17A) and table 16] in 24 h while for disperse red 167.1, the consortium of bacteria showed highest 82.76±0.25% decolorization capability for disperse red 167.1 at 50 mg/L in 24 h [Figure (17B) and table 16]. The other bacterial isolates such as, *Bacillus sphaericus*, *Paenibacillus* sp. C2, *Bacillus subtilis* displayed moderate percent decolorization i.e. 70.23±0.25%, 76.06±0.55% and 70.16±0.20% for disperse red 167 at 50 mg/L [Figure 17(A)] and 73.15±0.44%, 72.86±0.32% and 71.13±0.32% for disperse red 167.1 at 50 mg/L [Figure 17(B)]. With increase in concentration of dye, the percent decolorization decreases with time. The minimum rate of decolorization for disperse red 167 was exhibited by *B. panacihumi* (58.83±0.76%) at 50 mg/L (table 16), 52.40±0.45% at 150

mg/L (table 17) in contrast at 250 mg/L the lowest rate of decolorization was displayed by *B. cereus* 45.63±0.55% (table 18). In contrast, *Bacillus cereus* displayed decolorizing ability up to 74.93±0.90% and 73.51±0.46% in 24 h for disperse red 167 and disperse red 167.1 at 50 mg/L respectively (table 16). The consortium exhibited 71.23±0.25% rate of decolorization at 50 mg/L [Figure 17(A)], 70.10±0.25% at 150 mg/L [Figure 19(A)] and 59.40±0.40% at 250 mg/L for disperse red 167 respectively [Figure 20(A)]. The results obtained in present study are in agreement with the previous studies wherein *Pseudomonas* sp. SUK1 showed 63% decolorization efficiency in 30 h for Rubine GFL dye at 100 mg/L (Lade et al., 2012) and decolorization of Rubine GFL dye (50 mg/L) in 30 h by consortium GC-BL containing *Brevibacillus laterosporus* MTCC 2298 bacterium was reported by Waghmode et al., (2012). The use of mixed culture in the form of bacterial consortium offers significant advantages over using individual bacterial isolates for the decolorization and degradation of chemical based synthetic dyes. This enhanced decolorization potential of consortium of bacteria in contrast to individual bacterial isolates is attributed to the reason of synergistic activities among them (Chen and Burns, 2006). Further, the degraded metabolites produced by individual bacteria is captured by other bacterial isolates in consortium, and results into its further degradation, resulting into less toxic products (Moosvi et al., 2005). The study conducted by Jadhav et al., (2008) clearly revealed that the consortium containing species of *Bacillus* VUS and *Galactomyces geotrichum* MTCC 1360 exhibited 100% decolorization ability in 2 h at pH 7 while individual bacteria decolorized in 5 h for 100% decolorization of the same dye. Earlier studies indicated that *Brevibacillus laterosporus,* was capable to decolorize disperse brown 3REL up to 86% and disperse brown 118 up to 96% at 50 mg/L in 48 h (Kurade et al., 2012) and 100% decolorization was recorded in anoxic conditions (Dawkar et al., 2008). Moreover, the consortium HM4 (comprises four bacterial isolates) developed by Khehra et al., (2005) was capable to decolorize Acid Red 88 dye in 20 h, whereas the individual bacterial isolates were taking more than 50 h to achieve decolorization. Therefore, use of microbial consortium offers considerable potential over the use of individual cultures for the degradation of dyes since individual cultures may lack the ability to degrade the azo dyes completely. The highest rate of decolorization for disperse blue 60 (blue BG) was exhibited by *B. cereus* 76.29±0.51% [Figure 17(C) and table 16] in 24 h at 50 mg/L, 67±0.95% at 150

mg/L [Figure 19(C) and table 17] and 43.70±0.20% at 250 mg/L [Figure 20(C) and table 18] in 24 h While the lowest decolorization ability was possessed by *P. glaciei*, 65.22±0.68% [Figure 17(C) and table 16] in 24 h at 50 mg/L, 52.96±0.95% at 150 mg/L [Figure 19(C) and table 17] and 37.46±0.52% at 250 mg/L [Figure 20(C) and table 18] in 24 h.

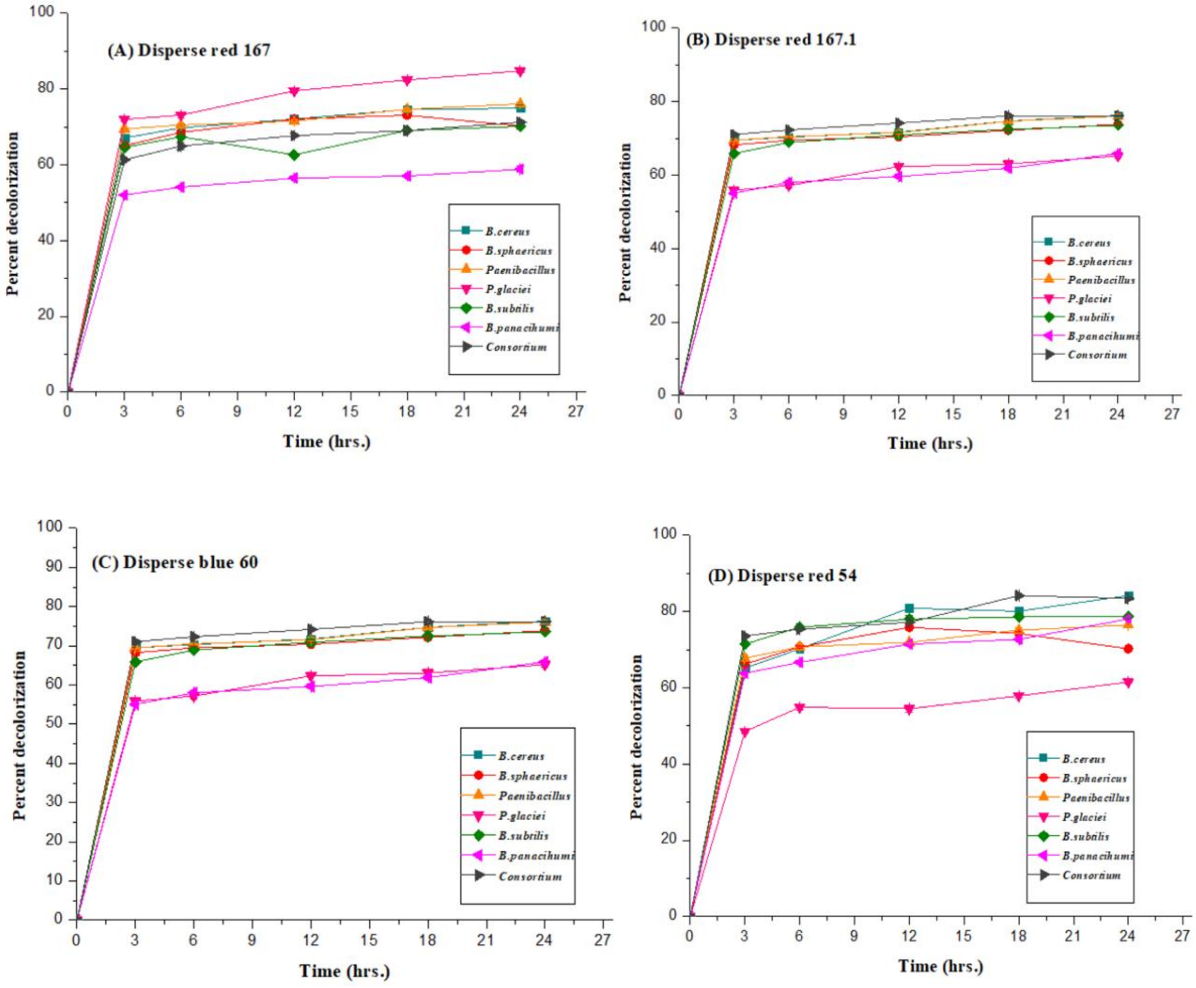


Figure-17: Percentage decolorization of four selected dyes (50 mg/L) by selected bacterial isolates individually and with consortium in 24 h

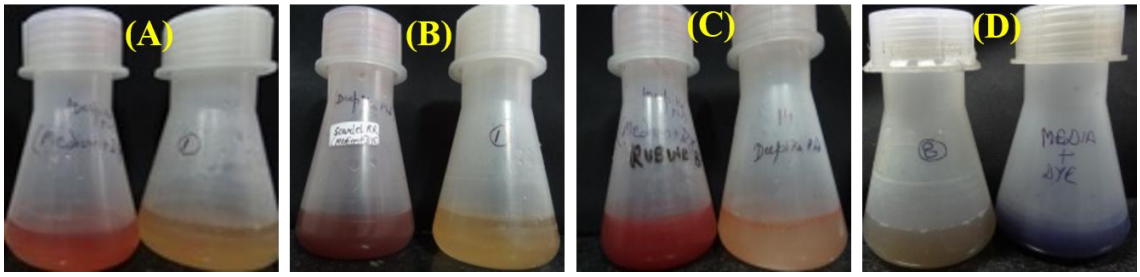


Figure-18: Image analysis of dye decolorization by bacterial isolates after 24 h. 18(A) comparison of decolorized MSM broth inoculated with isolate 4 and control dye (disperse red 167); 18(B) comparison of decolorized MSM broth inoculated with isolate 1 and control dye (disperse red 54); 18(C) comparison of decolorized MSM broth inoculated with consortium and control dye (disperse red 167.1); 18(D) comparison of decolorized MSM broth inoculated with isolate 3 and control dye (disperse blue 60)

Table-16: Decolorization rate of different bacterial isolates with consortium for four selected dyes at 50 mg/L in 24 h. All the experiments were performed in triplicates and the average was calculated to represent the decolorization rate in percentage (%).

Bacterial sp.	**Disperse red 167**	**Disperse red 167.1**	**Disperse blue 60**	**Disperse red 54**
B. cereus	74.93± 0.90	73.51± 0.46	**76.29± 0.51**	**84.17± 0.16**
B. sphaericus	70.23±0.25	73.15±0.44	73.80±0.30	71.93±0.11
Paenibacillus	76.06±0.55	72.80±0.32	73.20±0.43	76.50±0.42
P. glaciei	**84.80±0.34**	72.16±0.28	65.22±0.68	61.50±0.49
B. subtilis	70.16±0.20	71.13±0.32	73.55±0.40	78.61±0.53
B. panacihumi	58.83±0.76	78.13±0.37	65.90±0.17	78.10±0.36
consortium	71.23± 0.25	**82.76± 0.25**	76.12± 0.97	83.46± 0.26

Table-17: Decolorization rate of different bacterial isolates with consortium for four selected dyes at 150 mg/L in 24 h. All the experiments were performed in triplicates and the average was calculated to represent the decolorization rate in percentage (%)

Bacterial sp.	Disperse red 167	Disperse red 167.1	Disperse blue 60	Disperse red 54
B. cereus	58.93 ± 0.35	60.03 ± 0.15	**67.00 ± 0.95**	**68.06 ± 0.20**
B. sphaericus	61.76 ± 0.49	58.53 ± 0.47	59.70 ± 0.60	60.53 ± 0.75
Paenibacillus	62.63 ± 0.56	58.06 ± 0.11	54.63 ± 0.55	55.36 ± 0.47
P. glaciei	**72.20 ± 0.43**	54.33 ± 0.41	52.96 ± 0.95	60.20 ± 0.51
B. subtilis	54.43 ± 0.37	58.63 ± 0.55	57.70 ± 0.60	64.96 ± 0.15
B. panacihumi	52.40 ± 0.45	57.90 ± 0.81	60.96 ± 0.95	62.63 ± 0.55
consortium	70.10 ± 0.25	**67.00 ± 0.45**	66.53 ± 0.47	66.43 ± 0.40

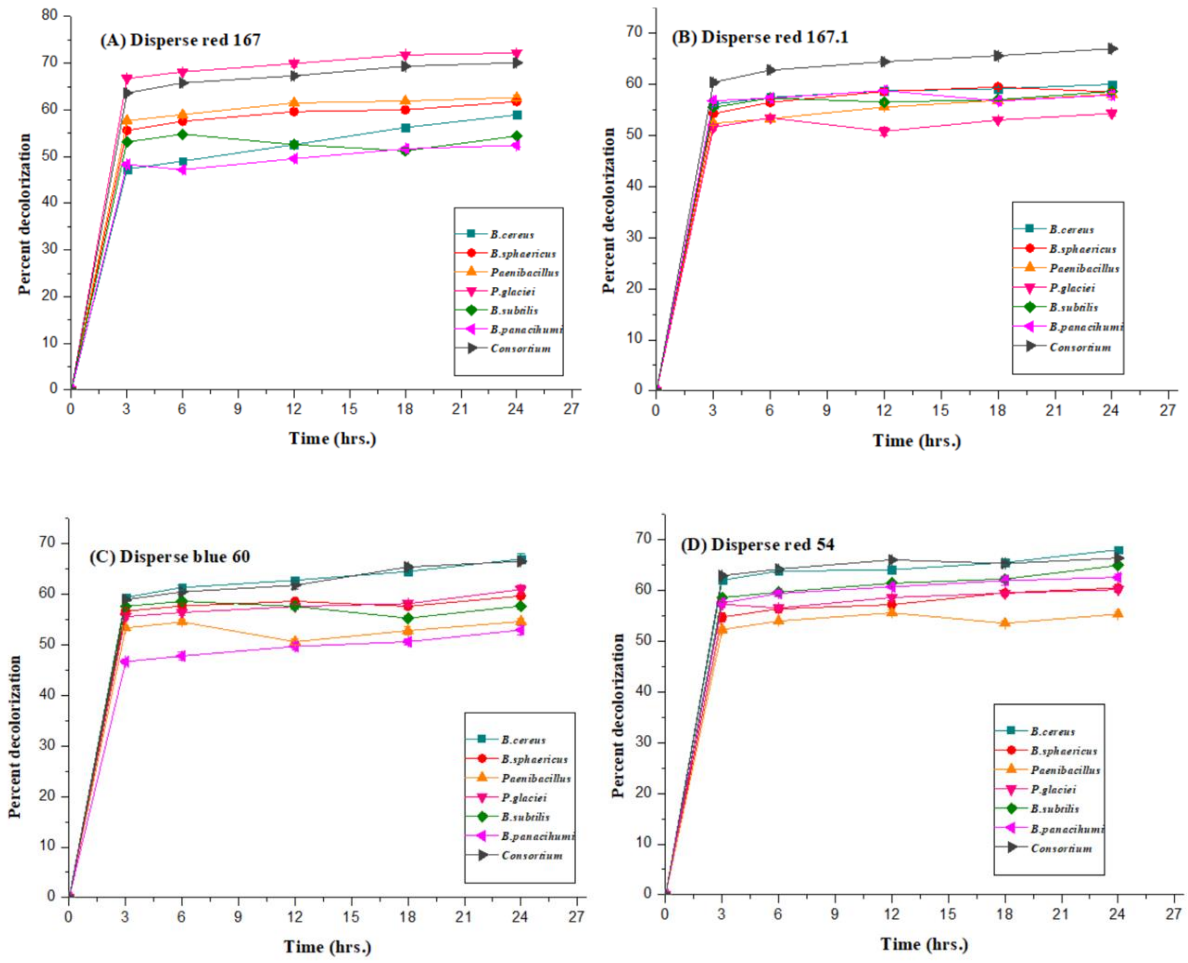


Figure-19: Percentage decolorization of four selected dyes (150 mg/L) by selected bacterial isolates individually and with consortium in 24 h

Table-18: Decolorization rate of different bacterial isolates with consortium for four selected dyes at 250 mg/L in 24 h. All the experiments were performed in triplicates and the average was calculated to represent the decolorization rate in percentage (%)

Bacterial sp.	**Disperse red 167**	**Disperse red 167.1**	**Disperse blue 60**	**Disperse red 54**
B. cereus	45.63 ± 0.55	44.96 ± 0.95	**43.70 ± 0.20**	**55.26 ± 0.55**
B. sphaericus	51.56 ± 0.55	43.63 ± 0.55	39.86 ± 0.23	49.60 ± 0.79
Paenibacillus	56.50 ± 0.43	39.76 ± 0.66	39.50 ± 0.45	46.56 ± 0.51
P. glaciei	**67.60 ± 0.52**	37.60 ± 0.52	37.46 ± 0.41	53.66 ± 0.58
B. subtilis	49.83 ± 0.28	40.06 ± 0.97	40.50 ± 0.76	44.96 ± 0.94
B. panacihumi	47.10 ± 0.10	43.00 ± 0.28	39.23 ± 0.49	47.56 ± 0.49
consortium	59.40 ± 0.40	**45.60 ± 0.51**	42.63 ± 0.55	54.53 ± 0.46

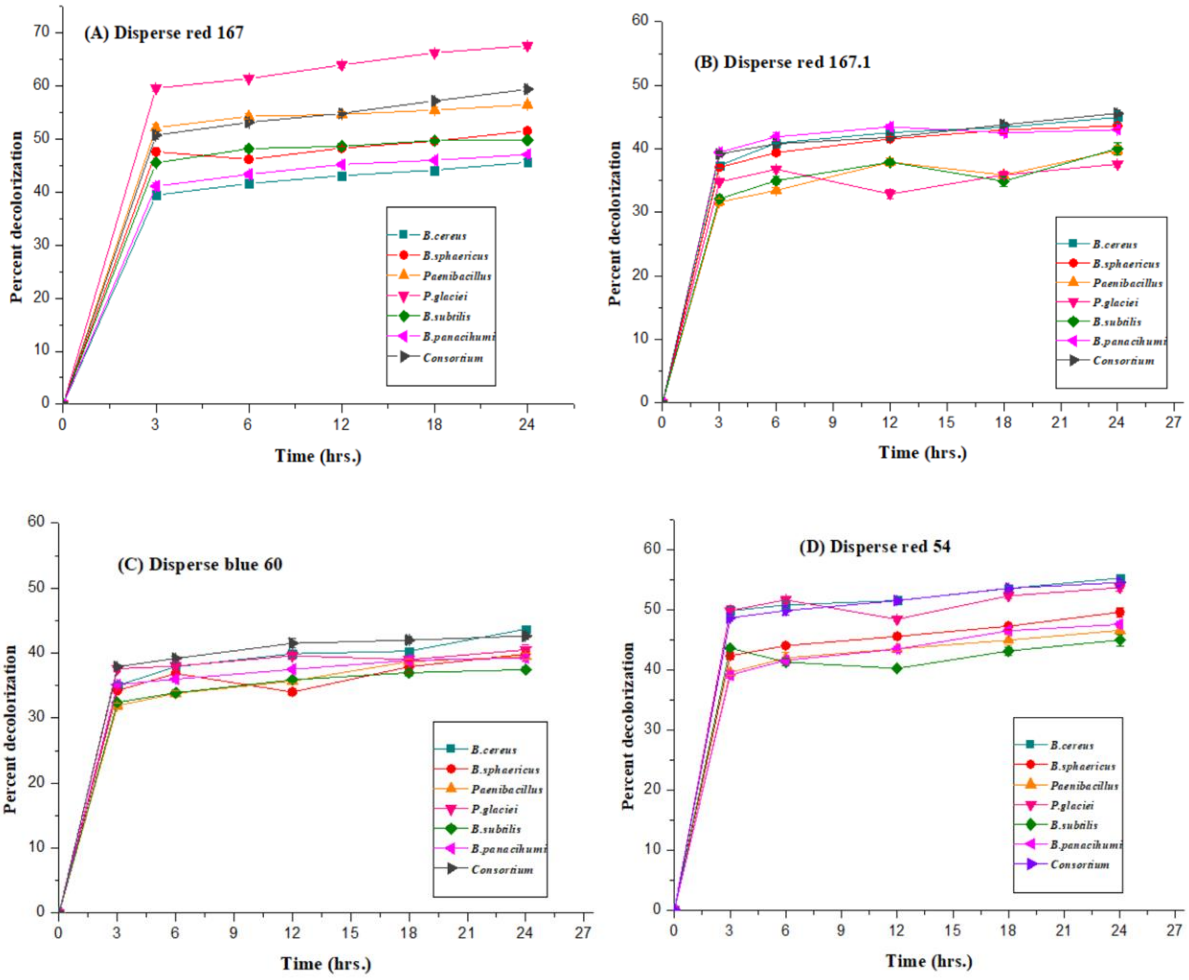


Figure-20: Percentage decolorization of four selected dyes (250 mg/L) by selected bacterial isolates individually and with consortium in 24 h

The other bacterial isolates displayed intermediate rate of decolorization in the range of 65-76% at 50 mg/L (table 16), 54-66% at 150 mg/L (table 17) and 39-42% at 250 mg/L (table 18). Our results are similar to the previous study conducted by Lade et al., (2012) for the decolorization of reactive blue 172 by employing *P. rettgeri*-HSL1. These results indicated that the particular bacteria were capable to decolorize the reactive blue 172 completely in 20 h with significant decrease in chemical oxygen demand (85%) and total organic carban (52%). Earlier studies reported 90% decolorization efficiency of *Bacillus fusiformis* for disperse blue 79 in 32 h at 75 mg/L concentration (Kolekar et al., 2008). Ponraj et al., (2011) investigated the potential of various bacterial species isolated from the dye effluent, such as *Bacillus* and *Pseudomonas* sps. possessing 89% decolorization of Orange 3R dye followed by *Salmonella* sp. (80%) and *Klebsiella* sp. (76%) after 144 h Waghmode et al., (2012) reported that glucose and yeast extract act as electron donors for higher decolorization of textile dyes. Previous study conducted by Saratale et al., (2009) ascertained that that the use of yeast extract in nutrient medium is responsible for the maximum decolorization of navy blue HER by *T. beigelii*. Chen et al., (2003) also observed the effect of that yeast extract and reported, increase in percent decolorization of red RBN dye after incorporating yeast in nutrient medium.

