Studies on stable azoreductase enzyme from *Chromobacterium violaceum*: possible application in industrial effluent dye degradation

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DOCTOR OF PHILOSOPHY

By

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Dedicated to
My ever amazing parents,
inspiring mentors
and curious researchers

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

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STATEMENT

I hereby declare that the matter embodied in this thesis titled **"Studies on stable azoreductase enzyme from *Chromobacterium violaceum*: possible application in industrial effluent dye degradation"** is the result of investigations carried out by me in the Centre for the Environment, Indian Institute of Technology Guwahati, Assam, India under the supervision of **Prof. Vikash Kumar Dubey** and **Dr. Lal Mohan Kundu.**

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work of other investigators are referred. Further the data in the thesis are collected by me. I certify that there is no fabrication or manipulation of data in the thesis.

Date: August, 2019

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CERTIFICATE

It is certified that the work described in this thesis **"Studies on stable azoreductase enzyme from *Chromobacterium violaceum*: possible application in industrial effluent dye degradation''** by **Mr. Kamalesh Verma** (Roll No. 146152009), submitted to Indian Institute of Technology Guwahati, India for the award of degree of Doctor of Philosophy, is an authentic record of results obtained from the research work carried out under my supervision at the Centre for the Environment, Indian Institute of Technology Guwahati, India and this work has not been submitted elsewhere for a degree.

Prof. Vikash Kumar Dubey
(Thesis Supervisor)

Dr. Lal Mohan Kundu
(Thesis Co- Supervisor)

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Kamalesh Verma
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Abbreviations

ANS	:	8-Anilino-1-naphthalene sulfonic acid
BLAST	:	Basic Local Alignment Search Tool
BOD	:	Biological Oxygen Demand
BSA	:	Bovine Serum Albumin
CD	:	Circular Dichroism
COD	:	Chemical Oxygen Demand
DCIP	:	2,6-dichlorophenol-indophenol
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic Acid
ED	:	Electron donor
EDTA	:	Ethylene Diamine Tetra Acetic Acid
EDX	:	Energy Dispersive X-ray
ETAD	:	Dyestuff Manufacturing Industry
FAD	:	Flavin adenine dinucleotide
FADH	:	Flavin adenine dinucleotide hydrogen
FBS	:	Fetal Bovine Serum
FESEM	:	Field Scanning Electron Microscopy
FMN	:	Flavin mononucleotide
FTIR	:	Fourier-transform infrared spectroscopy
GdmCl	:	guanidine hydrochloride
GI	:	Germination Index
HPLC	:	High-performance liquid chromatography
IPTG	:	Isopropyl β-D-1-thiogalactopyranoside
K_d	:	Dissociation Constant
kDa	:	Kilodalton
KI	:	Potassium iodide
LB media	:	Luria-Bertani media
Lip	:	Lignin peroxidase
MnP	:	Manganese peroxidase
mRNA	:	Messenger Ribonucleic Acid

MTCC	:	Microbial Types culture collection
MTT	:	[(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)]
NaCl	:	Sodium chloride
NAD	:	Nicotinamide adenine dinucleotide
NADH	:	Nicotinamide adenine dinucleotide hydrogen
NADPH	:	Nicotinamide adenine dinucleotide phosphate hydrogen
NCBI	:	National Center for Biotechnology Information
NCCS	:	National Centre for Cells Science
NEB	:	New England Biolabs
PBS	:	Phosphate Buffered Saline
PCR	:	Polymerase Chain Reaction
PDB	:	Protein Data Bank
PPO	:	Polyphenol Oxidase
RNA	:	Ribonucleic Acid
SDS	:	Sodium Dodecyl Sulphate
SDS-PAGE	:	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TDS	:	Total Dissolved solids

List of Figures

List of Tables

Contents

CHAPTER 1

Review of literature and scope of the work: Effect of industrial wastes on the environment and their degradation with emphasis on azo dyes *

ABSTRACT

"Dyes are chemically coloured substances having a strong affinity towards the substance on which they are applied." Dyes have been utilized by people around the world since ancient time for various purposes. More than 3600 types of dyes are available which are manufactured by using more than 8000 types of chemicals. In every year, 700,000 tons of the commercial dyes are being produced and more than 100,000 tons of dyes are being utilized only in textiles industries. These dyes are discharged into the water body after utilization without proper treatments. Such dyes are persistent in the environment for a very long time and converted into toxic and carcinogenic compounds. All these discharged dyes affect the water body, agriculture, human health and the surrounding environment. For the degradation of such dyes, various methods are available; each of the methods have some pros and cons. Thus, the complete degradation of an azo dye needs very effective and economically feasible methods. For complete degradation of azo dye, enzymatic method is very effective among all available methods. The work reported in the thesis focussed on cost effective method for purification and immobilization of azoreductase enzyme from *Chromobacterium violaceum* in order to develop effective method for the complete degradation and decolourization of azo dye.