For disperse red 54 (scarlet RR), the highest decolorization rate possessed by *B. cereus* 84.17±0.16% at 50 mg/L [Figure 17(D) and table 16], 68.06±0.20% at 150 mg/L [Figure (19D) and table 17] and 55.26±0.55% at 250mg /L [Figure (20D) and table 18]. While the lowest decolorization ability was found in *P. glaciei*, 61.50±0.49% at 50 mg/L (table 16). In comparison *Paenibacillus* showed lowest rate of decolorization i.e., 55.36±0.47% at 150 mg/L (table 17) and *B. subtilis* displayed minimum rate of decolorization 44.96±0.94% at 250 mg/L (table 18). Our results are in agreement with the study conducted by Jadhav et al., (2009) for the decolorization of scarlet RR by *G. geotrichum* at 100 mg/L. The results indicated that *G. geotrichum* was capable to decolorize scarlet RR 100 % in 18 h incubation. In our study, the other bacterial isolates such as *B. sphaericus* displayed intermediate rate of decolorizing ability for disperse

red 54 viz., 71.93±0.11% at 50mg/L (table 16), 60.53±0.75% at 150 mg/L (table 17) and 49.60±0.79% at 250 mg/L (table 18) and and *B. panacihumi* displayed 78.10±0.36% at 50 mg/L (table 16), 62.63±0.55% at 150 mg/L (table 17) and 47.56±0.49% at 250 mg/L (table 18). The similar trend of decreasing rate of decolorization of disperse red 54 by other bacterial isolates was observed when concentration of dye was increased up to 250 mg/L. Similar results were obtained for the decolorization of textile effluent with maximum decolorization of Scarlet RR by the consortium (BLGG) developed by Kurade et al., (2012). In contrast to individual microorganisms, the developed consortium was able to reduce the color produced by Scarlet RR (50 mg/L) upto 98% within 18 h, whereas the individual pure culture of *B. laterosporus* and *G. geotrichum* could achieve decolorization only upto 61% and 34% respectively. Similar results of higher rate of decolorization exhibited by consortium of bacteria and fungal cells for the degradation of textile dye (reactive navy blue) have been reported by Kadam et al., (2011). Barsing et al., (2011) also reported efficient biotransformation of sulfonated aromatic amines by bacterial consortium. Earlier eighteen bacterial isolates belonging to *Bacillus sp*., *Acinetobacter sp*., *Staphylococcus sp*., and *Pseudomonas sp*., were found to posses the potential to decolorize textile effluent containing mixture of dyes (Olukanni et al., 2006). Ajibola et al., (2005) investigated the capability of *S. aureus*, *B. fragilis*, *B. subtilis*, *B. cereus*, *C. perifringens*, *E. coli* and *Peptostreptococcus sp*. to decolorize and degrade indigo Blue dye present in textile effluent. The previous studies conducted by Joe et al., (2008) suggested that *C. biofermentans* (isolated from dye contaminated site) for the effective decolorization of reactive azo bonded dyes (reactive red 3BA, reactive black 5 and reactive yellow 3BA) was possessing 90% decolorization efficiency in 36 h incubation. The results indicated that the *C. biofermentans* was found to be the suitable microorganisms for the decolorization of dye contaminated wastewater. The effect of various carbon sources, nitrogen sources and agricultural waste on decolorization of rubine GFL was studied by Waghmode et al., (2012). They found that glucose (alone) as a carbon source was exhibiting 93% of decolorization for rubine GFL but in combination with NH_4Cl and yeast extract, the rate of decolorization was increased upto 100%.

6.7 Screening of bacterial isolates for the activity of dye degrading enzymes

6.7.1 Preparation of cell free extract

After decolorization assay, the decolorized culture medium was used as a source to prepare cell free extract for the screening of isolates for the activity of dye degrading enzymes. The bacterial cells were subjected to centrifugation (10,000 rpm for 15-20 min at 4°C) and the resulted supernatant was used for screening of enzyme activities. The separated biomass of bacterial cells was suspended in 50 mM potassium phosphate buffer (pH 7.4), mildly homogenized and further sonicated by giving 12 strokes each of 30sec with one min interval based on 60 amplitudes keeping sonifier output below 4 °C. The subsequent extracts were then used as enzyme source (Telke et al., 2008).

6.7.2 Screening for enzymatic activities: Azoreductase and laccase

Azo dyes are synthetic dyestuff, which are characterized by the presence of one or more color imparting groups (chromophores). These synthetic dyes persist in the natural environment for longer period of time, as they are not degraded easily, making them recalcitrant (Chequer et al., 2013). Azoreductase is the key enzyme which takes part in the reduction of azo bond present in the synthetic dyestuffs. Many bacteria initiate the cleavage of the azo bonds by using NADH/NADPH as an electron donor. Laccases belongs to the multi copper oxidase family, biocatalyzes the oxidation reaction of electron rich synthetic chemical based organic substrates and degrades them into harmless species without releasing toxic metabolites. Laccases are industrially important enzymes which show potential to degrade azo bonded, polymeric and heterocyclic organic pollutants and used for bioremediation process (Lade et al., 2012). Laccases have been broadly studied for their ability to degrade azo bonded dyes (Novotny et al., 2006). Many workers reported significant role of oxido-reductase enzymes from bacteria for the degradation of textile dyes (Kalme et al., 2007). In the present study, further getting insight about the decolorization and degradation mechanism of selected dye, screening of azo-reductase and laccase enzyme acitivites were checked (Table 19 and table 20) respectively. The cell free extract was used to study the enzyme activities involved in the biodecolorization and biodegradation of selected textile dyes. Results clearly indicated the major role in initial biodegradation of Disperse Red 167 by azo reductase enzyme for the disruption of azo bond. The

bacterial isolate *P. glaeci* showed maximum activity for azoreducatse (6.916±0.063) as compared to other bacterial isolates, likewise it showed maximum activity for laccase as well (2.338±0.006). The minimum activity for azoreducatse and laccase was shown by *B. subtilis* (1.16 ± 0.01) and (0.87 ± 0.15) respectively. In comparison to other bacterial isolates, consortium showed (6.77 ± 0.04) more activity for azo-reducatse for the degradation of disperse red 167 (table 23). For the degradation of disperse red 167.1, the consortium of bacterial isolates showed maximum activity for both azo-reductase and laccase (6.85 ± 0.04) and (2.86 ± 0.03). Similar induction in enzyme activity was observed in bacterial consortium that involved in the removal of reactive orange 16 (Jadhav et al., 2010). In the previous studies, azoreductases have been identified and characterized from various bacteria, such as *Xenophilus azovarans, Pigmentiphaga kullae k24, Bacillus sp. OYI-2* and *Enterobacter agglomerans* (Maier et al., 2004). For the proposed degradation pathway of disperse red 167.1, the involvement of azo-reductase for the cleavage of dye is clearly indicated to produce three different intermediates. The rest of the bacterial isolates were showing intermediate activities for both the enzymes. Likewise, for the degradation of anthraquinone based disperse blue 60, asymmetric cleavage has been occurred to produce two intermediates. The maximum azoreductase and laccase activity was showed by *B. cereus* (5.48 ± 0.02) and (2.75 ± 0.17) respectively. Our findings are in accordance with the previous findings for the degradation of disperse blue 79, by (Kolekar et al., 2008), which indicated the induction of azoreductase activity (16.01 ± 0.56) by *Bacillus fusiformis* KMK5. In the present study, the bacterial isolate 1, (*B. cereus*) initiated the asymmetric cleavage of disperse red 54, by possessing maximum activities for azoreductase (5.26±0.03) and laccase (2.49±0.01). Our results are in agreement of previous study conducted by Pan et al., (2017), for the biodecolorization and biodegradation of disperse blue 2BLN by *Aspergillus sp.* with the induction of intracellular as well as extracellular ligninolytic enzymes activities (laccase, lignin peroxidase and Mn-peroxidase). Previous studies on enzymes that catalyzed the asymmetric reductive cleavage of anthraquinone based dyes are available (Khan et al., 2013). Jadhav et al., (2009) also reported the involvement of laccase enzyme (0.067±0.006) for the effective degradation of scarlet RR (disperse red 54). The results indicated the increase in the activity of enzyme with increase in incubation time. The

consortium (GGBL) was reported by Waghmode et al., (2012) for the activities of reductive enzymes (azoreductase, riboflavin reductase and NADH reductase) to degrade rubine GFL. The enzyme azoreductase was induced in consortium GGBL as compared to individual microbial sp. (*G. geotrichum*) after decolorization. *B. laterosporus* showed higher azoreductase activity as compared to consortium, but in contrast the decolorization rate was faster in consortium as compared to the individual organism. The earlier report confirms the use of azoreductase for degradation of dyes (Asad et al., 2007). The NADH reductase and riboflavin reductase activity were induced after 15 h of decolorization in consortium as compared to control, but it was reduced drastically after complete decolorization. Saratale et al., (2009) also reported similar kind of study for the degradation of scarlet RR and mixture of eight dyes by consortium comprises (*P. vulgaris and M. glutamicus*) with the indiction of oxidative and reductive enzymes. The bacterial consortium of four different bacterial sp. (*B. cereus, P. putida, P. fluorescence and S. acidaminiphila*) for the degradation of Acid red 88, acid red 119, acid red 97 and acid blue 113 by the action of azoreductase was reported by Khehra et al., (2005). Asad et al., (2007) isolated halophilic and halotolerant bacteria for the biodecolorization of mixture of textile dyes (5000 mg/L) by azoreductase enzyme activity and reported 100% decolorization in 96 h.

Table-19: Screening of bacteria isolates and consortium for azo-reductase activity. Values are mean of three experimental sets Mean ± SD. Data was analysed by oneway analysis of variance (ANOVA) with Tukey–Kramer multiple comparison test (JMP

software) Mean values followed by different alphabetical letters are significantly different at *P < 0.05, **P < 0.01, ***P < 0.001

Bacteria culture	Azo-reductase activity (µM of azo dye reduced mL^{-1} min^{-1})			
	Disperse red 167	Disperse red 167.1	Disperse blue 60	Disperse red 54
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
B. cereus	4.21 ± 0.01^{D}	3.81± 0.05^{E}	5.48 ± 0.02^{B}	5.26 ± 0.03^{B}
B. sphaericus	3.50 ± 0.01^{E}	3.16 ± 0.05^{B}	4.16 ± 0.04^{D}	4.47 ± 0.02^{D}
Paenibacillus	5.33 ± 0.03^{B}	4.76 ± 0.04^{D}	2.85 ± 0.06^{F}	3.73 ± 0.06^{E}
P. glacei	6.91 ± 0.06^{A}	6.12 ± 0.10^{A}	4.89 ± 0.01^{C}	4.78 ± 0.08^{A}
B. subtilis	1.16 ± 0.01^{F}	0.91 ± 0.04^{F}	2.18 ± 0.01^{F}	2.37 ± 0.01^{E}
B. panacihumi	5.33 ± 0.05^{B}	4.92 ± 0.03^{C}	4.86 ± 0.03^{A}	4.22 ± 0.05^{E}
Consortium	6.77 ± 0.04	6.85 ± 0.04	4.79 ± 0.03	5.13 ± 0.04

Table-20: Screening of bacteria isolates and consortium for laccase activity. Values are mean of three experimental sets Mean ± SD. Data was analysed by oneway analysis of variance (ANOVA) with Tukey–Kramer multiple comparison test (JMP software). Mean values followed by different alphabetical letters are significantly different at *P < 0.05, **P < 0.01, ***P < 0.001

Bacteria culture	Laccase activity (µM of ABTS oxidized mL^{-1} min^{-1})			
	Disperse red 167	Disperse red 167.1	Disperse blue 60	Disperse red 54
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
B. cereus	1.12 ± 0.01^{E}	0.96 ± 0.07^{F}	2.75 ± 0.17^{A}	2.49 ± 0.01^{A}
B. sphaericus	1.14 ± 0.03^{E}	1.16 ± 0.03^{E}	1.94 ± 0.04^{E}	1.62 ± 0.02^{C}
Paenibacillus	2.03 ± 0.005^{B}	1.95 ± 0.02^{E}	2.12 ± 0.07^{B}	1.96 ± 0.03^{E}
P. glacei	2.33 ± 0.006^{A}	2.03 ± 0.05^{B}	1.69 ± 0.06^{E}	1.50 ± 0.09^{B}
B. subtilis	0.87 ± 0.15^{F}	0.79 ± 0.03^{F}	0.85 ± 0.04^{F}	1.07 ± 0.03^{F}
B. panacihumi	1.97 ± 0.02^{C}	1.83 ± 0.70^{C}	1.97 ± 0.03^{C}	1.62 ± 0.02^{C}
Consortium	1.64 ± 0.02	2.86 ± 0.03	2.20 ± 0.03	2.17 ± 0.04

6.8 Analysis of dye degraded metabolites: characterization of degraded products of dye

6.8.1 Analysis of degraded products of disperse red 167: FTIR analysis

The FTIR spectra of control disperse Red 167 is presented in Figure 12(A). After decolorization experiment FTIR, spectra were again recorded for 18 h and 24 h and extracted metabolites were obtained by *Paenochrobactrum glacei* (Figure 21A and 21B). The spectra revealed the presence of peaks at 1575.46 cm^{-1} for H-N-H bending. The stretching of (CO)-H was reported at 2852.81 cm^{-1} and the peak at 1609.65 cm^{-1} showed carbonyl stretching vibration. After 24 h decolorization, some new peaks appeared which showed deformation and C-H out of plane bending vibrations at 961 cm^{-1}. In 24 h spectra, the peak at 1039.67 cm^{-1} represented the formation of aryl alkyl ether (symmetric stretching). In control, the peaks at 1560.46 cm^{-1} and 1621.22 cm^{-1} is due to the presence of azo groups –N=N– stretching. The disappearance of peaks at 1621.22 cm^{-1} indicated the reductive cleavage of dye at azo bond position in 18 h and 24 h extracted metabolites. Our results are in aggrement with the similar kind of studies done by Ghodake et al., (2011) while studying the FTIR of amaranth (mono-azo dye). FTIR spectra of control amaranth dye displayed peaks in the fingerprint region (1,500–500 cm^{-1}) for mono-substituted and para-di-substituted naphthalene rings which are supported by a peak at 677 and 1,369 cm^{-1} for –S=O stretching of naphthalene rings, along with these peaks for aromaticity, a peak at 1105 cm^{-1} for the C–OH stretching vibrations for secondary alcohol group and a peak at 882 cm^{-1} for C–H stretching. Dawkar et al., (2008) also reported the FTIR spectra of textile dye disperse brown 3REL. Peaks in the control disperse brown 3REL presented its functional groups present in the structure in the form of: stretching vibrations of N–O (807 cm^{-1}), stretching at C–OH (1037 cm^{-1}), C–H deformation (1339 and 1138 cm^{-1}), O–H deformation (1392 cm^{-1}) and C–N stretching (1195 and 1531 cm^{-1}). While the FTIR spectrum of 8 h decolorized extracte showed a significant change in positions of peaks when compared to control dye spectrum. A new peak at 1458 cm^{-1} represented C–H asymmetric deformation of alkane, where as peak at 1667 cm^{-1} was observed for C=N stretching. Peak at 3421 cm^{-1} represented free N–H group.

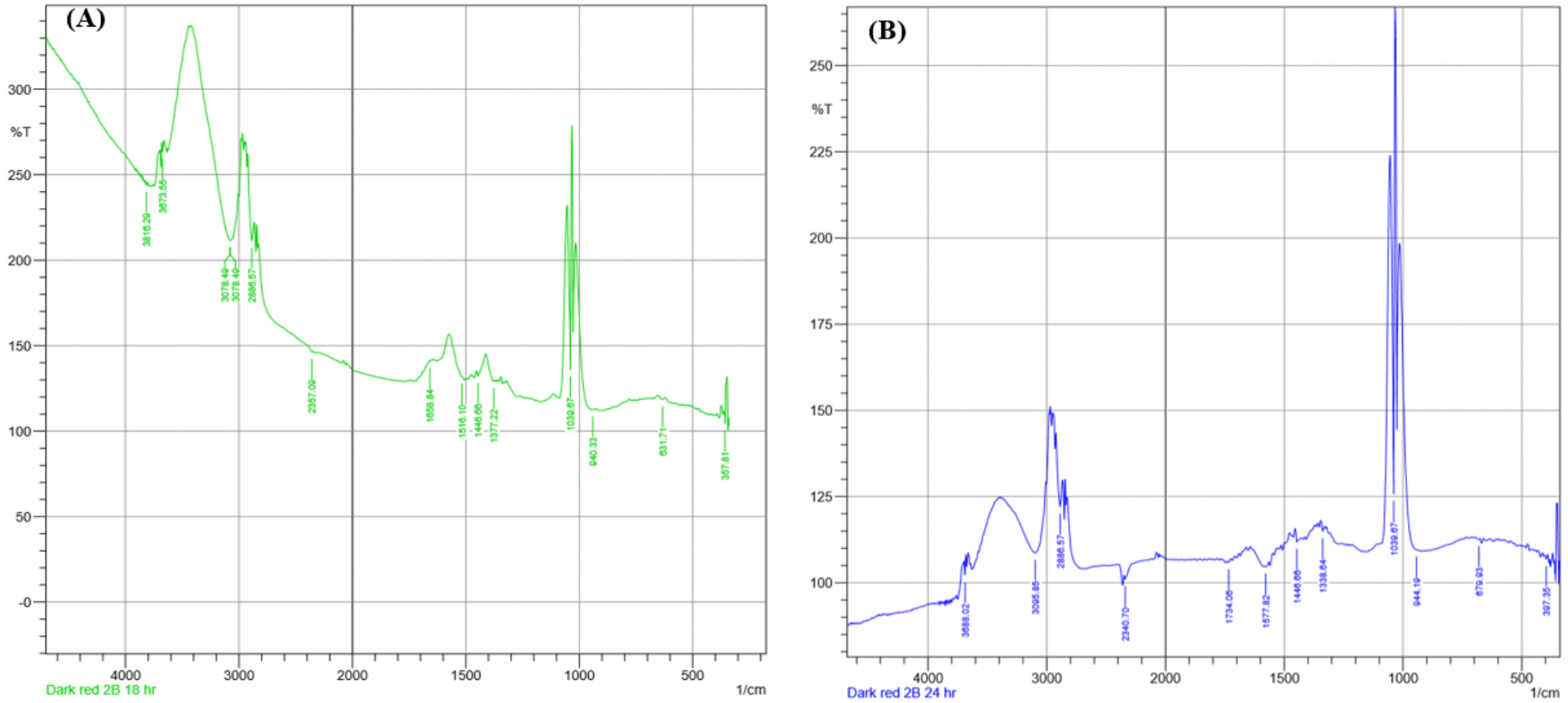


Figure-21: FTIR spectrum of decolorized disperse red 167. 21(A) after 18 h and 21(B) after 24 h

6.8.1.1 GC-MS analysis of dye decolorized medium: Proposed degradation pathway for disperse red 167

The gas chromatrograph and mass spectrum of disperse red 167 is presented in Figures 22A and 22B, respectively. The GC-MS profile showed that at retention time 16.64, asymmetric cleavage of disperse red 167 mediated by cleavage of azo bond and by the action of azo-reductase, yielded two intermediates (Figure 23), intermediate I (2-chloro-4-nitro-phenylamine; mol wt. 172) and intermediate II (Acetic acid2-[(2-acetoxy-ethyl)-(4-amino-3-propionylamino-phenyl)-amino]-ethyl ester; mol wt. 351). Intermediate II after azo-reductase action and dealkylation yielded the metabolite formic acid 2-[(4-amino-3-formylamino-phenyl)-(2-formyloxy-ethyl)-amino]-ethyl ester (mol wt-295; M/Z- 292. Furthermore, this metabolite reduced to form the final metabolite N4, N4-bis(2-methoxylthyl)-.N2-methyl benzene-1,2,4-triamine (mol wt-253; M/Z-250). Similar kind of study was conducted by Jadhav et al., (2009) for the degradation of disperse dye scarlet RR by *Galactomyces geotrichum* MTCC 1360. These results clearly indicated that the asymmetric cleavage of scarlet RR by the action of peroxidase enzyme leads to the formation of reactive products. The resultant products after demethylation and reduction reaction yields two stable intermediates i.e. 2-[ethyl-(4 oxo-3,4-dihydro-quinazolin-2-yl methyl)-amino]-acetamide and 1-chloro-4-nitro-benzene. The reduction of 1-chloro-4-nitro-benzene give rise to produce 1-chloro-4nitroso-benzene, which was reduced again to form N-(4chloro-phenyl)-hydroxylamine, and finally reduced to give the metabolite 4-chloro-phenylamine. The other intermediate 2[ethyl-(4 oxo-3,4-dihydro-quinazolin-2-yl methyl)-amino]acetamide was broken down (asymmetrically) to give 2-methyl-3 H quinazoline 4-one and 2-ethylaminoacetamide.

The demethylation of 2-methyl-3 H quinazoline 4-one produces 3 H quinazolin-4-one.

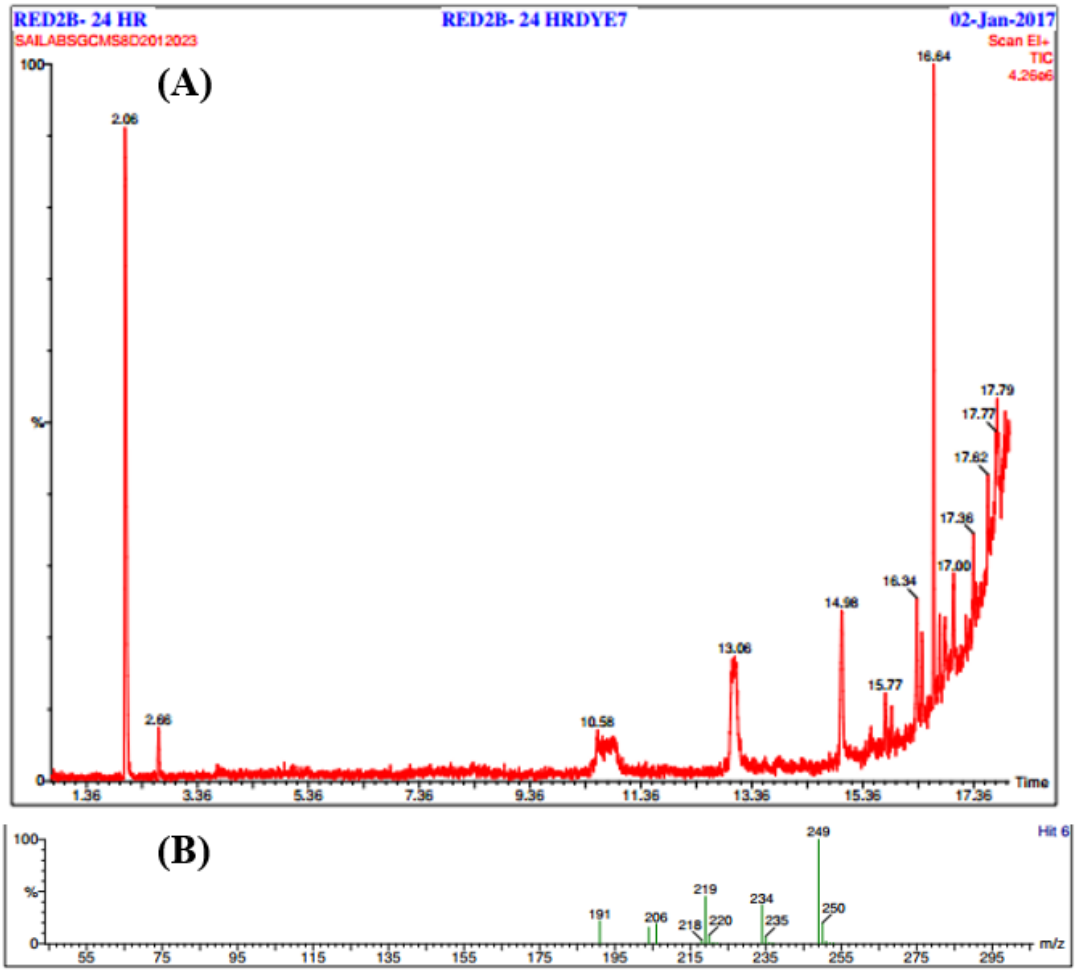


Figure-22: 22(A) Gas chromatogram of metabolites of disperse red 167 degradation after 24 h; 22(B) Mass spectrum of metabolites of disperse red 167 degradation after 24 h

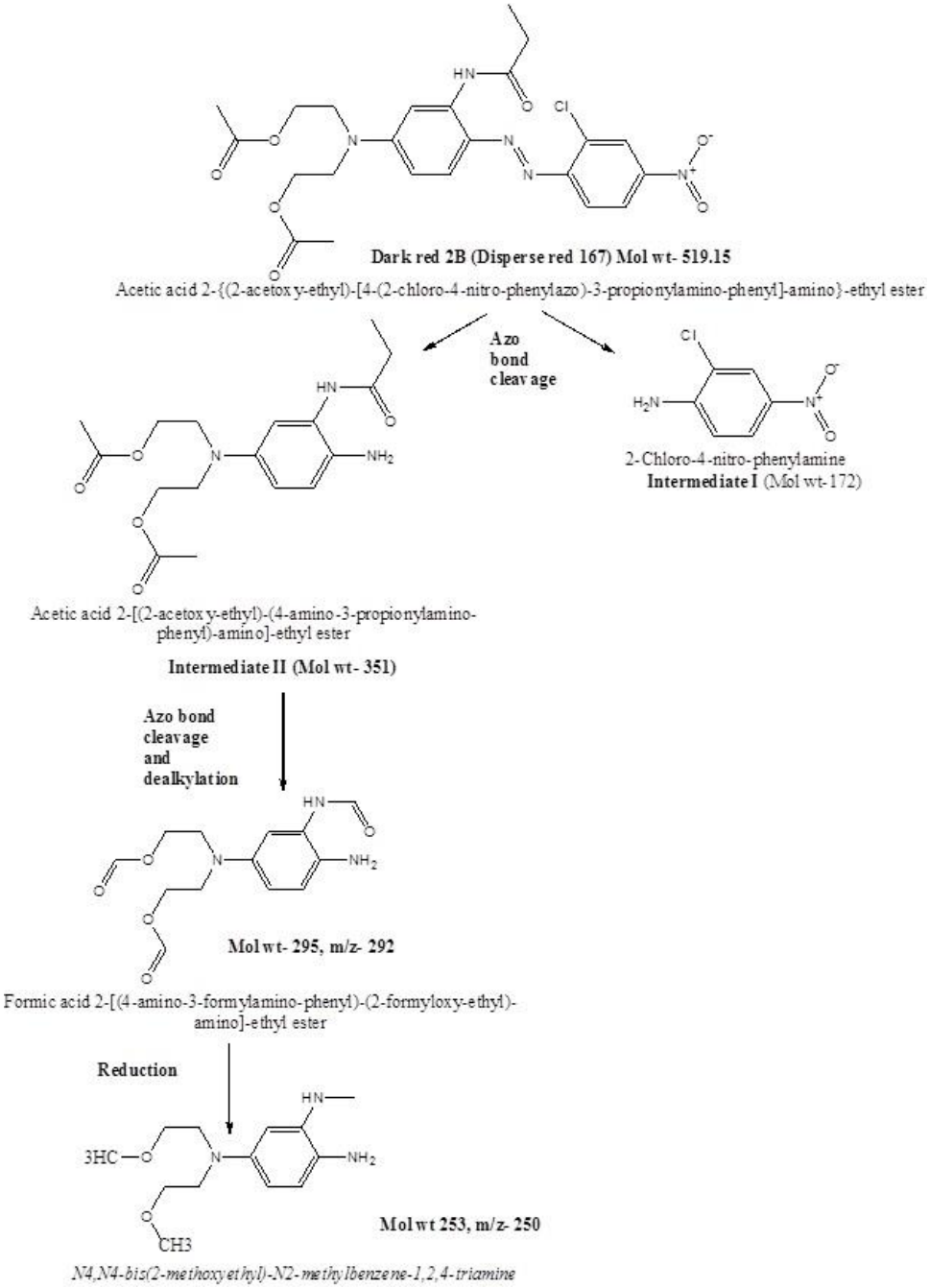


Figure-23: Proposed degradation pathway for degradation of disperse red 167

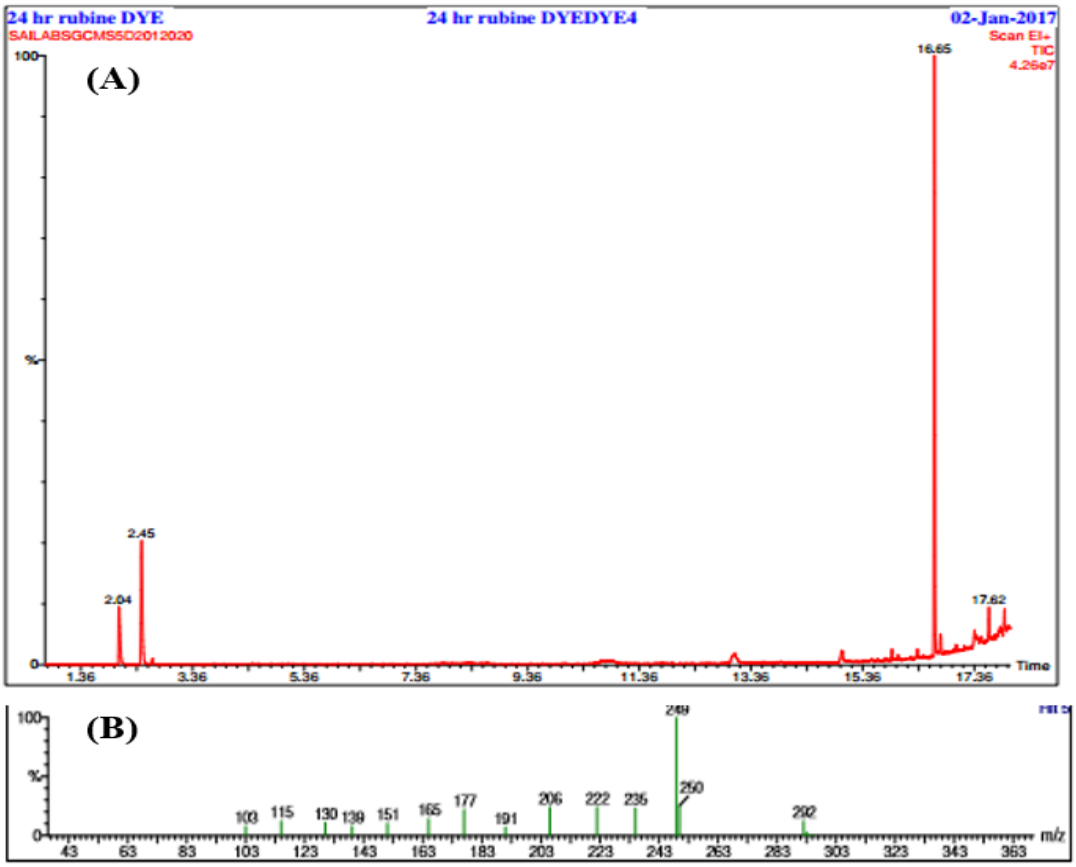


Figure-24: 24(A) Gas chromatogram of metabolites of disperse red 167.1 degradation after 24 h; 24(B) Mass spectrum of metabolites of disperse red 167.1 degradation after 24 h

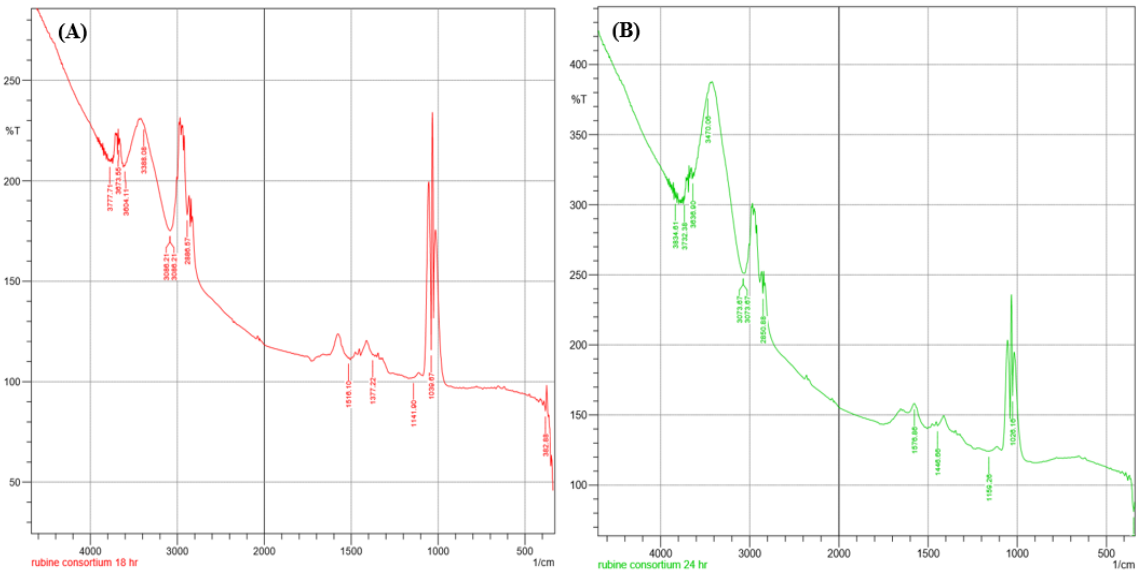


Figure-25: FTIR spectrum of decolorized disperse red 167.1; 25(A) After 18 h and 25(B) After 24 h

6.8.2 Analysis of degraded products of disperse red 167.1: FTIR analysis

The FTIR spectrum of control disperse red 167.1 (rubine BL) is displayed in Figure 12(B). The peak in the range of 1595-1600 cm^{-1} for the presence of azo bond (-N=N-), 831 cm^{-1} for C-H deformation in alkalanes and N-O stretching in nitrites, 734-750 cm^{-1} for the presence of benzene ring, 619.17 cm^{-1} for C–N stretching in acyclic compounds, C-N vibrations at 1120.68 cm^{-1}, peak at 3500–3400 cm^{-1} for -OH stretching vibration, peak at 1500–1400 cm^{-1} was attributable to aromatic C=C stretching frequency. Similar functional groups were found on Rubine GFL by Waghmode et al., (2012) and Pan et al., (2017). The FTIR spectra after degradation of disperse red 167.1 is presented in Figure 25(A and B). The metabolites found after degradation of disperse red 167.1 by consortium of bacteria showed peak at 1516.1 cm^{-1} for the presence of nitro (aromatic) compound –N=O- stretch, peak at 2,857.42–2,926.39 cm^{-1} for alkanes, 1141.9 cm^{-1} for C-H deformation (Jadhav et al., 2009). The disappearance of peak at (1595-1600 cm^{-1}) showed azo bond reduction by azo-reductase enzyme during decolorization of disperse red 167.1 by consortium (Waghmode et al., 2012). Absence of major peaks from FTIR spectra of control dye in contrast to the extracts obtained after decolorization of disperse red 167.1 displayed the action of enzymatic system of individual isolates in the form of consortium, responsible for the biotransformation of disperse red 167.1 (Waghmode et al., 2012).

6.8.2.1 GC-MS analysis of dye decolorized medium: Proposed degradation pathway for disperse red 167.1

The gas chromatrograph at retention time 16.65 and mass spectrum of disperse red 167.1 is presented in Figure 24(A) and 24(B) respectively. The proposed metabolic pathway for the degradation of disperse red 167.1 by bacterial consortium after 24 h is shown in Figure 26). Initially, disperse red 167.1 was scavenged asymmetrically by the action of laccase to form intermediate I, N,N-Diethyl-benzene-1,4-diamine (mole wt-164 and m/z at 167) and intermediate II, 2-Chloro-4-nitro-phenylamine (mole-wt-172). On the other hand, by the action of azo-reductase and after dealkylation, intermediate III, {[4-amino-3-(formylamino)phenyl]imino}diethane-2,1-diyl diformate was formed

(mole wt-295 and m/z at 292). Further after reduction of intermediate III, final product was formed N4, N4-bis(2-methylbenzene-1,2,4-triamine (mole wt-253 and m/z at 250). Similar study was conducted by Waghmode et al., (2012) by using consortium of two microbial cultures viz., *Galactomyces geotrichum* and *Brevibacillus laterosporus,* by altering the experimental conditions for the biodegradation of Rubine GFL dye. They ascertained consortium was showing better activity as compared to the individual organisnsm for the decolorization. The consortium was showing 100% decolorization of Rubine GFL in 50 mg/L in 30 h with substantial decrease in chemical oxygen demand i.e. 79% and 68% reduction in TOC (total organic carbon). Wagmode et al., (2012) proposed three different degradation pathways for rubine GFL by individual microbial cultures of *Galactomyces geotrichum* and *Brevibacillus laterosporus* as well as in the form of their consortium. After degradation by *G. geotrichum*, tyrosinase (oxidative enzyme) mediated assymetric cleavage occurred, to form two different metabolites 4-nitrobenzonitrile (mole wt-148 and m/z at 150) and other was unidentified. In case of *B. laterosporus*, assymetric cleavage by the induction of azo-reductase yields two metabolites, 2-(amino methyl)-5-nitroaniline (mole wt- 167 and m/z at 169) and other was unidentified. Further deamination yields 1-(4-nitrophenyl) methanamine (mole wt- 152 and m/z at 154) as final product. In comparison, after degradation by the consortium (*G. geotrichum* and *B. laterosporus*), assymetric cleavage by veratryl alcohol oxidase, gave two primary intermediates (mole wt- 148 and 202) respectively. Furthermore, after activity of reductase enzyme, the intermediate with mole-wt-148 was converted to 1-(4-nitrophenyl) methanamine (mole wt- 152 and m/z at 154). After induction of laccase mediated demethylation, the other intermediate with mole-wt- 202, produced secondary intermediate viz., 3-[(4-diazenylphenyl) amino] propanenitrile (mole wt- 173 and m/z at 170). Furthermore azo reductase carried out the reduction of secondary intermediate to produce N-(3-aminopropyl) benzene-1,4-diamine (mole wt- 165 and m/z at 168). Previous studies also reported the action of wild plants in the form of consortium was displaying fast biodegradation of remazol orange 3R dye. Moreover, the intermediates and products formed after decolorization of dye was less toxic and environmental friendly as compared to the parent dye (Kabra et al., 2011). Similarly, Telke et al., (2008) reported the biodegradation of reactive red 141 by *Rhizobium radiobacter,* the oxidative enzymes initiated the cleavage and further

desulfonation occurred to yield naphthalene diazonium (m/z at 155), 1, 3, 5- triazine 2, 4-diol (m/z at 112), p-dinitrobenzene (m/z at 168) and 2-nitroso naphthol (m/z at 171). Likewise, Jain et al., (2012) conducted the biodegradation of reactive violet 5 by mixed bacterial culture through enrichment technique on minimal salt media upto 1500 mg/L dye concentration and 20g/L salt concentration. Mixed bacterial culture was contained six strains of bacteria namely, (four different species of *Bacillus sp*.), *Lysinibacillus sp*., and *Ochrobacterium sp*. They proposed the degradation pathway for the mineralization of reactive violet 5 dye by using mixed bacterial culture and confirmed the formation of confirmed the formation of four intermediates viz., 1-diazo-2-naphthol, 4-hydroxybenzenesulphonic acid, 2-naphthol and benzene sulphonic acid**.** Jadhav et al., (2008) also reported the effect of incubation temperature as a process variable for the effective degradation of methyl red (azo dye) by employing *Galactomyces geotrichum.* They proposed the degradation pathway for the respective dye on the basis of GC-MS data and confirmed the formation of anthranilic acid as a final product at 5°C incubation temperature, N-N' dimethyl-p- phenylenediamine as a final product at incubation temperature 50°C. But both the products were absent at 30°C. Moreover, *Bacillus laterosporus* was also considered for the degradation of methyl red into high molecular mass intermediates by asymmetric cleavage by azo-reductase enzyme and laccase mediated reactions (Gomare and Govindwar, 2009).