***Manucript covering the part of the work is under preparation for publication.**

1.1 Introduction

The environment is the sum total of all surroundings, which comprises of both living and nonliving components, either tangible or intangible. It is an amalgamation of four spheres, the atmosphere, hydrosphere, lithosphere and biosphere. In all the sphere of environments, contaminants are being added at every moment that disturbs the basic composition of nature. Adding this contaminant into the environments creates pollution. The environmental pollution can be defined as "when the magnitude of the contaminants increases to such a level that its hazardous effects are reflected on living beings then it is referred to as pollution" and "the undesirable alteration in the physical, chemical and biological characteristics of the environment, which tends to be injurious to human health" (*Egboka et al., 1989*).

Pollution created by human beings as well as nature is prevailing in the environment. Pollution from natural sources has been from times immemorial, but among the existing sources of anthropogenic pollution emerge out as the most prominent ones. The substance like solid, liquid, or gas emitted out from a source hampering the balance of the environment is termed as a pollutant. Human beings have the capability to harness the natural resources in the maximum possible ways and as an efficient manner. But the utilization of natural resources leaves behind a multitude of waste products which acts as "pollutants" and disturbs the natural equilibrium. Generation of manmade pollution has gained its pace with the geometric rise in population. The pollutants generated as a result of human activities are surpassing the natural ones and therefore pose a severe threat to the natural environment. Pollution has extended to all spheres of life and it has not even spared the hydrosphere (water bodies) and air, which has been affected in the worst manner.

Industries are the prominent sources of water pollution, as they use a high amount of water. According to World Bank's reports, total consumption of water for industrial purposes in India is about 13% of total freshwater of the country and water demand for industrial uses grows at a rate of 4.2% per year *(World Bank, 1998)*. The amount of wastewater is generated by the industries is proportionate to its consumption by the same. The data in Table 1.1 reflects the quantity of water consumed and wastewater generated by Indian industries.

Table 1.1: Water consumption and wastewater generation of Indian industries *(CSE, 2004)*

Industrial Sector	Annual wastewater discharge (million cubic meters)	Annual consumption (million cubic meters)	The proportion of total water consumed in the industry (%)
Thermal power plant	27,000.9	35,157.4	87.87
Engineering	1551.3	2019.9	5.05
Pulp and paper	695.7	905.8	2.26
Textiles	637.3	829.8	2.07
Steel	396.8	516.6	1.29
Sugar	149.7	194.9	0.49
Fertilizer	56.4	73.5	0.18
Others	241.3	314.2	0.78
Total	30,729.2	40,012.0	100.0

It is clearly reflected from this table that the textile industry contributes to a significant proportion of water pollution in India. Textile industry in India has grown from a very indigenous form to very modern and sophisticated forms in the present day. The main component in the textile industry is the use of "Dyes" because the colour is the main attraction of fabrics. There are more than 3600 types of individual textile dyes that are being manufactured by the industries to colour the fabric by using almost more than 8000 kinds of different chemicals *(Kant, 2012).* The dye used in different industries is not completely utilized and released with wastewater resulting in the pollution of both surface and groundwater *(O'Neill et al., 1999)*. Apart from textile industries, pulp and paper, distilleries and tanneries also release coloured wastewater, but the pollution from textile industries is more prominent in terms of chemical dye compound, creating an unacceptable level of colour and BOD (Biological Oxygen Demand) *(Wang et al., 2007).* Apart from aesthetic complexities, dyes reduce light penetration into water bodies, thus preventing aquatic plants from photosynthesis.