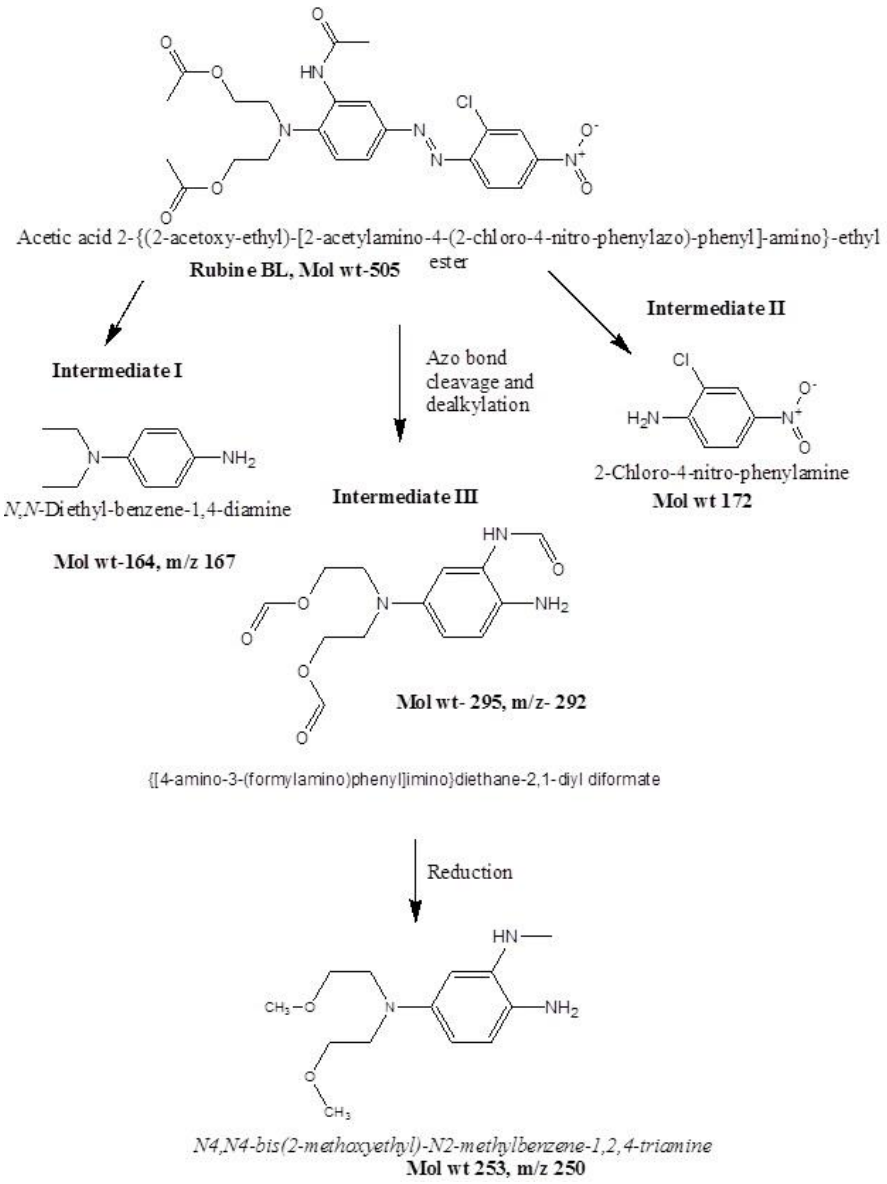


Figure-26: Proposed pathway for degradation of disperse red 167.1

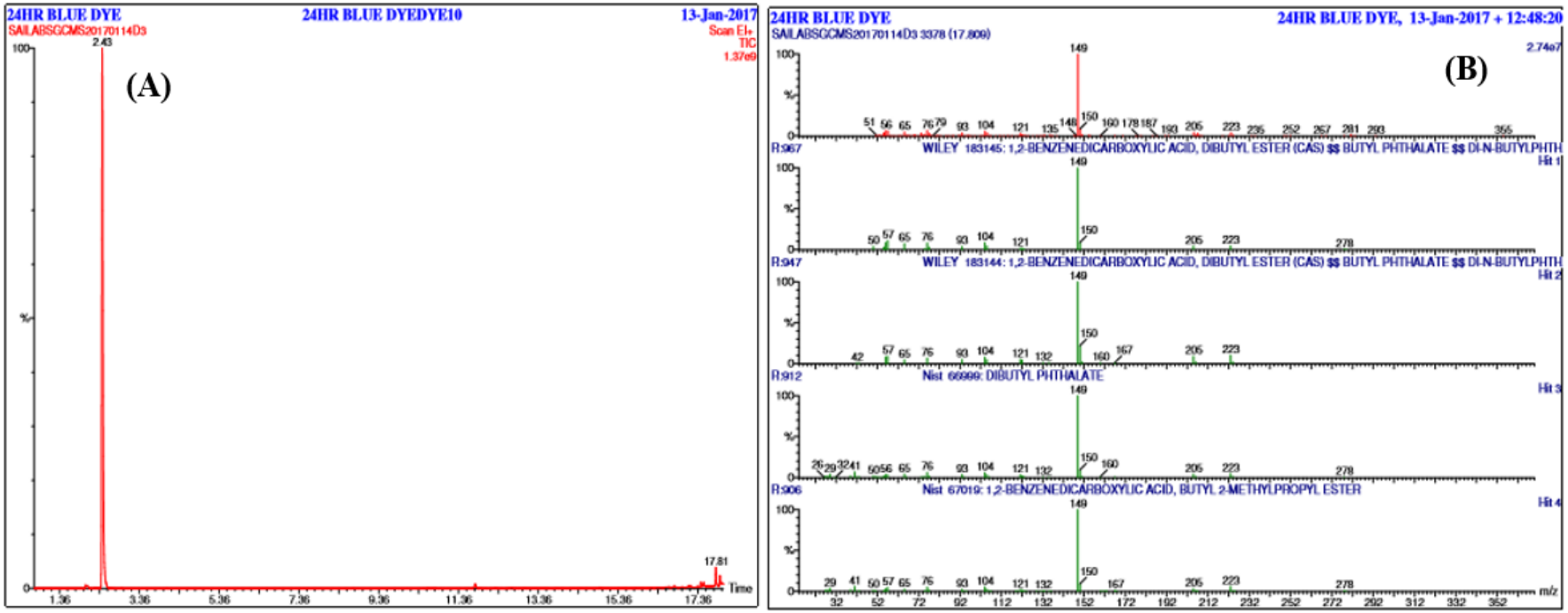


Figure-27: 27(A) Gas chromatogram of metabolites of disperse blue 60 degradation after 24 h; 27(B) Mass spectrum of metabolites of disperse blue 60 degradation after 24 h

6.8.3 Analysis of degraded products of disperse blue 60: GC-MS analysis and proposed degradation pathway for disperse blue 60

The GC-MS analysis revealed the formation of various metabolites after the decolorization of disperse blue 60. The GC chromatograph and mass spectrum of disperse blue 60 is shown in Figure 27(A and B respectively). Therefore, a pathway has been elucidated for the degradation of disperse blue 60 by *B. cereus* (Figure 29). The analysis of biodegradation products of DB60 showed that there is asymmetric cleavage which leads to the formation of 11-aminopent,6,10,12,16-oxy, 8,14 amino-10-dihydroanthracene and finally reduced to 11-aminopent, 6,16-oxy,8,14 amino-10-dihydroanthracene having molecular weight 279 which shows m/z at 281. Further reduction and deamination reaction occurs, to produce 11 aminopent,10-dihydroanthracene (molecular weight-221 and corresponding m/z at 223. The deamination reaction occurred to give 2,3-dimethyl-9,10-dihydroanthracene (molecular weight-208, corresponding m/z at 205. Earlier studies conducted by Kolekar et al., (2008) for the degradation of disperse blue 79 showed that there was asymmetric cleavage of the azo-bond by the action of azo-reductase which leads to the formation of hydrazine, and other product is debrominated with removal of bromine, further removal of nitro groups leads to form dinitrobenzene. Weber and Adams, (1995) studied chemical and sediment mediated reduction of disperse blue 79, to form 2-bromo-4,6-dinitroaniline. The degradation pathway of disperse blue 79 obtained after decolorization by *B. fusiformis* KMK5 is totally dissimilar from those obtained by chemical and sediment mediation reduction (Weber and Adams, 1995) and by sequential batch process (Melgoza et al., 2004).

Similarly, the degradation of anthraquinone based disperse blue 2BLN dye was studied by Pan et al., (2017), which revealed the cleavage of anthraquinone chromophoric group and partial mineralization of target dye. The results indicated that more than one degradation pathways have been reported for disperse blue 2BLN dye by *Aspergillus sp*. XJ2. The cleavage started by the oxidation reaction initiated by enzymes and opening of ring by hydroxylation, oxidation polymerization and finally decarboxylation. The metabolic pathway confirms the formation of ethyl propionate at m/z at 102; 1,3-indanone at m/z at 146 and the formaqtion of hydraquinone at m/z at 110. Earlier study conducted by Rajkumar et al., (2007) also confirmed the formation of 1,3-indanone (m/z 146) in the metabolites of ozonation reactive blue 19 by electrochemical degradation. Dhanve et al., (2008) studied the degradation pathway of di-azo, reactive blue 172 by *Exiguobacterium sp.* RD3, and proposed the degradation pathway initiated by laccase and azo-reductase to form naphthalene as a final product at m/z 128.

6.8.3.1 Analysis of degraded products of disperse blue 60

To investigate the possible mechanism or pathway of decolorization of dye, the aliquots obtained at different time intervals after 18h and 24h, during decolorization process were scanned in the range of 4000–400 cm^{-1} wave numbers using FTIR. Results of FTIR after decolorization were shown in Figure 28(A and B). The IR spectra of parent DB 60 was shown in Figure 12(C). The parent dye (DB 60) displaying various peaks in the finger print region for para-disubstituted and meta-disubstituted benzene rings as well as a peak at 1741.38 cm^{-1} for C=0 stretch and peak at 2945.22 cm^{-1} for asymmetric CH_3 stretch, a peak at 1454.38 cm^{-1} designate C-C stretch in aromatics, a peak at 945.15 cm^{-1} for O-H bend of carboxylic acid and presence of peaks at 677.04 cm^{-1}, 736.83 cm^{-1},763.84 cm^{-1} indicates aromaticity or the presence of benzene ring (Kolekar et al., 2008). A peak at 3439.19 cm^{-1} for –OH stretching vibration and -NH stretching vibration, a peak at 1651 cm^{-1} shows -C=C- stretching. Other peaks at 1516.1 and 1560.46 cm^{-1} are characteristic of >C=C< (anthraquinone); while peaks at 1651 cm^{-1} to >C=O (anthraquinone) (Ortiz et al., 2017; Pan et al., 2017). While after degradation the FTIR spectra showed that the peaks at 1363.72 cm^{-1} to C-C-C (alkanes) and peaks at 1039.67 cm^{-1} to C-O-C (ester). The IR spectrum of 24 h, decolorized medium displayed a band at 1489.1 cm^{-1} for $–CH_2$ asymmetric bend. Moreover, the significant change in the FTIR spectrum of metabolites compared to control spectrum suggest the biotransformation of complex dyes present in the mixture into simple form (Ortiz et al., 2017).

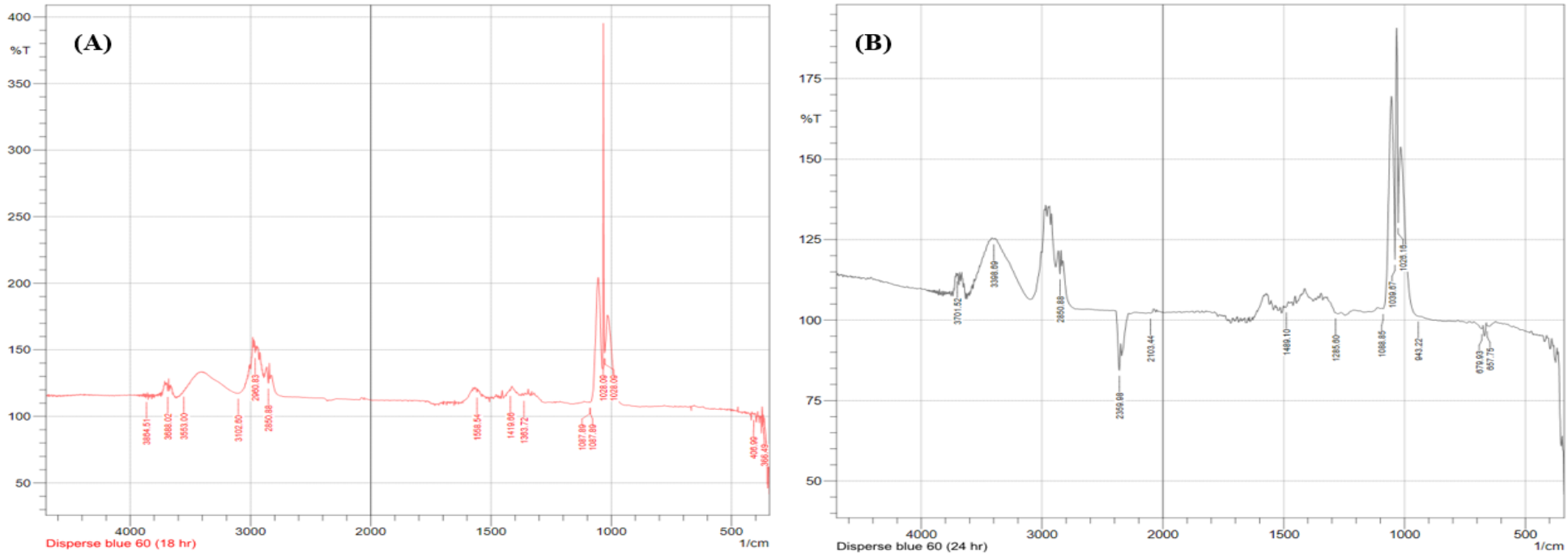


Figure-28: FTIR spectrum of decolorized product of disperse blue 60 after 18 h and 24 h

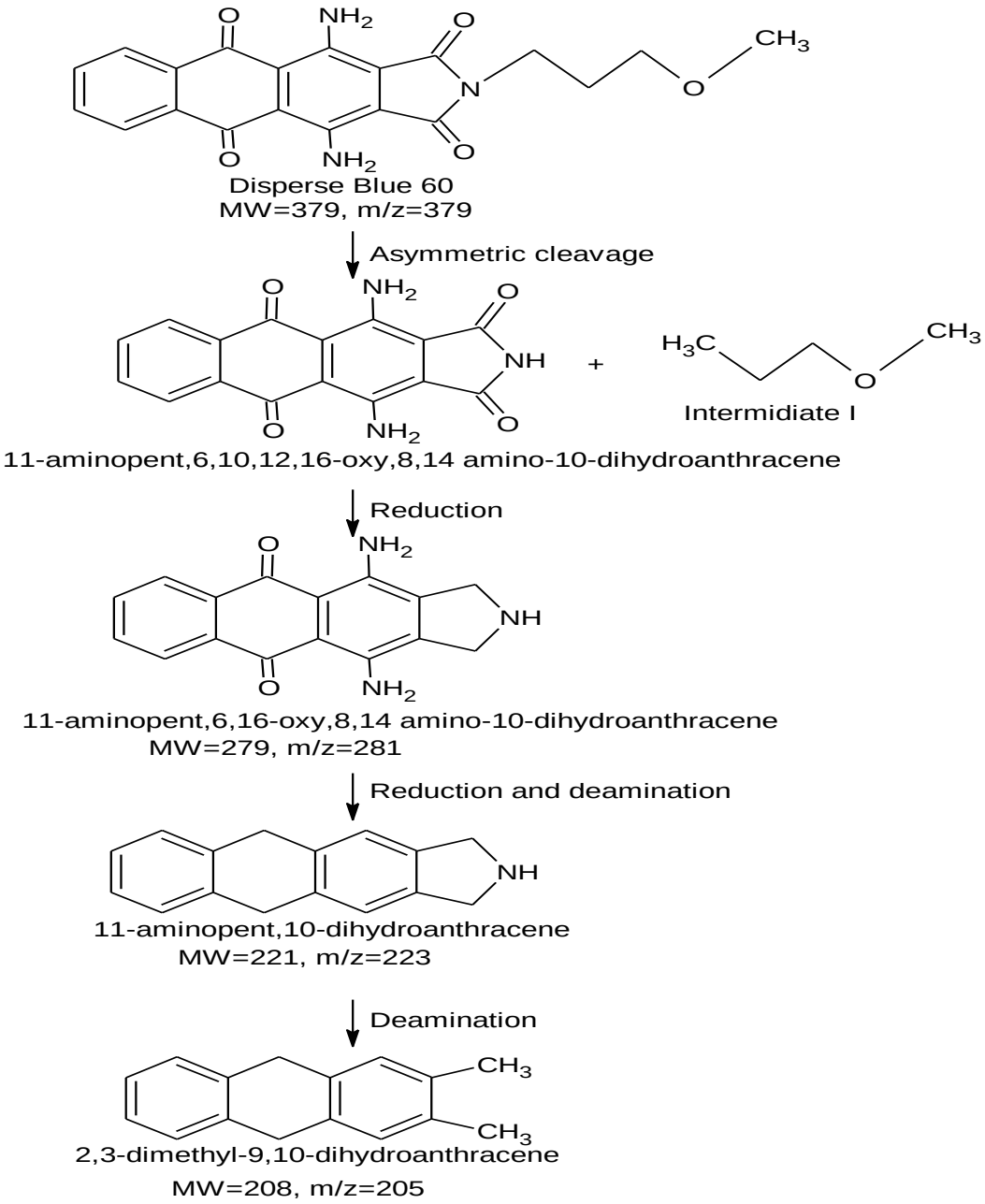


Figure-29: Proposed pathway for degradation of disperse blue 60

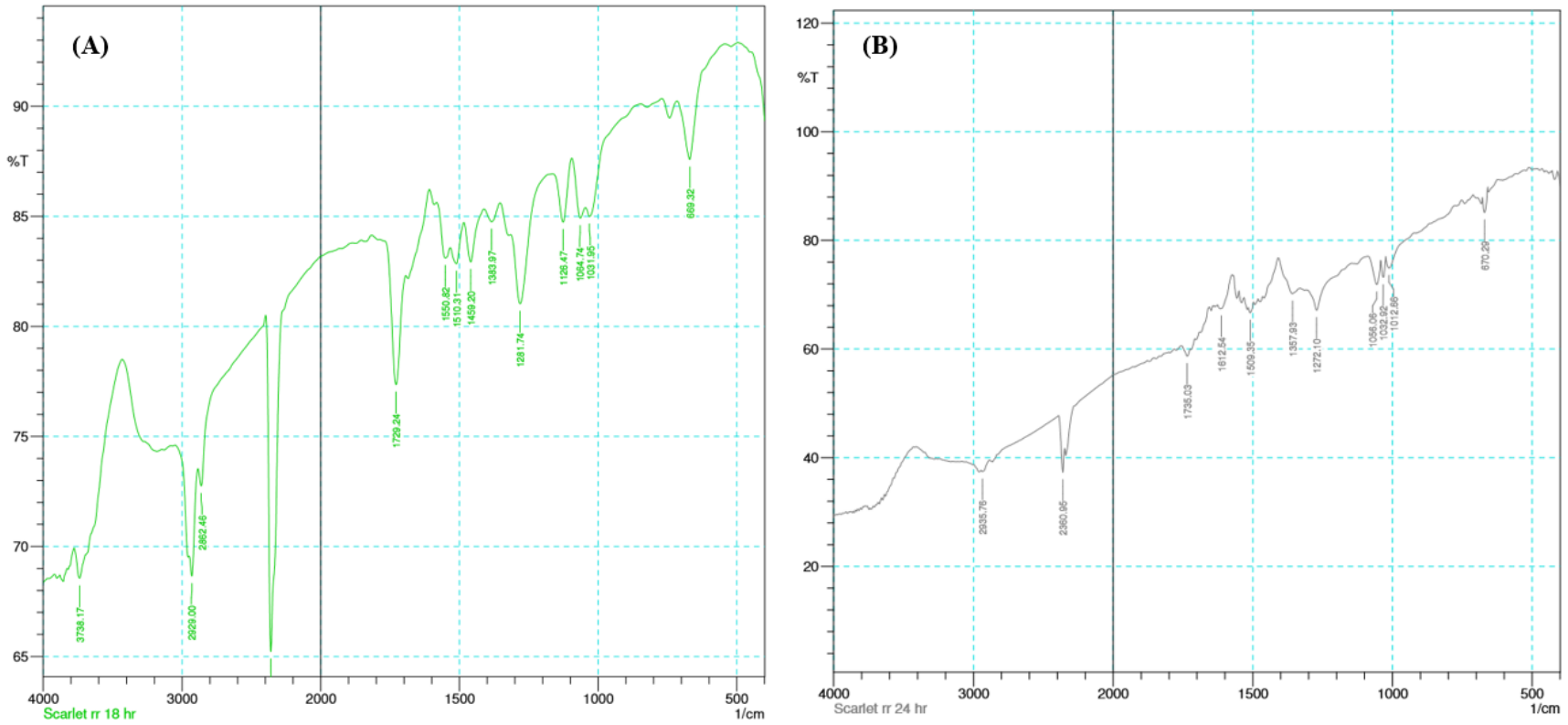


Figure-30: FTIR spectrum of metabolites of disperse red 54 degradation 31(A) after 18 and 31(B) after 24 h

6.8.4 Analysis of degraded products of disperse blue 60: FTIR analysis

The FTIR spectra of control disperse red 54 dye is represented in Figure 12(D) which confirms the structure of parent dye. The peaks at 3321.53cm^{-1} corresponds to -N-H stretching as in amines, 1599.04 cm cm^{-1} for N=N stretching as in azo groups and -C-N stretching as in amides, 1114.89 cm^{-1} for C–O-H stretching as in alcohols, 1041.6 cm^{-1} for S–O stretching which displayed the sulfoxide nature of dye, and 682.82 cm^{-1} for C–Cl stretching, 2953.13 cm^{-1} for –C-H stretching, 1363.72 cm^{-1} for CH_3 vibration, 1442.8 and 1473.66 cm^{-1} corresponds to CH_2 bends and 1737.92 cm^{-1} for –C=O (ketonic) vibration (Jadhav et al., 2009; Kurade et al., 2012) whereas significant change in the FTIR spectrum was observed after decolorization. The FTIR spectrum of decolorized medium after different time interval is presented in Figure 30(A and B). In contrast, the FTIR analysis of the dye metabolites confirmed the CH_2 as C-H stretching at 2935.76 cm^{-1}, and the formation of cross conjugates possessing C=O at 1612.54 cm^{-1} (Jadhav et al., 2009). Moreover, the peaks at 1510.31 cm^{-1} and 1509.35 cm^{-1} corresponds to the presence of NO_2 stretch. This is in the agreement with the results obtained by GC-MS of the respective dye, which confirmed the formation of nitrobenzene as one the metabolite at m/z 125. These results clearly indicated that appearance of new peaks after decolorization with disintegration of some peaks in control dye, displayed the bonding interactions which supports the biotransformation of parent dye.