1.2 Dyes: Historical perspective

“Dyes are chemically coloured substances having a strong affinity to the substance on which they are applied." The use of the dyes started from the ancient time likely from the Indus Valley Civilization at 2500 B.C. But the Marco Polo mentioned that the use of the natural dyes in India during the 1300 AD form the indigo plant. These natural dyes are purified from the plant's bark, leave, wood and root, etc. The production of natural dyes was limited because of seasonal changes, unavailability of the plants throughout the year and also the natural dyes can not be stored for longer period of time. Due to all these reasons, people thought about the use of synthetic dyes and in 1856 the Lavender dyes were discovered artificially from coal tar by William Henry Perkin. The discovery of lavender dyes attracted the people in the production of synthetic dyes industry throughout the world. More and more people are attracted in the synthesis of synthetic dyes because it can be stored for a longer period of time and also due to the very low cost. Finally, in 1897, synthetic dyes also came into the market. Nowadays more than 700,000 tons of the commercial dyes are being produced annually and more than 100,000 dyes are being utilized only in textiles industries *(Lucas et al., 2007)*. But the annual consumption rate of the dyes was found to be 7 x 10^5 tonnes *(Karthikeyan et al., 2010)*. After utilization, these all dyesstuff is discharged into the water body as waste where they persist in the environment and creates pollution to the water. To save the environment from dye pollution, many international committees were formed to maintain the regulations. In 1974, the Ecological and Toxicological Association of the Dyestuff Manufacturing Industry (ETAD) committee made some regulations to fully cooperate with government and public concern on the toxicological impact of their product. To minimize the environmental pollution many countries applied a very strict rule for the protection of the environment from the dye pollutants *(Anliker,1979)*. Whereas, many European countries made a very strict rule that zero synthetic chemical should be released into the marine environment *(Willmott et al., 1998; O'Neill et al., 1999)*? Due to the presence of the chromophoric group in the dyes, a very small amount of the dyes can produce different colour in the liquid medium, which can be detected in visible range by different techniques like spectrophotometers and chromatography. These chromophoric groups absorb light at different absorption range. The commonly available chromophoric groups are –N=N- (azo), =C=O (carbonyl), =C=C=, NO or N-OH (nitroso), C=S (sulfur), etc.

and absorption range for a different colour of dyes are available in table 1.2 *(Sen et al., 2015).*

Table 1.2: Detection range of different colours with different wavelength

S. No.	Colour	Detection range in wavelength(nm)
1.	Red	700-635
2.	Orange	635-590
3.	Yellow	590-560
4.	Green	560-490
5.	Blue	490-450
6.	Violet	450-400

1.3 Types of dyes

Dyes have been classified into two categories based on their origin: natural and synthetic. Natural dyes are either inorganic or organic in nature. Natural dyes were commonly used in the earlier times, but nowadays synthetic dyes are gaining popularity and also available in various forms. Dyes can be classified into different groups according to their chemical constituents and based on their usage. Textile industries are one of the major areas in which most of the dyes have been utilized. Apart from all dyes, azo group of dyes is prominently used in the textiles industries *(Hashem et al., 2018)* but also used into the food industries, pharmaceutical, paper, leather, agriculture and cosmetics, etc.