6.8.4.1 Analysis of degraded products of disperse red 54: GC-MS analysis and proposed degradation pathway for disperse red 54

The mass spectra of disperse red 54 after decolorization is presented in Figure 31. On the basis of this mass spectrum data, the degradation pathway of the dye has been elucidated (Figure 32). After 24 h of decolorization, disperse red 54 was mineralised by the action of azo-reductase to cleave azo bond to form two primary intermediates i.e. 2-chloro-4-nitroaniline (molecular weight-172 and corresponding m/z at 172 and aniline (molecular weight-91 and corresponding m/z at 91). On the other side, asymmetric cleavage was also occurred by the action of laccasse to form third primary intermediate, methyl 3-[(2-cyanoethyl) amino]propanoate (molecular weight-156 and

corresponding m/z at 156). Further the first primary intermediate underwent deamination reaction with the removal of amine group to form 1-chloro-3-nitrobenzene (mole. wt- 157 and m/z at 157) to form final product nitrobenzene (mole. wt-123 and m/z at 125) with the removal of chlorine. The results are in accordance with the previous studies conducted by Jadhav et al., (2009) in which 1-chloro-4-nitro-benzene were formed after the asymmetric cleavage of scarlet RR by peroxidase enzyme. In our study, the second intermediate underwent oxidation reaction to form phenol (mole. wt-94 and m/z at 92) to form final product i.e. benzene (mole wt. 78 and m/z at 75) after reduction initiated by azo-reductase. Further reduction of third intermediate by the same enzyme to form secondary metabolite, 3-(propylamino)propanenitrile (mole wt- 112 and m/z at 114).

After deamination, disintegration of this secondary metabolite occurred to give final product N-propylpropan-1-amine (mole.wt- 101 and m/z at 101). Jadhav et al., (2009) reported different intermediates of disperse red 54 (scarlet RR) degraded by *G. geotrichum.* The difference in the formation of intermediates might be due to the different operating conditions provided to the microbes i.e. shaking condition at 150 rpm and pH-12.0, temperature-50° C. The degradation pathway of scarlet RR was also proposed by Kurade et al., (2012) by bacteria-yeast consortium and individually. Initially, scarlet RR was cleaved asymmetrically by action of veratryl alcohol oxidase to give primary intermediate [BL-A], 2-{ethyl[(4-oxo-3,4-dihydroquinolin-2-yl)methyl]amino} acetamide, (mole.wt-259 and m/z at 257]. Further this intermediate underwent deamination reaction to form secondary intermediate [BL-B], {ethyl[(4-oxo-3,4-dihydroquinolin-2- yl)methyl]amino}acetaldehyde (mole.wt-244 and m/z at 244]. The final product [BL-C], 2-(aminomethyl)quinolin4(3H)-one (mole.wt- 174 and m/z at 172] formed by the removal of aliphatic chain from this secondary intermediate. The degradation by consortium resulted into asymmetric cleavage of dye by veratryl alcohol oxidase and laccase to form {ethyl[(4- oxo-3,4-dihydroquinolin-2-yl)methyl]amino}acetaldehyde (mole wt-244 and m/z at 244] along with the formation of an unidentified reactive intermediate (Allegre et al., 2006). Further disintegration of the intermediate by laccase gave the final product, 3,4- dihydroquinoline (mole. wt-131, m/z at 129). Similar studies were conducted by Saratale et al., (2009) for the

degradation of scarlet RR by consortium (GR) comprises two different bacteria, *Proteus vulgaris* and *Micrococcus glutamicus*. The results indicated reductive cleavage of azo bonds of dye followed by demethylation reaction to form final product 1,4-benzenediamine (mole. wt-108 and m/z at 109).

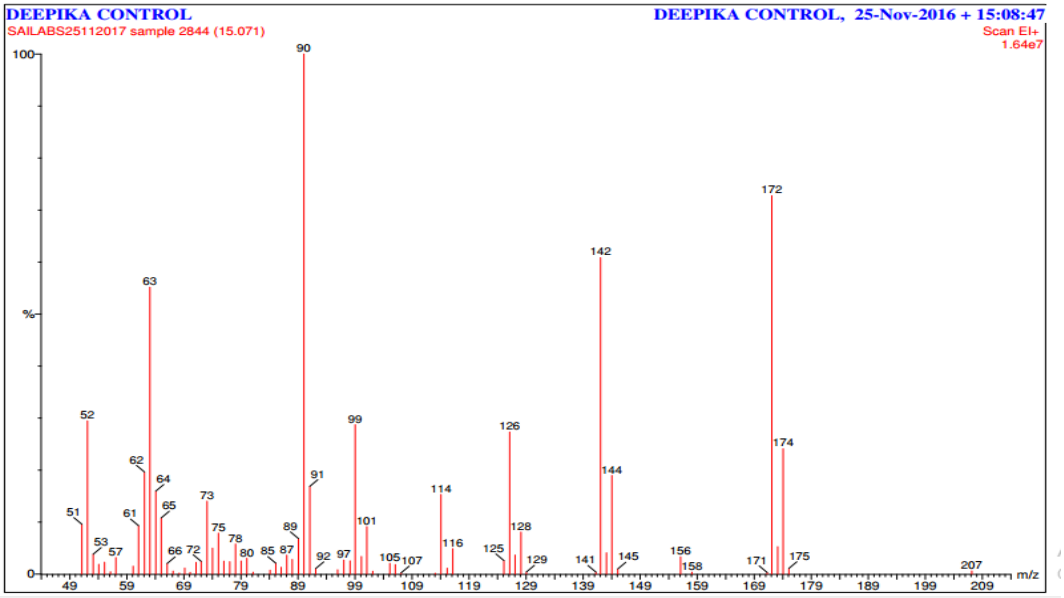


Figure-31: Mass spectrum of disperse red 54 after degradation

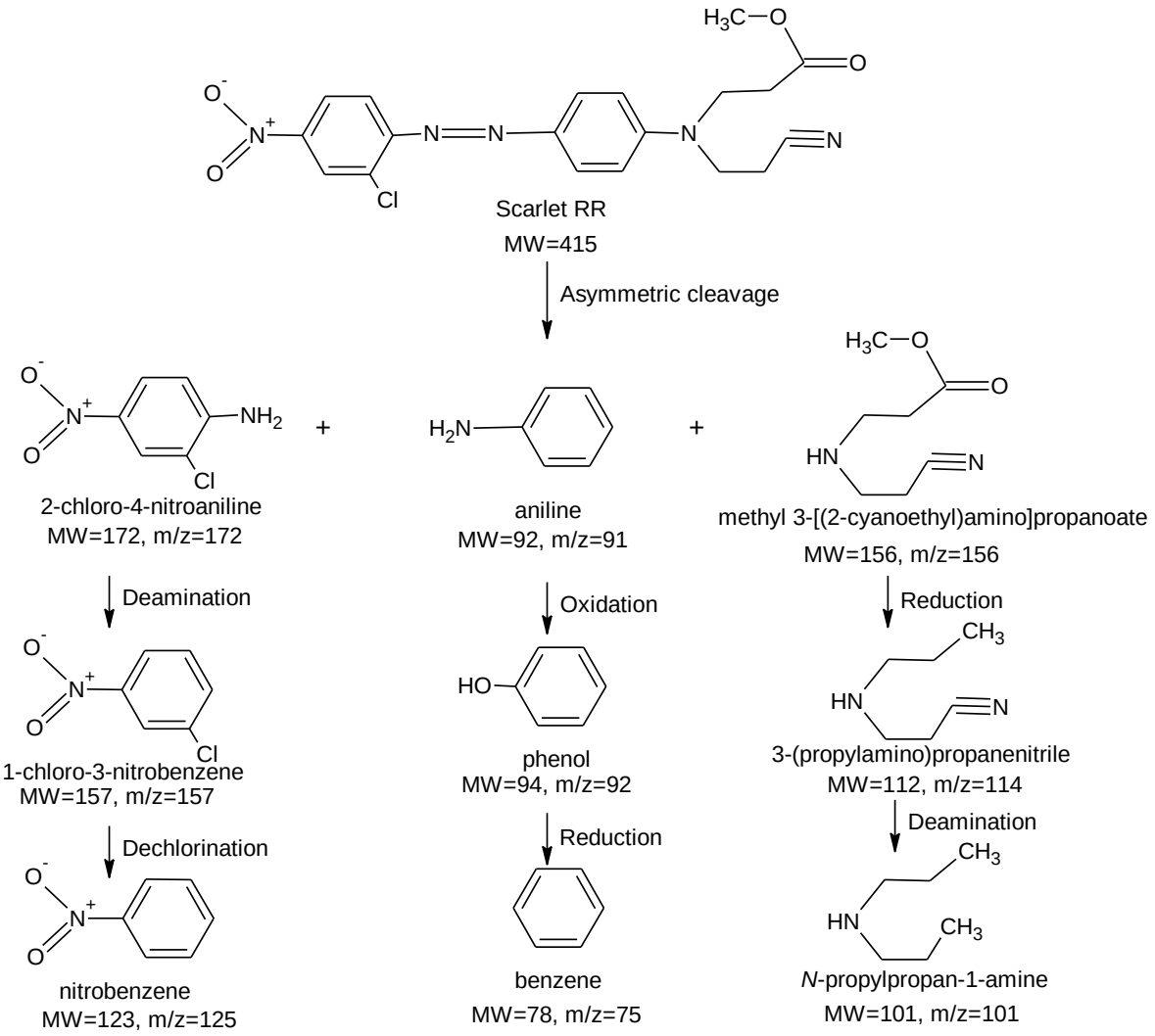


Figure-32: Proposed pathway for degradation of disperse red 54

6.9 Toxicology study of dyes: Phytotoxicity assay

There has been a global awakening during the last few decades regarding the proper management of natural resources. Among them, irrigation water is one which becoming costlier due to increasing demand from agriculture to feed the increasing human population. Simultaneously, the demand for food is also increasing, which has brought more and more land under cultivation and focused the attention on the use of fertilizers and irrigation water at an alarming rate. With these certain limitations, one has to turn to non-conventional recourses to meet the irrigation water demand. Among others, one of the most important source of water for irrigation is industrial waste water, which consists of about 95% water and the rest as organic and inorganic nutrients. Since, disposal of wastewater is a big problem in urban areas, applying the textile industry wastewater after treatment to agricultural fields, instead of disposing off in lakes and rivers, can be better source of irrigation water due to presence of various nutrients (like N, P, K, Ca, Mg etc.) in wastewater (Saravanamoorthy et al., 2007). There can be both beneficial and damaging effects of irrigation with waste water on various crops including vegetables (Saratale et al., 2009) especially the textile industry effluent water that contains much of undesirable qualities like opacity due to suspended particles, alkaline pH and toxic synthetic chemicals such as the dyes. The need is to assess wastewater quality and plant species requirements before using treated waste water for crops production (Saravanamoorthy et al., 2007). It is very important to know whether biodegradation of a dye leads to detoxification of the dye or not. This can be done by performing phytotoxicity and microbial toxicity tests of the original dye and its biodegradation products. In phytotoxicity studies, the seeds of model plants can be treated with a particular concentration of the original dye and also with its biodegradation products. The effect of the treatment on percent germination and length of plumule and radicle can be evaluated and the results compared with those of the control (without treatment with dye and its biodegradation products). Improper disposal of dyeing effluents containing reactive azo dyes causes serious environmental and health hazards, they are being disposed off in water bodies and this water is being used for an agriculture purpose. Use of untreated and treated dyeing effluents containing water for the agriculture purpose has direct impact on the fertility of soil (Telke et al.,

2008). Therefore, it is of concern to assess the phytotoxicity of the textile dye effluent before and after degradation by any mode of treatment. Seed germination and plant growth bioassays are the most common techniques used to evaluate the phytotoxicity (Saratale et al., 2009). Though, chemical treatment is being currently carried out in the industries that are under pressure to reduce pollutant load, the water that is drained into the streams is not significantly improved in its qualities. Biological treatment is not only eco-friendly but also economically friendly. However, there is a need for a careful examination of presence of any undesirable toxic metabolites. Since, water is a commodity of high demand in the country, it is imperative to carryout toxicity tests atleast for the plants as it may be pumped for irrigation purposes (Jadhav et al., 2010). Therefore, any efficient treatment methodology needs to be evaluated for its phytotoxicity.

Aromatic sulfonic azo dyes and their metabolic intermediates (sulfonated and unsulfonated aromatic amines) represent an important group of environmental pollutants having a toxic nature (Anburaj et al., 2011). Improper disposal of the dye effluents containing reactive groups cause a serious problem to environment and health. Such effluents when disposed in the water bodies are of major concern particularly when such water is to be used in irrigation.

6.9.1 Effect of selected azo-disperse dyes (three different concentrations) and decolorized/degraded dye solutions on *Triticum aestivum* seed germination, root length and shoot length

The last part of this study was to determine the effects of disperse dyes and decolorized dye metabolites on environmental ecology. This was studied by conducting experiments on the effects of dye metabolites on seed germination, shoot and root length using *T. aestivum* seeds. Phytotoxicity study of selected dyes and the degraded metabolites obtained after its decolorization is presented in (Table 21). The results of the toxicity study showed that there was a reduction of rate of germination of seeds treated with different concentration of dye. Less germination was observed on highest concentration of dye (250 mg/L). However, relatively more germination was observed in the seeds treated with dye degradation metabolites. The seeds of *Triticum aestivum* moistened with 50, 150 and 250 mg/L disperse red 167 solution exhibited 90%, 80%

and 50% germination (33A) respectively while the seeds moistened with similar concentrations of disperse red 167.1 solutions exhibited 80%, 70% and 60% germination, respectively (table 21). In case with disperse red 167, the germination index was found to be reduced to half (50%) at 250 mg/L dye concentration as compared to control (treated with distilled water). Whereas the germination index was 80% after 24 h with extracted metabolites of the respective dye. Thus, remediated dye solution was less toxic in comparison to untreated dye solution. Likewise, the seeds soaked in similar concentration (50, 150 and 250 mg/L) of disperse blue 60 and disperse red 54 displayed 90%, 70% and 60% germination index for both dyes at respective concentration. However, 100% seed germination was observed with control (distilled water). In case of seeds soaked in decolorized/degraded dye solutions of disperse red 54 displayed 80% germination (33D) while 90% germination was observed with the metabolites of disperse red 167.1 and disperse blue 60 (Figure 34(B) and 34(C). Similarly, Pourbabaee et al., (2006) reported 100% inhibition of *Triticum aestivum* seed germination, when soaked in 150 mg/L of untreated dye solution, which reduced to only 10% inhibition for seed germination in presence of remediated dye solution.

Disperse Red 167 affected significantly the elongation of root and shoot compared to its metabolites obtained after decolorization and degradation (Figure 34A). Similarly, the reduction in shoot and root length was noticed on increasing the concentration from 50 to 250 mg/L. The shoot length (cm) was 3.73 $\pm$ 0.20 (50 mg/L); 3.33 $\pm$ 0.20 (150 mg/L) and minimum 2.76 $\pm$ 0.15 was noted (250 mg/L) in comparison the extracted metabolite shows the growth of shoot 3.20 $\pm$ 0.20, which was significantly different in contrast to that of parent dye. Likewise, in case of root length, it was maximum at 50 mg/L (5.96 $\pm$ 0.20), decreased up to 2.63 $\pm$ 0.15 (250 mg/L) and 4.66 $\pm$ 0.15 (extracted metabolite) as represented in (Figure 34A). This specifies the less toxic nature of degradation metabolites to the plants.

Disperse Red 167.1 also affected the length of root and shoot compared to its metabolites obtained after decolorization and degradation (Figure 34B). This effect was noticed on increasing dye concentration from 50 mg/L to 250 mg/L. The shoot length (cm) was 3.00$\pm$ 0.10 (50 mg/L); 2.53$\pm$ 0.05 (150 mg/L) and minimum 1.83$\pm$ 0.05 was noted (250 mg/L) in comparison the extracted metabolite shows the growth of shoot

3.20± 0.20, which was significantly different in contrast to that of parent dye. Likewise, in case of root length, it was maximum at 50 mg/L (4.73± 0.20), decreased up to 2.40± 0.10 (250 mg/L) and 4.73± 0.15 (extracted metabolite) as represented in (Figure 34B). In the case of disperse red 54 and disperse blue 60, the same trend of reduction in shoot and root length was observed with increase in dye concentration. But for the extracted metabolites for both above mentioned dyes, better results were obtained for elongation of root and shoot. Disperse red 54 (Figure 34D) showed shoot length 3.56±0.20 (50mg/L), 2.70±0.26 (150 mg/L), 1.73±0.20 (250mg/L). Likewise disperse blue 60 (Figure 35C) displayed shoot length, 3.46±0.30 (50 mg/L), 2.53±0.30 (150mg/L), 1.93±0.05 (250mg/L). The minimum root length was observed at 250 mg/L for disperse red 54 and disperse blue 60, (1.96±0.47) and (1.63±0.32) respectively. In contrast the extracted metabolites, have shown 3.23±0.25 (shoot length) and 4.03±0.15 root length for disperse blue 60. Similarly, 3.20±0.20 (shoot length) and 4.10±0.10 (root length) for disperse red 54. Our results are in accordance with the study conducted by Dawkar et al., (2008), they reported 80% and 70% germination inhibition and 2-5% reduction in shoot and root length in *T. aestivum* upon exposure of untreated Navy blue 2 GL dye. Ogugbue and Sawidis, (2011) and Parshetti et al., (2006) observed the similar results of reduced germination rate in *T. aestivum* with malachite green dye in contrast to its degradation product and distilled water. Jadhav et al., (2008) also reported 80% and 100% inhibition of seed germination of *Sorghum biocolor* and *Triticum aestivum* upon exposure of untreated scarlet RR dye (disperse red 54) and 10% reduction in germination upon exposure of tetrazine dye described by Raju et al., (2015). Konwar and Jha, (2010) reported maximum plant and shoot height in reference plants in comparison to dye exposed plants. Similar influence of textile industry effluent on peanut seed germination and shoot length were also reported by Saravanamoorthy et al., (2007).

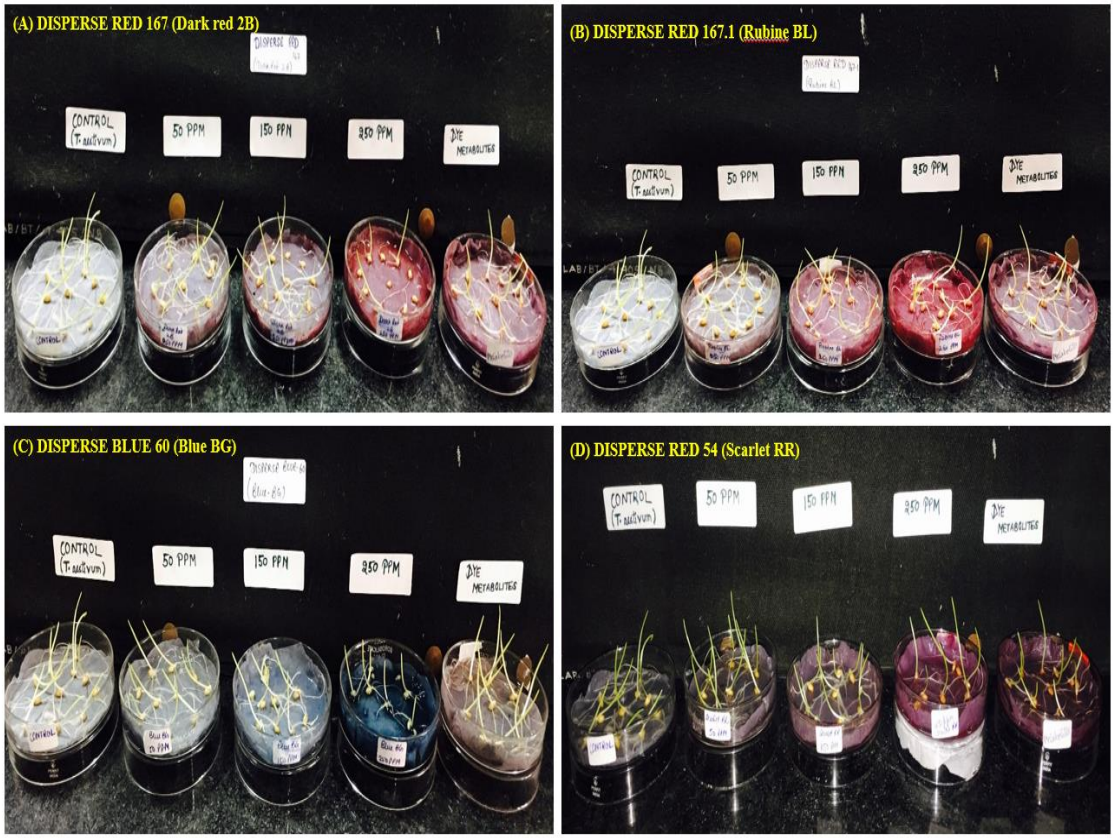


Figure-33: Phytotoxicity assay. 33(A) Plates showing the growth of *T. aestivum* in the presence of disperse red 167 (dark red 2B), dye metabolites and control (distilled water); 33(B) Plates showing the growth of *T. aestivum* in the presence of disperse red 167.1 (rubine BL), dye metabolites and control (distilled water); 33(C) Plates showing the growth of *T. aestivum* in the presence of disperse blue 60 (blue BG), dye metabolites and control (distilled water); 33(D) Plates showing the growth of *T. aestivum* in the presence of disperse red 54 (scarlet RR), dye metabolites and control (distilled water)

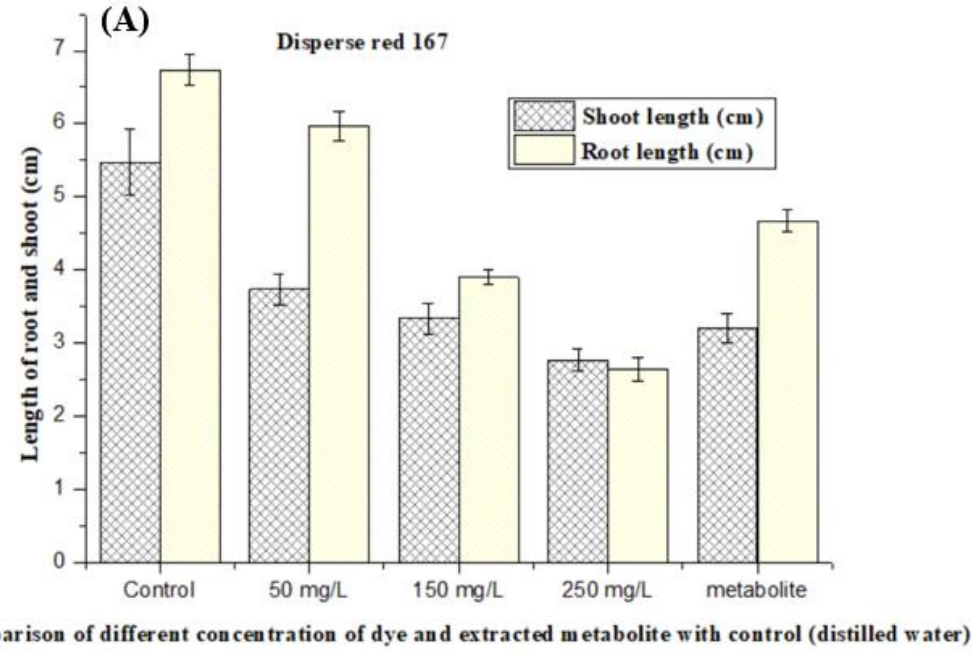


Figure-34: Phytotoxicity assay. 34(A) Effect of disperse red 167 and dye metabolites on root and shoot length of *T. aestivum*

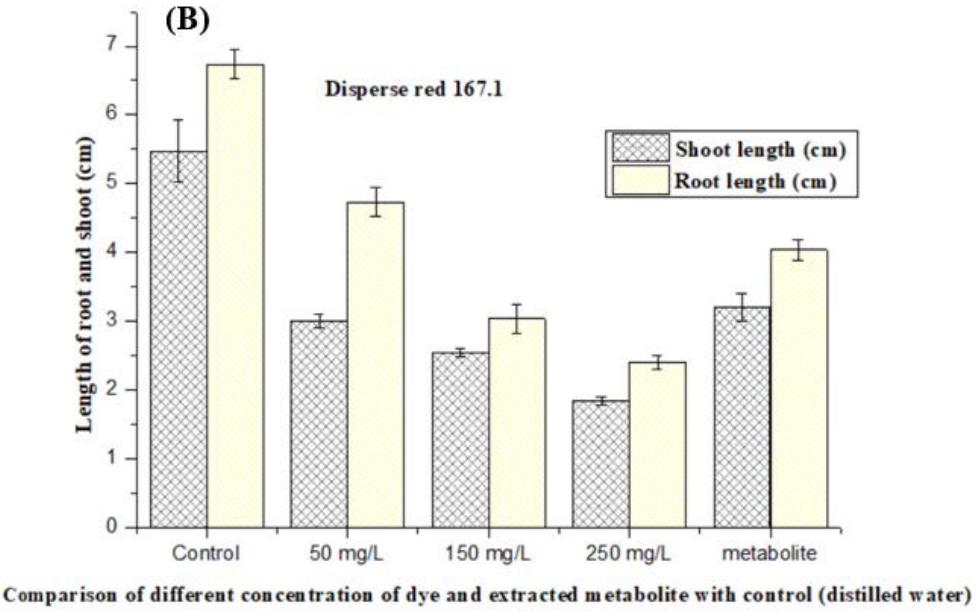


Figure-34: Phytotoxicity assay. 34(B) Effect of disperse red 167.1 and dye metabolites on root and shoot length of *T. aestivum*

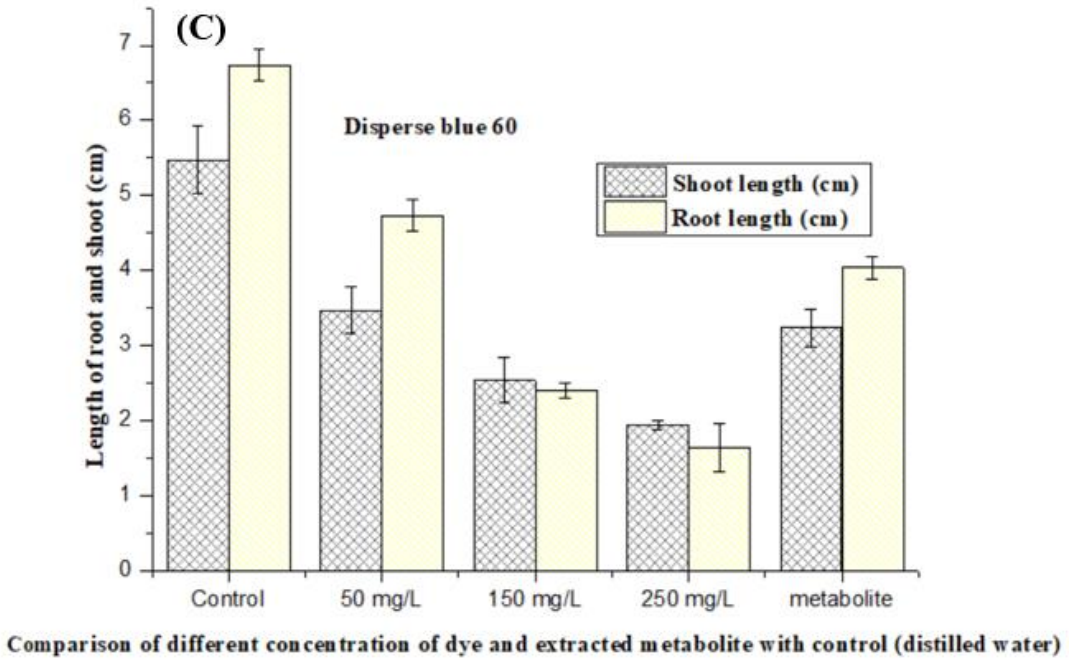


Figure-34: Phytotoxicity assay. 34(C) Effect of disperse blue 60 and dye metabolites on root and shoot length of *T. aestivum*

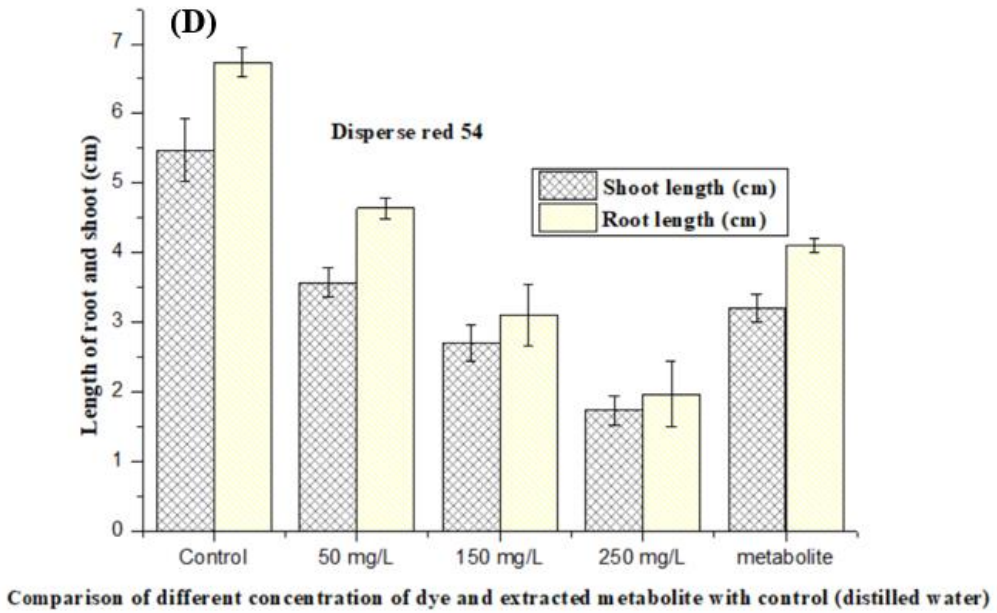


Figure-34: Phytotoxicity assay. 34(D) Effect of disperse red 54 and dye metabolites on root and shoot length of *T. aestivum*

Table-21: Effect of selected dyes (different concentration) and decolorized/degraded dye solution on the growth of *Triticum aestivum.* Data was analysed by oneway analysis of variance (ANOVA) with Tukey–Kramer multiple comparison test (JMP software) using mean values of different experiments. Seeds germinated in parent dye at three different concentrations are significantly different from the control (seeds germinated in distilled water) at *P < 0.05, **P < 0.01, ***P < 0.001 and the seeds germinated in extracted metabolites are significantly different from the seeds germinated in parent dye at three different concentrations $^{\$}P < 0.01$, $^{\$\$}P < 0.001$

Plant species	**Treatment**	**Concentration**	**Germination (%)**	**Shoot length (cm)**	**Root length (cm)**
Triticum aestivum (Wheat)	Control (Distilled water)		100	5.46±0.45	6.73±0.20
Triticum aestivum (Wheat)	Disperse red 167 (Dark red 2B)	50 mg/L	90	$3.73\pm0.20^{***}$	$5.96\pm0.20^{***}$
		150 mg/L	80	$3.73\pm0.20^{***}$	$3.90\pm0.10^{***}$
		250 mg/L	50	$2.76\pm0.15^{***}$	$2.63\pm0.15^{***}$
	Metabolites after degradation		80	$3.20\pm0.20^{\$}$	$4.66\pm 0.15^{\$\$}$
Triticum aestivum (Wheat)	Disperse red 167.1 (Rubine BL)	50 mg/L	80	$3.0\pm0.10^{**}$	$4.73\pm0.20^{***}$
		150 mg/L	70	$2.53\pm0.05^{***}$	3.03±0.20
		250 mg/L	60	$1.83\pm0.05^{***}$	$2.4\pm0.10^{***}$
	Metabolites after degradation		90	$3.2\pm0.20^{\$}$	$4.03\pm0.15^{\$\$}$

Triticum aestivum (Wheat)	Disperse blue 60 (Blue BG)	50 mg/L	90	3.46±0.30***	4.73±0.20***
		150 mg/L	70	2.53±0.30***	2.42±0.10***
		250 mg/L	60	1.93±0.05***	1.63±0.32***
	Metabolites after degradation		90	3.23±0.25$	4.03±0.15$$
Triticum aestivum (Wheat)	Disperse red 54 (Scarlet RR)	50 mg/L	90	3.56±0.20***	4.63±0.15***
		150 mg/L	70	2.70±0.26***	3.10±0.43***
		250 mg/L	60	1.73±0.20***	1.96±0.47***
	Metabolites after degradation		80	3.20±0.20$	4.10±0.10$$

In the present investigation, after development of consortium on analysing dye decolorization assay, the consortium was found to show the highest rate of decolorization for disperse red 167.1 (82.76± 0.255%) within 24 h in comparison to rest of three selected dyes. The purpose of the development of consortium is to further develop biofilters by using low cost agricultural waste as a substrate/matrix for the immobilization of consortium for removal of dyes from aqueous solution. The studies being conducted as a part of fourth objective are extending the previous research or results obtained from objective 3, which allowed us to immobilize bacterial consortium on corncob for the effective treatment of disperse red 167.1 (rubine BL). This will help us to determine, if the design of biofilter and equations developed from lab-based column studies can describe conditions that occur in the "real world."

6.10 Analysis of surface characterization and morphology of corncob

6.10.1 Physico-chemical characteristics of corncob

Physico-chemical characteristics such as pH and electric conductivity of corncob represents significant properties to enhance adsorption capacity (Liu et al., 2014). The corncob is alkaline in nature (pH= 5.68±0.6) with bulk density of 0.2088 g/cm^3. The electric conductivity was measured by using HANNA (HI 8733) and found to be 3.20±0.37 mS/cm (table 22).

Table-22: Physico-chemical analysis of corncob

Sample	Bulk Density (g/cm^3)	Particle density (g/cm^3)	pH	Electrical Conductivity (mS/cm)
Corncob	0.1374	0.2088	5.68±0.6	3.20±0.37

The bulk and tapped densities measure the flowability of a material. In general, the higher the densities, the better the potential for a material to flow and re-arrange itself under compression. The results obtained from this study are moderate and are consistent with previos findings (Azubuike and Okhamafe, 2012).

6.10.2 SEM characterization

The results obtained after scanning electron microscopy indicates the change in surface topology of corncob after immobilization and after adsorption treatment. The SEM of raw corncob (35A) shows robust surface morphology and represents the area where immobilization

will take place. The SEM images of raw corncob obtained show the robust surface and heterogeneous morphology of corncob powder with cellulose fiber of various sizes and forms. Immobilization of bacterial consortium on the surface of corncob changed its morphology (35B), immobilized bacteria can be seen on the surface of corncob (rod shape bacteria) as seen in magnified portion of circled area. It was found that the bacterial immobilization causes the swelling of biomaterial, thereby promotes the penetration of bacteria into the micropores of lignin fibres of biomass. The bacterial cells in the consortium has been immobilized on rough surface of corncob by a natural phenomenon called adhesion (Genisheva et al., 2011). It is evident from the SEM image (Figure 35A) that the raw corncob cube has enough rough and porous surfaces, which provided a natural entrapment for the cells. Scanning electron micrograph of the bacterial co-immobilized corncob (biomaterial) fig (35B), demonstrated that immobilization did not occur in a homogenous form on the material structure, but it was more favoured in specific regions, such as rough and porous structures only. The rough and porous structure of raw biomaterial allowed microorganisms to attach more firmly than the smooth structures. This phenomenon has also been reported in previous studies but with single culture (Branyik et al., 2004; Yu et al., 2010; Genisheva et al., 2011). Previous studies have revealed that immobilization technology is advantageous in maintaining the degradation efficiency of cells for wastewater treatment (Chen et al., 2007; Chen et al., 2008; Pakshirajan and Swaminathan, 2009; Xu et al., 2012). After adsorption experimentation of immobilized corncob with dye solution, results in the disintegration of corncob surface morphology, as seen in the SEM images (35C and 35D) it can be concluded that the bacteria were able to biodegrade corncob and disintegrate the dye after certain period of time. Enzymatic hydrolysis of corncob has been occurred.

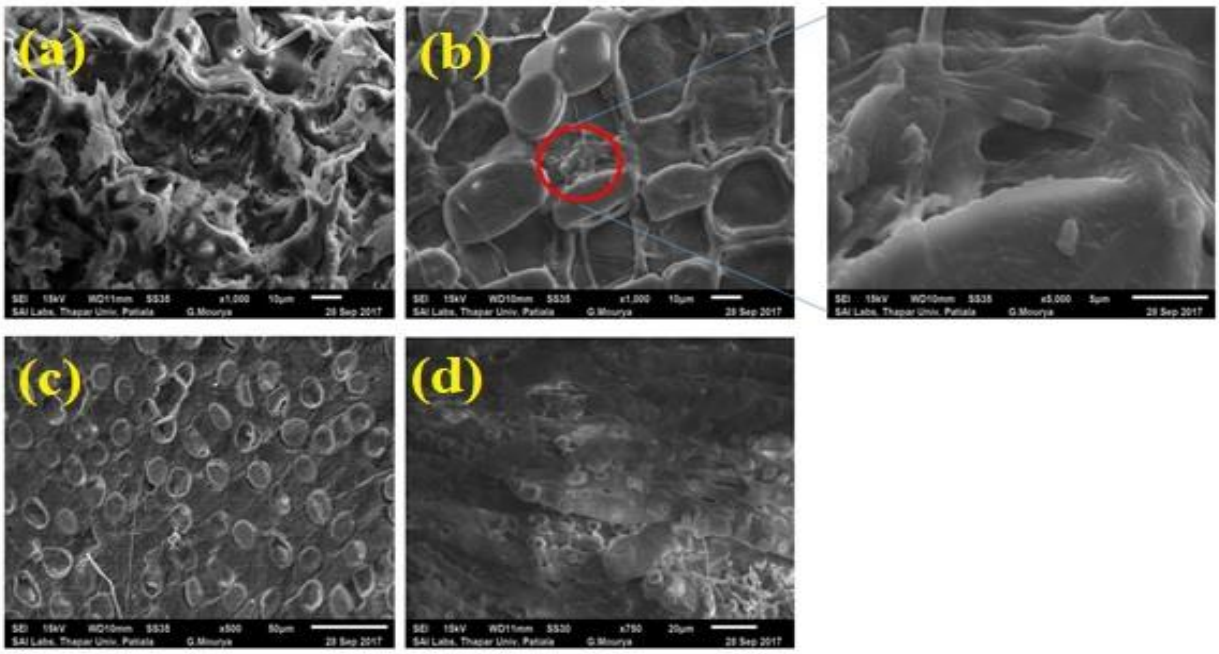


Figure-35. SEM images of corncob. 35(A) Raw corncob; 35(B) corncob after immobilization of bacteria consortium, and (35C and 35D) after adsorption of dye solution

6.10.3 XRD Interpretation of corncob after immobilization and adsorption of dye

The XRD pattern of raw corncob (Figure 36), immobilized corncob with consortium (Figure 37) and after dye adsorption is depicted in (Figure 38). The major peak of raw corncob at bragg 2θ angles were observed in XRD pattern at 22.25° and 34.80° are indexed to (002) and (023) or (004) planes, respectively which confirms the presence of crystallographic plane. These peaks are characteristic for the crystal form of cellulose I polymorph, this behavior has been reported by other authors (Favier et al., 1995; De Souza and Borsali, 2004). The major part of cellulose is in the crystalline form due to intra and intermolecular hydrogen bonding of hydroxyl groups. Significantly reduced intensity peaks were observed in the crystalline pattern of the immobilization and after dye adsorption.