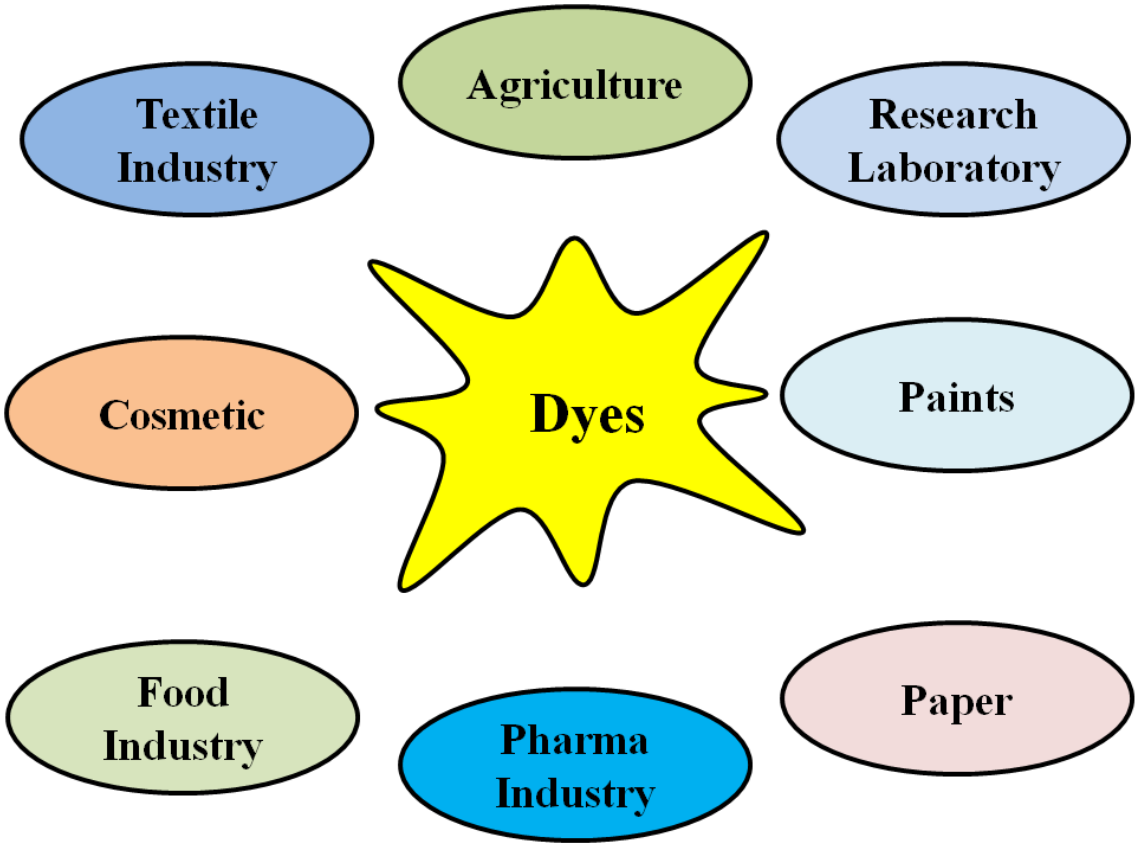


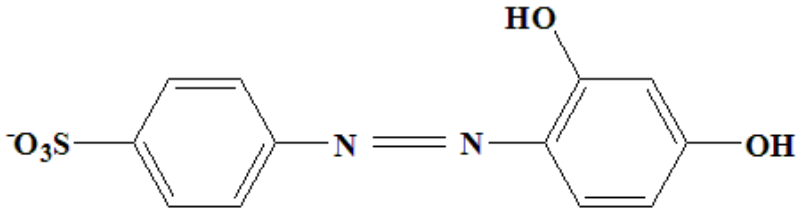
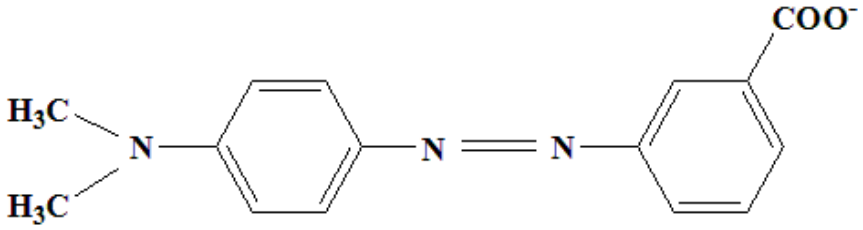
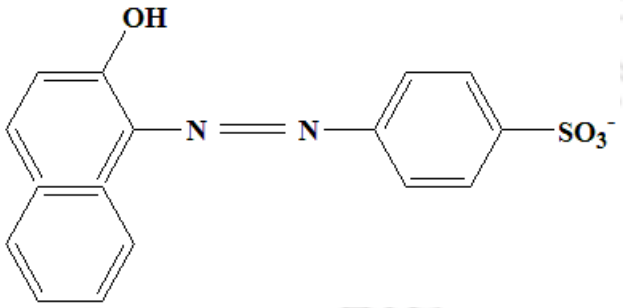
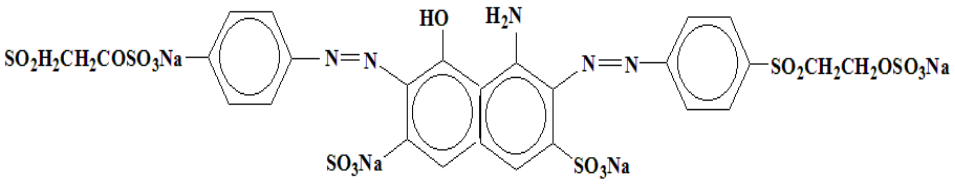
Figure 1.1: Utilization of dyes in different Industries

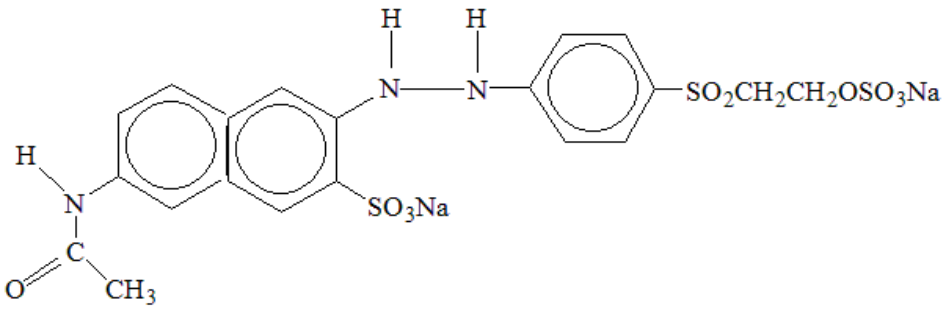
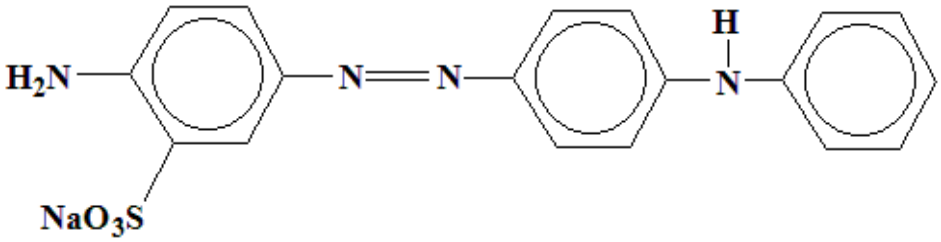
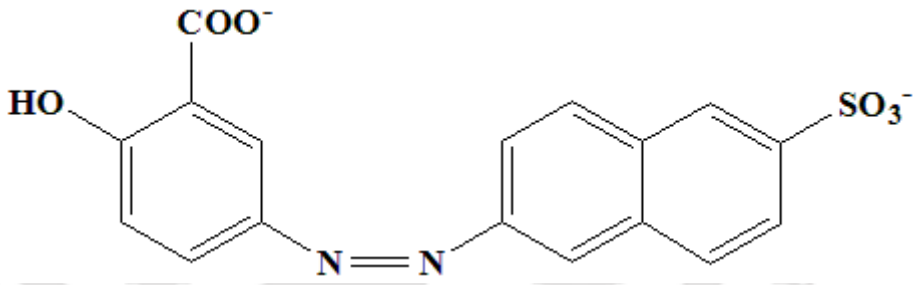
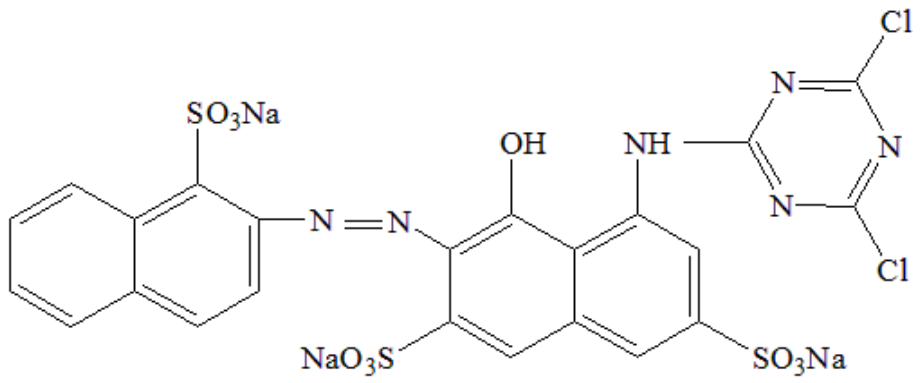
1.4 Azo dyes

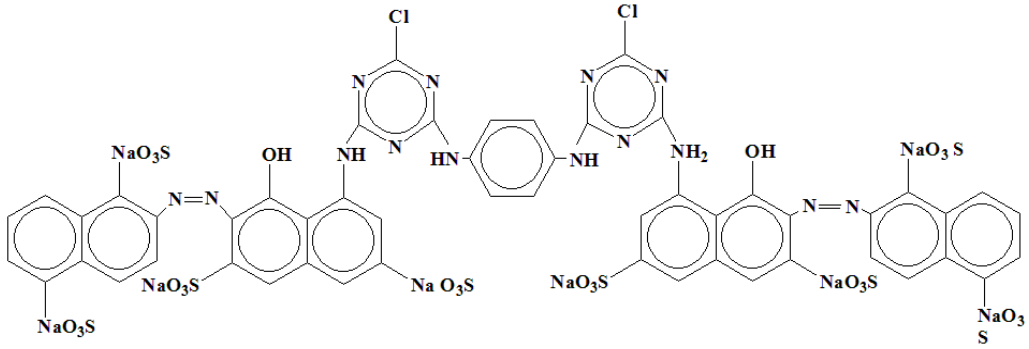
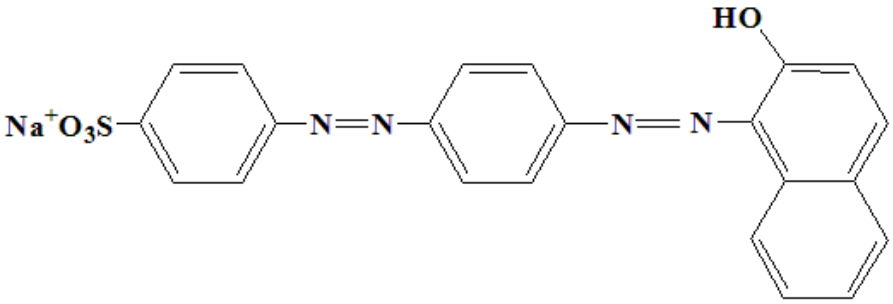
Azo dyes are organic substance containing an azo group (-N=N-). Azo dyes are mainly of two types: naphthol and non-naphthol. Naphthol type of azo dyes includes the reactive black, reactive blue 171, reactive green and red, but the non-naphthol group includes direct reactive yellow and direct yellow dyes. They are mainly used in cotton, polyester, cellulose and rayon, etc. *(Hunger, 2003; Wackett et al., 1991; Hsueh et al., 2009).* We have seen that many dyes have been used in different industries. Apart from that azo group of dyes is maximally used, which comprise 60-70% of all dyes. The reason behind the maximum use of the azo dyes is their cost-effectiveness, structural diversity and high molar extinction coefficient *(Bafana et al., 2011).* Around 70% of the produced dye belongs to the azo group *(Zollinger, 1987).* However, these dyes are not devoid of their negative impacts to the environment as most of them have been identified as carcinogenic and mutagenic due to the direct action of the agent itself or to aryl amine derivatives generated during the reductive biotransformation of the azo bonds *(Rajaguru et al., 1999).* Presence of even the trace amounts of dyes in the water bodies can cause human