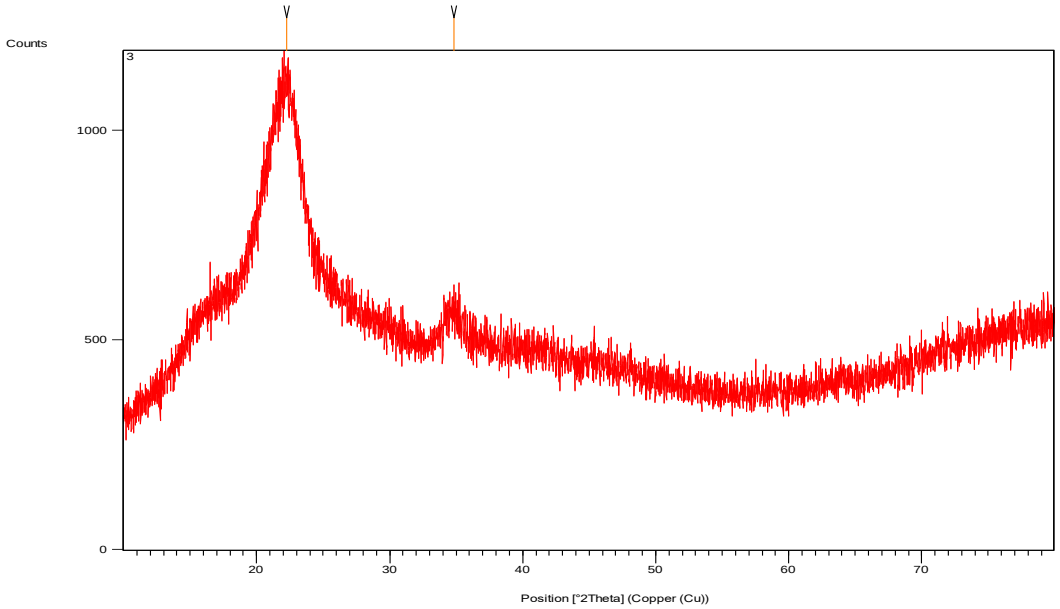


Figure-36: XRD patterns of raw corncob

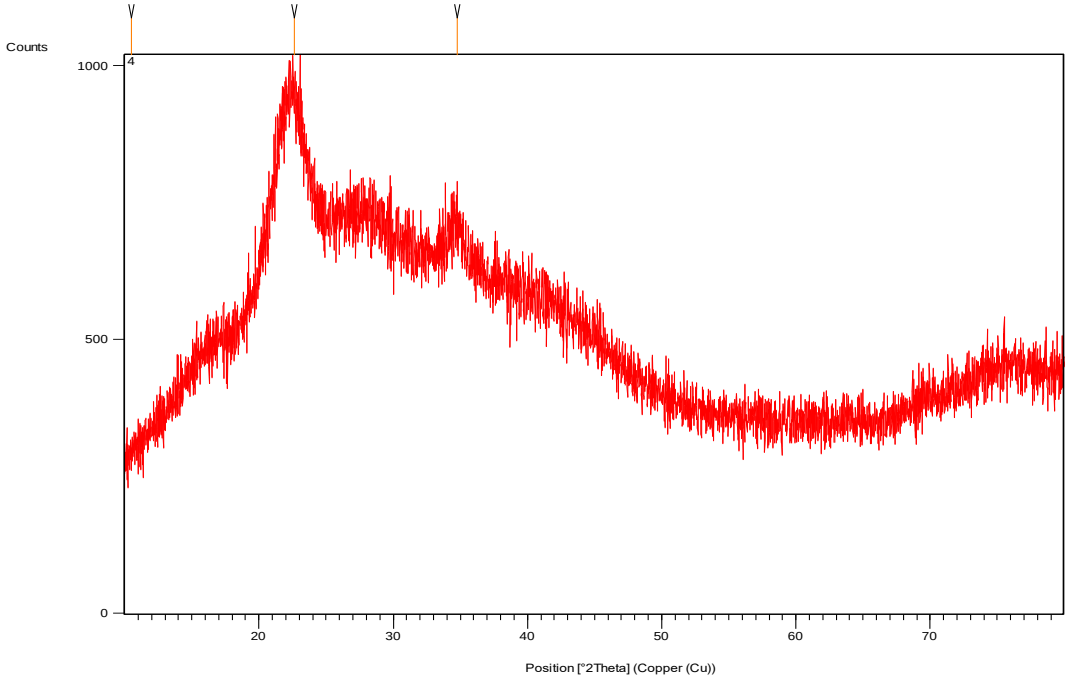


Figure-37: XRD patterns of immobilized corncob

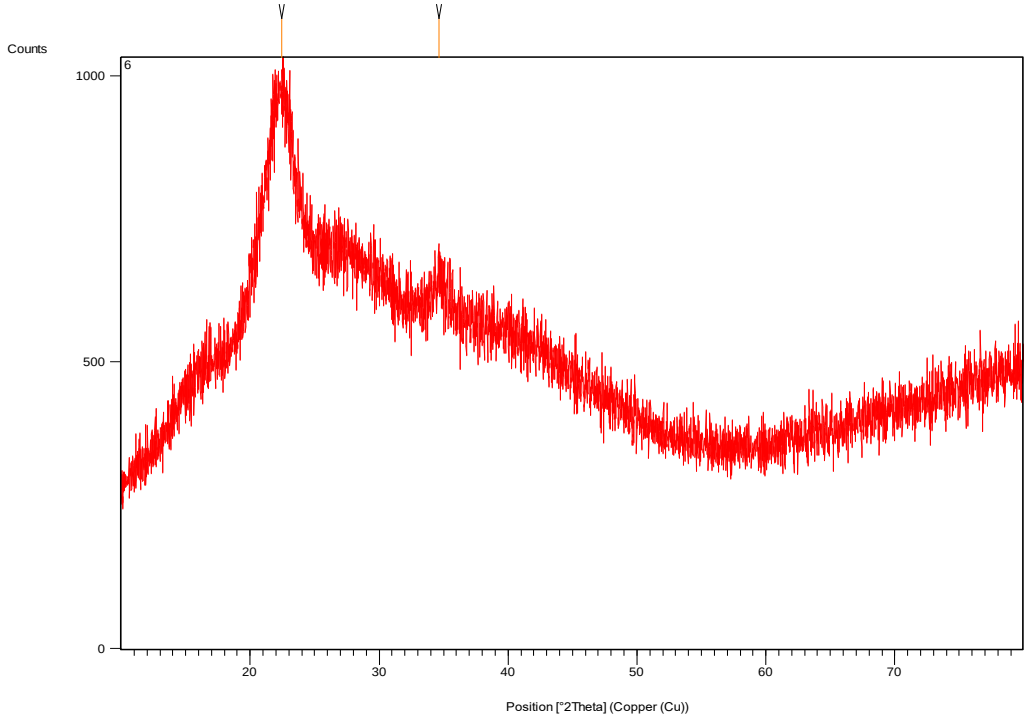


Figure-38: XRD patterns of immobilized corncob after dye adsorption

6.10.4 FTIR Characterization

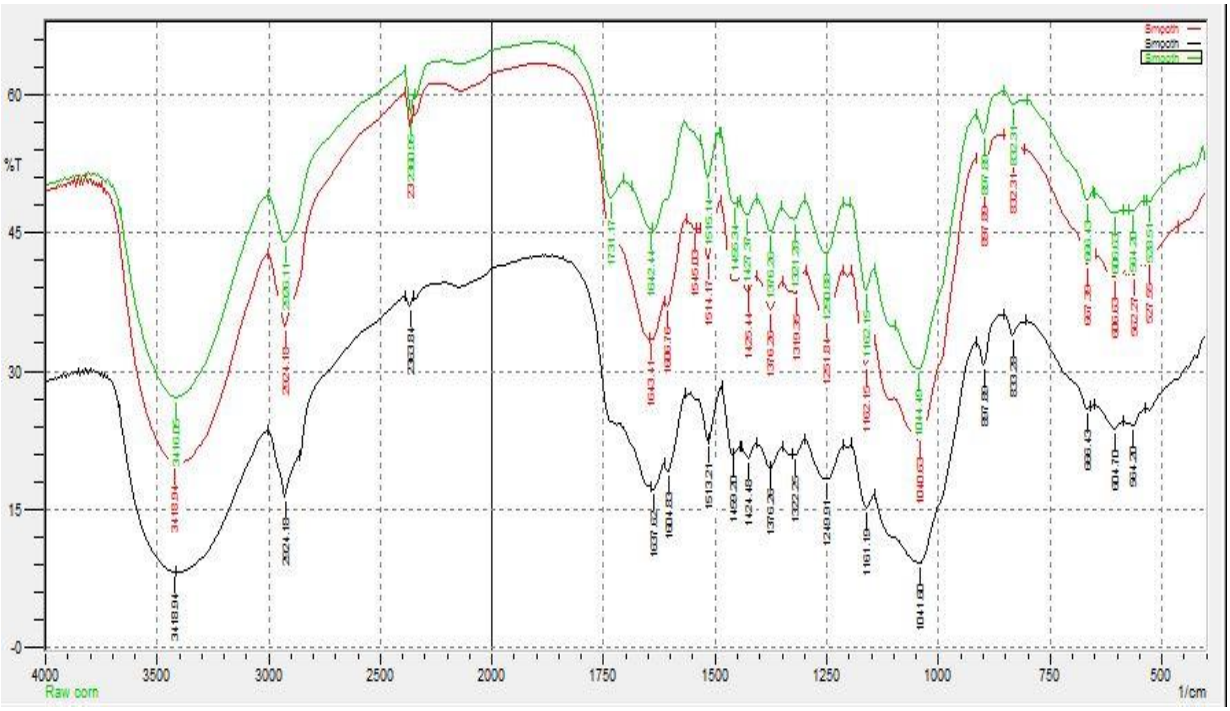


Figure-39: FTIR spectra of corncob. 39(A) raw corncob; 39(B) after immobilization of bacteria consortium and 39(C) after adsorption with dye

In order to investigate the surface chemistry of the sample, Fourier Transform Infrared Spectroscopy (FTIR) analysis was carried out to reveal the presence of several peaks or functional groups. FTIR spectra of raw corncob (Figure 39A), after immobilization of bacteria consortium (Figure 39B) and after adsorption with dye are shown in Figure 39C. The Figure reveals that some peaks in raw corncob are shifted and disappeared which may be due to the adsorption of dye on immobilized corncob. The broad absorption peak in the range of 3400 cm^{-1} corresponds to the O–H stretching vibration of alcohols, phenols and carboxylic acids as in pectin, cellulose, and lignin, thus showing the presence of "free" hydroxyl groups on the surface of corncob (Li et al., 2007; Liang et al., 2010). It can also be attributed to the -OH stretching associated to polar groups linked through intra and intermolecular hydrogen bonding (Sun et al., 2005). The presence of peak at 564.2 cm^{-1} indicates the -C≡C-H-C-H bending functional groups, 1427.37 cm^{-1} due to –C-H bending, 1642.44 cm^{-1} due to –C=O stretching, 1515.14 cm^{-1} due to –N-O stretching, 1162.15 cm^{-1} due to –C-O stretching, 2926.11 cm^{-1} due to –CH stretching and 1250.88 cm^{-1} –C-N functionalities in raw corncob. The characteristic peak at 1604.83 cm^{-1} indicates the presence of aromatic rings and C–H deformation bonds in the corncob after adsorption of dye (Pasquali and Herrera, 1997). FT-IR data of raw corncob also shows that the peak at 1731 cm^{-1} that is assigned to ester acetyl and uronic acid groups in hemicelluloses or to the ester group of ferulic and p-coumaric acids in lignin and/or hemicelluloses (Sain and Panthapulakkal, 2006; Sun et al., 2005). This peak practically disappears in the spectra of corncob after adsorption due to the removal of hemicelluloses after hydrolysis. A sharp band at 1638 cm^{-1} in corncob after dye adsorption was also detected and attributed to H-O-H stretching, measured in KBr which belongs to the absorbed water molecules associated with the cellulosic fibers (Xiao et al., 2001, Oliveira et al., 2010; Sain and Panthapulakkal, 2006), which is in aggrement with the XRD pattern of corncob after adsorption. The peaks at 1424.48 cm^{-1} and 1322.25 cm^{-1} corresponded to the C–H in-plane bending vibrations in methyl group of disperse red 167.1 (Yang and Qiu, 2010 and due to vibration binding of C–O respectively (Dahri et al., 2014).

The other peak at 1162.2 cm^{-1} is due to C–O and C–N stretching. The peaks at 2926.11 cm^{-1} and 1642.44 cm^{-1} are shifted to 2924.18 and 1637.62 cm^{-1}, respectively. This shift in the absorption peak suggested the interactions of dye molecules with the functional groups of corncob (Liang et al., 2010).

6.11 Immobilization of bacterial consortium on corncob

In recent times, the application of immobilized bacterial cell has been receiving increasing attention in the field of textile effluent decolorization. The agricultural waste materials have

little or no economic value and often pose a disposal problem. The utilization of agricultural waste is of great importance. A number of agricultural waste materials are being studied for the removal of different dyes from aqueous solutions at different operating conditions. Agricultural waste such as rice husk (Malik, 2003), coconut coir (Kavitha and Namasivayam, 2007), banana peel (Annadurai et al., 2002), wheat straw (Kannan and Sundaram, 2001), corncob (Paliwal et al., 2015) can be used as an adsorbant or matrix on to which microbial consortium can be immobilized. Various researchers have studied the effect and application of immobilized whole microbial cells and enzymes on decolorization characteristics, since immobilization provides distinct stability over free cells in the medium (Paliwal et al., 2015). Immobilization of bacteria on substrate/support matrix will increase the density of bacteria which in terms will increase the rate of degradation within the bioreactor (Chen et al., 2005). In the present investigation, it is evident from the immobilized corncob SEM image (Figure 36B) that the bacteria consortium has been immobilized on rough and robust surface of corncob by adhesion (Genisheva et al., 2011). Our results of immobilization of bacterial consortium on the surface of corncob are in agreement with the previous findings of Patowary et al., (2016). Their study revealed the biosorption of aqueous solution of Procion Blue 2G by using immobilized cells of *P. aeruginosa* and *P. chrysosporium*. It was found that maximum dye uptake is 1.648 mg g^{-1} of bead for *P. aeruginosa* and it is 1.242 mg g^{-1} of bead for *P. chrysosporium.* The study conducted by Paliwal et al., (2015) ascertained biodegradation of black liquor by immobilizing two bacterial sp., *B. megaterium* and *P. plecoglossicida* on corncob cubes. Results revealed significant reduction in color and lignin content of black liquor upto 913.46 Co-Pt and 531.45 mg/L, respectively. The decolorization of malachite green and methylene blue by free and immobilized *Desmodesmus* sp. was investigated by analyzing the reduction in absorbance of dye under different culture condition such as incubation time of incubation and initial concentration of dye. The results showed that the maximum decolorization (96%) of both dyes with immobilized *Desmodesmus sp*. after 6 days at initial concentration 20 mg/L (Al-Fawwaz and Abdullah, 2016). Previous results obtained by Sharma et al., (2016) clearly indicated the potential of immobilized cells of *Aeromonas jandaei* for the removal of methyl red dye under anaerobic and aerobic conditions. The results revealed complete decolorization of methyl red at concentration of 100 mg/L in 6 h for both anaerobic and aerobic suspended cultures.

6.12 Operation of continuous column: experimental set up

In the literature, two types of adsorption studies viz., batch mode and continuous flow mode or column studies has been reported for textile dye removal from aqueous solution. Most of the researchers prefer to conduct batch mode study because small amount of material (adsorbent) is required, less time-consuming method and easy to operate. But these batch mode studies are not convenient for field applications. In other words, the adsorption capacity data obtained from batch studies may not provide accurate scale-up information regarding the column operation systems. So, it is very important to check the practical applicability of the adsorbent in the continuous flow mode. The continuous flow system is an effective process for the treatment of large scale wastewater volumes and adsorption. So, in the present investigation, continuous column studies have been conducted for the removal of disperse red 167.1 by immobilized corncob to check the effect of various parameters viz., dye concentration and the bed height on adsorption (procedure has been explained in previous chapter under 5.11). The experimental set for column study is presented in Figure 40. The calibration curve for disperse red 167.1 (rubine BL) was plotted between absorbance and concentration of the standard dye solution (Figure 41).

6.12.1 Analysis and breakthrough modelling of column data

In the literature, there are many mathematical models available to describe the adsorption through a fixed bed continous column. These models use the results of the breakthrough curves. In the present investigation, two models, thomas and adams-bohart model, were applied to treat the experimental data in the column.

Figure 40: Experimental setup of continuous column

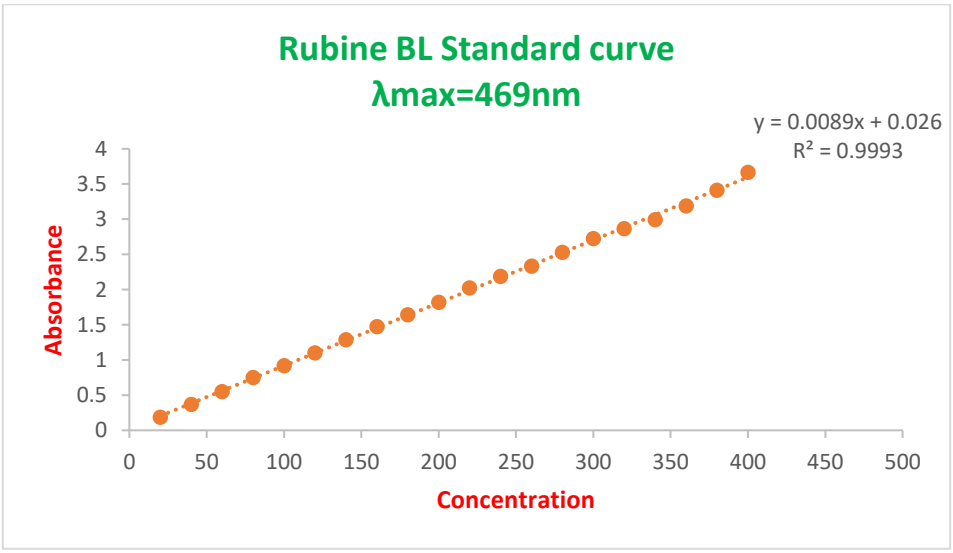


Figure-41: Standard calibration curve for disperse red 167.1

6.12.1.1 Effect of Bed Height on Adsorption

The effect of bed height on the adsorption of dye by varying the bed height from 10 cm to 15 cm was studied. The experimental set up for this study is shown in Figure 43. As bed height is increased, dye solution had more time to stay in contact with the adsorbent. This resulted in

higher dye removal from the textile industry effluent which further results in lower dye concentration in the effluent. From Figure 42, it can be observed that the slope of the breakthrough curve decreased with increase in bed height, which lead to achieve higher mass transfer zone. The total adsorption capacity of the bed increased with increase in bed height. The mass of the sorbent forming the homogeneous fixed bed is proportional to the bed height, and as a result the number of sorption sites increases with the increase in bed height leading to a larger sorption capacity of the reactor (Hamdaoui, 2006). At lower adsorbent bed height, axial dispersion phenomenon predominated and reduced the diffusion of dye (Foo and Hameed, 2012). Higher uptake was observed at the highest bed height due to an increase in the surface area of the biosorbent (corncob), which provided more binding sites for the sorption and this resulted in higher dye removal (Vijayaraghavan et al., 2004). Saha et al., (2012) reported, as the bed height is increased, the dye molecules have more time to contact with the biosorbent resulting in higher dye removal efficiency. The break through curve was steeper at lower bed height. The column parameters obtained from effect of bed height is listed in below table 23.