health disorders like hemorrhage, ulceration of the skin and mucous membranes and can cause severe damage to kidneys, reproductive system, liver, brain and central nervous system. Based on the above facts, it can be said that the discharge of azo dyes in the environment is becoming a major threat to flora and fauna. These dyes are classified according to their chemical structure shown in Table 1.3.

Table 1.3: Commonly used azo dyes and their structure

S. No.	Chemical Structure
1.	 **(Acid orange)**
2.	 **(Methyl red)**
3.	 **(Acid orange 7)**
4.	 **(Remazol black)**

5.	 **(Reactive orange)**
6.	 **(Acid yellow 36)**
7.	 **(Mordant yellow)**
8.	 **(Reactive red 11)**

9.	 **(Reactive red 141)**
10.	 **(Acid red G)**

There is no life without water and we are all aware of the ever-increasing demand for water in our daily lives. A large proportion of water is being utilized by the industry. This utilized water from industries is discharged in the forms of effluents, which contains a lot of waste and various types of pollutant. This contaminated water is drained out in water bodies without any prior treatment, which further pollutes our water bodies and also our environment. Even a very small amount of dyes contaminant into the water affects the water transparency, gas solubility and light penetration into the water bodies *(Banat et al., 1996; Robinson et al., 2001)*. After seeing all the literature survey, we have found that the decolourization of textile wastewater is a major environmental concern; even people are doing work in this field for a long time back. The problem of textile wastewater arises by increasing population, urbanization and industrialization and also with the lack of a proper method for decolourization. By using the conventional wastewater treatment system, it is very difficult to remove the wastewater contaminated with synthetic dyes. To overcome this serious environmental problem (wastewater contaminated with dye) we have to develop some methods which is very helpful in decreasing the dyes pollutant and also to save our life by cleaning the environment from the hazardous pollutant.

1.5 Dye as a pollutant

Now we know that more than 700,000 tons/annum of the commercial dyes are being produced throughout the globe and these dyes are being utilized by different industries. Apart from all these dyes, more than 100,000 tons of dyes are being utilized only in textiles industries *(Lucas et al., 2007).* But the annual consumption rate of the dyes was found to be the 7 x 10^5 tonnes *(Karthikeyan et al., 2010)*. Around more than 15% of the utilized dye is not able to bind with fabrics and directly discharged into the wastewater as effluent into the environment. The discharge of the unutilized dye is dependent on the various factors like the types of dyes that are being used during the dying process. If a basic dye is being used, then 2% of dyes are lost but in the case of reactive dye, 50% of used dyes are lost during the dying process. These unutilized dyes are released into the water body with wastewater generated from the industries. It is reported that the wastewaters of textile industries show extreme fluctuations in chemical oxygen demand (COD), biochemical oxygen demand (BOD), pH, colour and salinity *(Dos Santos et al., 2007).* The coloured water cuts down the light penetration into the water bodies thus trimming the photosynthesis of aquatic plants *(Aksu, 2003).* Most of the dyes are very toxic in nature. Triphenyl methane is one of the most commonly used dyes in textiles, fish farming industries, paper, leather, food, and medicines *(Azsmi et al., 1998).* Many other and Black *et al*. showed that triphenylmethane dye is carcinogenic in nature to the biotic environment and also mutagenic to the fishes *(Black et al., 1980)*. Precursors of many dyes are carcinogenic compounds like Benzidine, which accumulate into the environment and create problems *(Baughman et al., 1988)*. Many synthetic azo dyes are carcinogenic in nature due to the presence of -N=N- bond *(Mahmood et al., 2011).* Apart from these, the colour content makes the water aesthetically unacceptable and unsuitable for use in irrigation in agricultural fields.

1.6 Remediation Process

Environmental remediation is the process for the removal of the pollutant from the environment (soil, air and water). The industrial effluents containing textile wastewater is very difficult to degrade because of the complexity and chemical nature of dyes is very diverse. Due to this reason, it is very difficult to degrade the dyes by using conventional procedures *(Alinsafi et al., 2006).* Due to structural varieties, (basic, azo, acidic, disperse, diazo, metal complex and anthraquinone based dyes) of dyes, making them more

complex, and decolouration of textile dye effluent is very difficult by using municipal sewerage systems *(Willmotte et al., 1998).* This type of effluent includes suspended solid, surfactant, unused dyes, etc. Dyes containing wastewater are very harmful, mutagenic, and carcinogenic in nature. So that we have to focus very seriously and priority based method for the removal of dyes and make the environment clean from such contaminants. In order to proceed, we have to develop a cost-effective method is priority-based, which can remove all the available dyes from the wastewater. Till date, many methods have been proposed to remediate the dye pollution. These methods include physical, chemical and biological processes, which efficiently contribute to the remediation process. However, all these methods have some cons, which bring the attention of the scientific world to precede more studies in this area for the development of a suitable and efficient process. The presently available methods for the removal, degradation and mineralization of dye are physical, chemical and biological methods. In the following sections, all methods are discussed with their pros and cons.

1.6.1 Physical methods

Physical methods are very important methods for the degradation of an industrial effluent containing dyes. In the physical study, the various methods are presents, which include adsorption, ion exchange, coagulation, reverses osmosis and membrane filtration.

1.6.1.1 Adsorption

Adsorption process can be used for the removal of various types of dyes. It is utilized mostly because of their stability for the conventional treatment method. Due to the economic feasibility of this method, it attracts more and more people to use it *(Choy et al., 1999).* The adsorption process is mainly affected by physicochemical factor-like pH, temperature, sorbent surface area, dye and sorbent interaction, particle size and contact time *(Kumar et al., 1998).* In this method, the various adsorbent can be used like wood chips can be used for acid dyes, but it not the good adsorbent for others dye *(Nigam et al., 2000).* The role of various adsorbent used in adsorption of dyes is discussed bellow.

1.6.1.2 Activated carbon

In adsorption, the use of activated carbon is most commonly used for the removal of dye. Activated carbon can absorb most of the dyes but not suitable for all types like direct dye, vat dye, reactive and pigment dyes *(Raghavacharya, 1997; Rao et al., 1994).* If activated carbon is once used, again, we have to activate it for the next use; otherwise, the activity

of the adsorbent is affected and at every reactivation, 15-20% of the sorbent was lost. The performance of the activated carbon depends on the characteristic of wastewater and types of carbon used. Sometimes it is not very effective because the nature of activated carbon is amphoteric and pH of dye plays a major role in the adsorption process. Some time, low-cost adsorbents like rice husk, sugarcane bagasse, pinewood, chitosan, etc. that can absorb and accumulate dyes but not as efficiently as activated carbon *(Crini et al., 2016).* Use of the adsorption method is limited because of problems associated with the regeneration, high sludge production and high cost *(Yerramilli et al., 2005; Robinson et al., 2001).*

1.6.1.3 Membrane filtration

Membrane filtration is another very useful method for the separation of dyes from wastewater. This method has the ability to concentrate, clarify and continuously separate the dye from the effluents *(Mishra et al., 1993; Xu et al., 1999).* Filtration methods like membrane filtration, ultra-filtration and reverse osmosis are mainly used for chemical separation and water reuse, this method is also can be used for the removal of various type of dyes. In membrane filtration method, selection of membrane dependents on the chemical composition of wastewater *(Dos Santos et al., 2007).* This method has some advantages like resistant to microbe and temperature. But some disadvantages are also there like limitations for pore size of the membrane with a different molecular weight cut off (MWCO), for reverse osmosis (<1000 MWCO), nano-filtration (500-15000 MWCO) and ultrafiltration membranes (1000-100000 MWCO). This method is not very useful because of the high cost of the membrane and also the membrane needs to be changed or regenerated from time to time. In this method, dyes are not degraded, but we can concentrate the dyes and dispose at regular time intervals.

1.6.1.4 Electro kinetic coagulation

Coagulation is effective and economically feasible methods for the removal of dyes, which contain sulphur disperse and direct dyes. Coagulation is methods based on the destabilization of electrostatic interactions between dye and water molecules. These methods evolve the addition of many chemical reagents like $FeC1_3$, $FeSO_4$, $AlCl_3$, or $Al_2(SO_4)_3$, to remove the direct dye from the wastewater *(Allegre et al., 2004).* Many flocculating reagents like linear long-chain polymer and copolymer are also used with coagulating agents. This method is limited for a few selected types of dye pollutant and

produces a large amount of sludge. The removal of sludge puts extra disposal cost and the chemicals used for precipitation during the treatment also add extra cost to this method *(Gahr et al., 1994;Hao et al., 2000).* This method is not commonly utilized because of the extra cost involved.