Table-23: Effect of different bed height on adsorption

Bed Height (cm)	**q_{total} (mg)**	**M_{total} (gm)**	**V_{eff} (ml)**
10	17.57	220.5	4410
15	24.54	220.5	4900

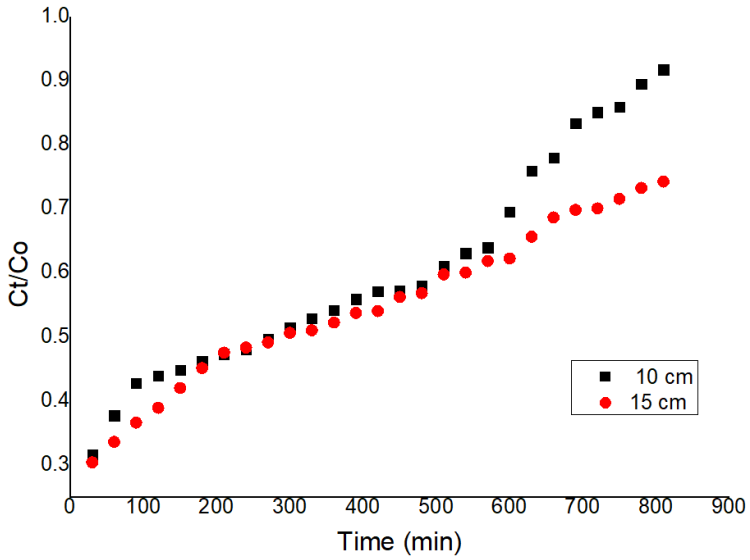


Figure-42: Effect of bed height (10 cm and 15 cm) on breakthrough curve

6.12.1.2 Effect of initial Dye Concentration on Adsorption

Effect of initial dye concentration on adsorption was studied by conducting experiments at different concentrations, 50 mg/L, 150 mg/L and 250mg/L of disperse red 167.1. The following trend was observed with increase in dye concentration (Figure 43). It can be seen that, at lower dye concentration, the break through curves are dispersed. The above graph demonstrates that, change in concentration gradient affects saturation rate and reduces break through time. This can be explained by the fact that; more adsorption sites will be covered with increase in dye concentration. The larger the concentration of dye, steeper is the break through curve. With increase in dye concentration, the driving force for mass transfer will increase. This resulted in a decreased adsorption zone. The column parameters obtained from effect of initial dye concentration are given in the table 24. This is due to the fact that high dye concentration leads to quick saturation of the sorbent, which in turn leads to the earlier breakthrough and exhaustion time (Hasan et al., 2010). From Figure 44, it can also be seen that more favorable and steep breakthrough curves were obtained at high influent dye concentration. These results demonstrated that the change in concentration gradient affects the saturation rate and breakthrough time (Goel et al., 2005). This can also be explained by the fact that more sorption sites were being covered as the concentration of the dye increases (Han et al., 2009). This indicates that with the change in concentration gradient, the saturation rate and breakthrough time are affected (Ko et al., 2000). In addition, as the influent concentration increases, the dye loading rate increases, so does the driving force for mass transfer, which resulted a decrease in the sorption zone length (Ko et al., 2000).

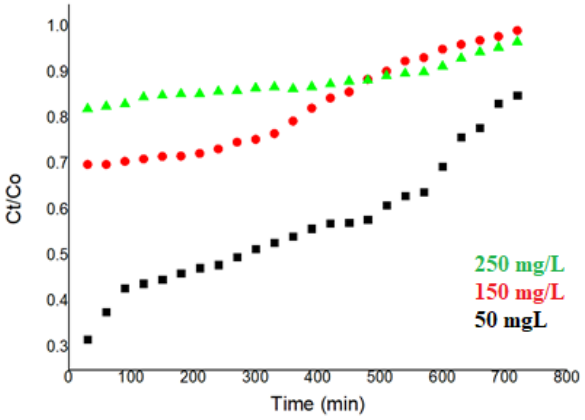


Figure-43: Effect of inlet dye concentration disperse red 167.1 on breakthrough curve

Table-24: Effect of different inlet dye concentration on adsorption

Inlet dye conc (mg/L)	**q_{total} (mg)**	**M_{total} (gm)**	**V_{eff} (ml)**
50	17.57	220.5	4410
150	72.91	661.5	4410
250	80.36	1102.5	4410

6.12.2Thomas Model

Thomas model was applied to the sorption kinetic data at three different inlet dye concentrations, i.e., 50, 150, and 250 mg L^{-1} and at bed height of 10 cm and 15 cm for the sorption systems of disperse red 167.1. The Thomas rate constant (k_{TH}) and the bed capacity (q_0) were calculated from the slope and intercept of the plot between ln (C_0/C_t-1) versus t at constant flow rate i.e., 7 ml/min. The calculated values of k_{TH} and q_0 along with the regression coefficients are presented in Table 25. The relatively high R^2 values at all the operating conditions suggested that the Thomas model was suitable for describing the column biosorption data of dye by immobilized corncob. From table 25, it is seen that values of determined coefficients (R^2) range from 0.57 to 0.87. As it can be seen**,** the value of qo increased with increase in bed height and corresponding K_{TH} values decreased. This is because, at higher bed

heights, more reactive sites were available. These results are similar to those obtained using different materials (Yahaya et al., 2011, Chowdhury et al., 2012). Likwise, biosorption of Acid Blue 15 (AB15) using fresh water macroalga *Azolla filiculoides* was investigated by Padmesh et al., (2006). The dye biosorption was favoured at increasing bed height and initial dye concentration, while the maximum dye biosorption was achieved at minimum flow rate. The optimum conditions for AB15 uptake were reported at 25 cm of bed height, 5 mL/min of flow rate and 100 mg/L of initial dye concentration. In the present study, as concentration of dye increased, qo (max) increased and K_{TH} decreased. This is because, with increase in concentration, the driving force for adsorption increased (Tan et al., 2008). Our results are in accordance with the previous study conducted by Han et al., (2009) by using of rice husk for the adsorption of Congo Red (CR) from aqueous solution in column mode. The effect of several important parameters such as pH, flow rate, initial dye concentration, existing salt and bed depth were studied. The results obtained by this study, indicated that biosorption of CR was initial dye concentration, bed depth and flow rate dependent. In this study, several models were applied. From the application of Thomas model, a less favourable adsorption of CR was observed at higher flow rate and lower influent concentration. A better column performance was obtained when the initial dye concentration increased, due to the increase of driving force for biosorption. Thus, Thomas model was suitable for adsorption processes where the external and internal diffusions will not be the limiting step. From Adam-bohart model, the model was valid for the relative concentration region up to 0.5 but a large discrepancy between the experimental and predicted curves was observed above this level. Han et al., (2009) studied the biosoption of methylene blue from aqueous solution by rice husk using fixed-bed column. The breakthrough curve was affected by pH, influent concentration, flow rate and existed salt. Results showed that, the breakthrough curves shifted from left to right when the pH of the influent increased. It was observed that breakthrough occurred faster with higher flow rate. In this study, two models were applied namely, Thomas and BDST models. By applying Thomas model, lower influent concentration and higher flow rate were unfavourable to the adsorption of MB on rice husk column. BDST and Thomas models were applied and both models were valid.

Table-25: Thomas model parameters using linear regression analysis under various operating conditions for adsorption of disperse red 167.1

Flow rate (ml/min)	Bed Height (cm)	Inlet dye conc. (mg/L)	K_{TH} (L/min.mg)	q_0 (mg/gm)	R^2
7	10	50	0.003	197	0.84
7	15	50	0.002	327.5	0.68
7	10	150	0.002	362.83	0.87
7	10	250	0.001	485.92	0.57

6.12.3 Adams-bohart Model

The values of adsorption capacity of the adsorbent, N_0 and kinetic constant of the model, K_{AB} determined by linear regression analysis are listed in table 26. The results show that as the bed height increased, the adsorption capacity (N_o) increased. The values of K_{AB} decreased. The adsorption capacity increased with increase in dye concentration. As the bed height increased from 10 cm to 15 cm, the adsorption capacity increased from 0.67 to 0.99 mg/L with K_{AB} decreased from 0.012 to 0.002 L/mg.min. From Table 26, it is seen that values of determined coefficients (R^2) range from 0.48 to 0.97.

Table-26: Adams-bohart model parameters using linear regression analysis under various operating conditions for adsorption of disperse red 167.1

Flow rate (ml/min)	Bed Height (cm)	Inlet dye conc. (mg/L)	K_{AB} (L/mg.min)	No(mg/L)	R^2
7	10	50	0.012	0.67	0.48
7	15	50	0.002	0.99	0.31
7	10	150	0.001	23.1	0.97
7	10	250	0.001	56	0.96

Han et al., (2009) studied the adsorption of MB by phoenix tree leaf powder in fixed-bed column. It was observed that the breakthrough time increased as the initial dye concentration and flow rate decreased, while the bed depth increased. Several models such as Thomas, Adam-Bohart, Yoon-Nelson, Clark and BDST models were applied to the experimental data. Thomas and Clark models were found suitable to describe the whole breakthrough curve, while Adam-bohart model was used to predict the initial part of dynamic process. In our present study, with

increase in bed height, the adsorption capacity increases from 0.67 to 0.99 mg/L. Our findings are in accordance with the study conducted by Ahmad and Hamed, (2010). They reported the fixed-bed adsorption of Reactive Black onto granular activated carbon prepared from bamboo waste. The adsorption system was found performed better with lower influent concentration, lower flow rate and higher bed height. The highest bed capacity of 39.02 mg/g was reported using 100 mg/L influent concentration, 80 mm bed height and 10 mL/min of flow rate. Adam-bohart, thomas and yoon-nelson models were used to fit the adsorption data, and the results fitted well to Thomas and Yoon-Nelson models with high R^2.

SUMMARY AND CONCLUSIONS

Chapter 7

SUMMARY AND CONCLUSIONS

The primary objective of this study was to investigate the pollution potential of textile industry effluents draining into Buddha Nallah stream located in Ludhiana, Punjab (India), and develop eco-friendly technologies determine to treat textile industry wastewater before it is discharged to water bodies. Therefore, this thesis focuses on the physico-chemical based characterization of textile industry effluents, isolation of efficient dye degrading microorganism and identification of microorganisms at molecular level, elucidation of mechanism of dye degradation, and evaluation of toxicity of control dyes with its degraded metabolites on plant (*T. aestivum*). This research further investigates the development of consortium of bacteria and checking the ability of consortium for decolorization of selected dyes compared to individual bacteria. The final objective of the thesis was to develop a biofilter by using bio-sorbent in the form of glass column in lab. The consortium was immobilized on corncob and used as a bio-sorbent for the treatment of textile dye. The overall goal of this research was to develop eco-friendly technologies for the treatment of wastewater discharged to waterbodies by the textile industries.

Following paragraphs give the conclusions of this research in relation to each objectives of the study:

1.Objective 1: to determine physico-chemical and biological characteristics of textile dyes in the untreated textile industry wastewater at selected discharge sites effluent. Wastewater samples from four textile industries were collected during the year 2014-2015 and were analyzed for various physio-chemical properties. The water quality parameters like BOD, COD, TSS and TDS of textile effluent were found to be significantly higher than the maximum permissible limit prescribed by WHO. The results of this study clearly show that textile industry in Punjab, India, is polluting the local surface water bodies and there is a need to develop simple, cost-effective and eco-friendly treatment systems for the bio-remediation of textile effluents.

2.Objective 2: to isolate, identify, and characterize dye-degrading bacterial strains from textile industry wastewater. To accomplish this objective of this study, study was conducted and we were successful to isolate and identify indigenously occurring bacteria in the textile industry effluents with potent dye degrading ability to degrade

the selected azo-disperse dyes (disperse red 167; disperse red 167.1; disperse blue 60 and disperse red 54). Results revealed less toxicity of extracted metabolites in comparison to parent dyes.

3.Objective 3: to develop bacterial consortium for enhanced dye degrading capability in textile industry wastewater. The studies conducted in response to this objective concluded that in the successful development of consortium on analysing dye decolorization assay. This consortium was found to show the highest rate of decolorization for disperse red 167.1 (82.76± 0.255%) within 24 h in comparison to rest of three selected dyes.

4. Objective 4: to design and develop clean-up technology (biofilters) for in-situ bioremediation of textile industry effluents. In this study, biofilters were developed by using low cost agricultural waste material (corn-cobs) as a substrate/matrix for the immobilization of consortium and for the removal of dyes from aqueous solution. This study concluded that adsorption of dye through a fixed-bed continuous flow column was dependent on the bed depth and inlet concentration. Also, with the increase in bed height, dye adsorption capacity of biofilter increases, due to more surface area and more available adsorption sites. The Thomas and Adams-bohart models were both successfully used to predict the breakthrough curves. Further studies are needed to scale up from lab scale to the pilot scale. The results obtained in the fourth objective, can help us to determine, if the design of biofilter and equations developed from lab-based column studies can describe conditions that occur in the "real world".

It can be concluded that, although decolorization is a challenging process to both the textile industry and the wastewater treatment, the results of this finding suggest a great potential for bacteria to be used to remove color from dye containing wastewater. The textile dye is degradable with a concerted effort of bacteria isolated from an effluent disposal site. Further, it can be suggested that the potential of the bacteria need to be demonstrated in its application for treatment of dye bearing wastewater using appropriate practice and through biotechnological approaches for removal of color on one hand and on another hand Government legislation standards should more stringent regarding the disposal of untreated textile effluents in natural water bodies. Moreover,

further research on these strains could explore new tools and techniques to evolve viable and eco-friendly microbial solutions for treatment of textile industrial effluent.

7.1 Future recommendations

In this thesis research, our studies were primarily conducted in the laboratory and we were successful in developing the conceptual design of a biofilter to treat and clean industry wastewater. This lab-based technology needs to be scaled up and tested under real world field conditions. This technology for the upscale process can be patented to commercialize the technology for helping textile industries.

Taking into consideration the advances in this field, we believe that future research activities should focus on below mentioned principal areas:

- In the present study, the bioremediation potential of individual bacteria and consortium has been checked for disperse dyes. In future, the bioremediation potential can be evaluated for all classes of dyes as different dyes contains variety of chromophores, auxochrome and complexity of structure.

- We have chosen textile dyes as a contaminant but in the future, the ability of bacterial consortium can be evaluated for other contaminants present in wastewater such as pesticides, hormones, heavy metals.

- In the current study, the bacteria have been screened for azo-reducatse and laccase enzymes for dye degradation. It can be suggested that, purification and characterization of other dye degrading enzymes can be done and demonstration of their role in treatment process by setting up immobilized cell and enzyme reactor for treatment of textile industry effluent. Optimization of process parameters can be carried out in future.
- We have analysed the toxicity of selected dyes and their metabolites on *T. aestivum*. But future research can focus on the extension of toxicity of dye degraded metabolites on cell line, *Daphnia* assay, fish toxicity etc. to address human health.
- Our study used corncob as a matrix to immobilize bacteria consortium. Future studies can use different biosorbents to support matrix in the form of immobilized packed bed reactor, where bacterial consortium can be impregnated in upscale process.

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Yu, J., Yue, G., Zhong, J., Zhang, X., and Tan, T. (2010). Immobilization of Saccharomyces cerevisiae to modified bagasse for ethanol production. Renewable Energy, 35(6): 1130-1134.

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LIST OF PUBLICATIONS

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Referred journal articles

- **Bhatia, D.,** Sharma, N. R., Singh, J., & Kanwar, R. S. (2017). Biological methods for textile dye removal from wastewater: A review. Critical Reviews in Environmental Science and Technology, 47(19): 1836-1876.
- **Bhatia, D.,** Sharma, N. R., Kanwar, R., & Singh, J. (2018). Physicochemical assessment of industrial textile effluents of Punjab (India). Applied Water Science, 8(3): 83.
- Daljeet Singh Dhanjal, Simranjeet Singh, **Deepika Bhatia**, Neeta Raj Sharma, Joginder Singh and Rameshwar S. Kanwar (2018). Pre-treatment of the mun.icipal wastewater with chemical coagulants. Pollution Research, 37: 32-38
- **Bhatia, D.,** Singh, S., Vyas, A., Rasool, H. I., & Kaur, P. (2014). Studies on Fungal Strains of Selected Regions of Ludhiana and their Biochemical Characterization. Current World Environment, 9(1): 192-202.
- Singh, Joginder, Simranjeet Singh, Parvinder Kaur, Priyanka Kapoor, Hakim Ishfaq Rasool, Jitender Sharma, Sadia Shabir Mir, Bilal Ibrahim, Deepak Vasist, and **Deepika Bhatia.** (2014). Biochemical characterization of *Tylophora indica* and its immuno-modulatary effect. South Asian Journal of Experimental Biology, 4(2): 61-67.

Conference presentations

1. Awarded International travel grant to attend Summer Institute 2016 (May 27-June 5) on Nanotechnology in the areas of infrastructure, water, and health at University of Alberta, Edmonton, Canada and presented **E-poster abstract entitled "Removal of textile dyes using nano biochar from corn cob"** w.e.f. May 27- June 5, 2016 on Nanotechnology in the areas of infrastructure, water, and health at **Summer institutes 2016, Edmonton, Canada.**
2. Attended 6th International Graduate Research Conference (IGRC) on **Feb 9-10, 2017 at Chiang Mai University, Thailand and** presented an oral paper from my thesis **entitled "Use of Naturally Occurring Bacteria to Clean Textile Industry Wastewater"**

3. Abstract has been selected to participate and present **oral presentation entitled "Removal of Disperse red 167 (dark Red 2B) from aqueous solution using sustainable adsorbent''** in World Biotechnology Congress (WBC-2017), on February 20-22, 2017 at Jawahar Lal Nehru University, New Delhi
4. Awarded International travel grant to attend Summer Institute 2017 (**June 1-June 11)** on Sustainable Communities in Low-Resource Settings at **University of British Columbia, Vancouver, Canada.**
5. Awarded International travel grant to attend one day Conference (**June 29)** on Sustainable Solid Waste Management (SuSWaM 2017) at **McGill University, Montreal, Canada.**
6. Abstract entitled **"Continuous column studies for the removal of Disperse red 167 dye by using low cost adsorbent"** has been selected to participate in Innovative Strategies for Sustainable Water Management Conference (ISSWM-2017) at Lovely Professional University, India
7. Abstract entitled **"Bio-Decolorization of Disperse Dyes by Using Naturally Bacteria"** has been selected to participate in Innovative Strategies for Sustainable Water Management Conference (ISSWM-2017) at Lovely Professional University, India
8. **Attended 12th Agricultural Science Congress, 2015** at National Dairy Research Institute **(NDRI),** Karnal, India, as a delegate.
9. **Presented Paper** entitled 'Studies on Physico-chemical characterization of surface water body in Ludhiana, Punjab, India, in conference on "Exploring Basic and Applied Sciences for Next Generation **(EBAS-LPU) on November 14-15, 2014.**
10. Attended **3rd Bharatiya Vigyan Sammelan (BVS) and Expo** as delegate at Lovely Professional University (2011)
11. Short term training on **"Plant Tissue Culture" at Plants Breeding Dept under the guidance of Dr S. S Gosal** at Punjab Agricultural University (PAU), Ludhiana, Punjab.
12. Short term training on "**Extraction of major metabolites from *Tylophora indica* under the guidance of Dr Harmanjeet Kaur** at Thapar Univerisity (TU), Patiala, Punjab.

Patents Filed:

- Ref No: IN201811002650 (Patent Applied) – Filtration Assembly for Wastewater Treatment (2018)
- Ref No: 201811009348 (Patent Applied) – A Process of Degradation of Textile Dyes (2018)
- Ref No: 201811009349 (Patent Applied) – A Process to Remove Heavy Metals from Industrial Effluent using Biochar (2018)

Gene Bank depositions:

- Bhatia,D., Kanwar,R., Das,A. and Singh,J., *Paenibacillus pocheonensis* strain DP2 from India 16S ribosomal RNA gene, partial sequence. GenBank Acession number: KX034565.1
- Bhatia,D., Kanwar,R., Das,A. and Singh,J., *Lysinibacillus* sphaericus strain DP2 from India 16S ribosomal RNA gene, partial sequence. GenBank Acession number: KX034564.1
- Bhatia,D., Kanwar,R., Das,A. and Singh,J., *Bacillus cereus* strain DP2 from India 16S ribosomal RNA gene, partial sequence. GenBank Acession number: KX034563.1
- Bhatia,D., Kanwar,R., Das,A. and Singh,J., *Bacillus subtilis* strain DB1 from India 16S ribosomal RNA gene, partial sequence. GenBank Acession number: KX034562.1
- Bhatia,D., Kanwar,R., Das,A. and Singh,J., *Paenochrobactrum glaciei* strain DB1 from India 16S ribosomal RNA gene, partial sequence. GenBank Acession number: KX034561.1
- Bhatia,D., Kanwar,R., Das,A. and Singh,J., *Escherichia coli* strain DB1 from India 16S ribosomal RNA gene, partial sequence. GenBank Acession number: KX034560.1
- Bhatia,D., Kanwar,R., Das,A. and Singh,J., *Brevibacillus panacihumi* strain DB1 from India 16S ribosomal RNA gene, partial sequence. GenBank Acession number: KX034559.1