1.6.1.5 Ion-exchange

Ion exchange is another method for the degradation of dye with various advantages over the available dye degradation methods like no regeneration problem, no loss of adsorbent and also can be used for the separation of dissolved dyes. In this method, ion exchange resin is used for the separation. The resin used during this method is cation-exchange resin (Amberlite IR 120) and anion- exchange resin (Amberlite IRA 400) *(Raghu et al., 2007).* The use of this method was found to be limited to a few types of dyes *(Slokar et al., 1997).* The major disadvantage of this method is the cost of the method. The various organic solvent is used for degradation, which put extra cost and makes the process more expensive *(Mishra et al., 1993).*

1.6.2 Chemical methods

Chemical method is very important for dye removal from wastewater effluent containing dyes. This method includes oxidization, ozonation, photochemical, sodium hypo chloride, and electrochemical destruction. The brief description of these methods is given below.

1.6.2.1 Oxidative process

This is an important and commonly used method because of the simplicity of the application and this process is much more common for the decolourization of dye from wastewater in chemical meaning. In this process, various oxidizing agents have been used. The most commonly used oxidizing agent is hydrogen peroxide (H_2O_2), ozone (O_3) and permanganate (MnO_4) *(Slokar et al., 1997).* In oxidation, the dye molecule is separated by oxidation and breakage of the aromatic ring takes place *(Raghavacharya et al., 1997; Field et al., 1995).* In this process, the activated oxidizing reacts to the dye molecule and brings about a change in the structure of the dye. Thus dye is decolourized and removed by chemical separation. The common pathway for the degradation of dyes by the hydroxyl radical is as follows.

$$OH + RH \longrightarrow H_2O + R$$

$$R + H_2O_2 \longrightarrow ROH + OH$$

$$R + O_2 \longrightarrow ROO$$

$$ROO + RH \longrightarrow ROOH + R$$

In the oxidation process, hydroxyl radical is used most commonly because the hydroxyl potential of hydroxyl radical found to be much more than other radical. This method has many disadvantages over advantage, like the generation of a large amount of sludge is a major problem for the disposal.

1.6.2.2 Use of H_2O_2–Fe salts (Fenton's reagent)

In this process, the use of Fenton's reagent is much more common and widely used for the removal of both soluble and nonsoluble dyes. Fentons reagents are resistant to the biological methods of treatment and also poisonous to live biomass in wastewater *(Slokar et al., 1997)*. In this reaction, the combination of ferrous and H_2O_2 performs their functions one by one, firstly the oxidation of dye molecules by OH radical and secondly the coagulation of dyes with the help of iron. Hydroxyl radical oxidizes the dye and converts itself to the Fe^{3+} moiety. It is very effectively decolorizing all the soluble and nonsoluble dyes *(Rauf et al., 2009)*. In this method, the generation of a large amount of sludge is a major problem for the disposal and also includes the cost on that and reaction is very slow so that it needs more time for completion *(Chung et al., 1993)*.

1.6.2.3 Ozonation Method

Ozonation is also an oxidation process for the decolourization of synthetic dyes from wastewater. Ozone has the capability to degrade phenols, chlorinated hydrocarbon, aromatic hydrocarbon and pesticides *(Xu et al., 1999; Lin et al., 1993)*. In this process, Ozone decolorizes dyes by degrading the conjugated bond presents in chromophore *(Peralto – Zamora et al., 1999)*. This process is very useful because, there is no generation of sludge and it also decreases the chemical oxygen demond (COD) of waste so that it can be directly discharged into the water bodies *(Ince et al., 1997)*. This method is mostly used for the azo dye molecule and also used in the gaseous state so that the volume of the waste and sludge does not increase *(Slokar et al., 1997)*. The major drawback of this method is the short half-life of ozone, which is around 20 minutes.

Ozonation needs to be done continuously for the complete treatment of dyes and this increases the overall cost of the process *(Xu et al., 1999)*. The ozonation process is affected by pH, salt and temperature, etc. and decomposition of ozone start in the alkaline condition, so we need to maintain the pH of wastewater carefully *(Slokar et al., 1997)*. Severe others drawback of this process is that ozone is a pollutant gas for our environment and usage of ozone results in the formation of toxic products. But if ozone is used for a longer duration, then that will reduce the effect of toxic products *(Wang et al., 2003)*.

1.6.2.4 Sodium hypochlorite (NaOCl)

Use of sodium hypochlorite (NaOCl) in the treatment of dyes is also a good option but not suitable for dispersing types of dyes. In this case, chloride (Cl) attacks the amino group of dye molecule cleaving azo bonds. Increase in decolourization of dye increases the amount of chloride concentration in the water bodies. The higher chloride concentration in water body has an adverse effect on the environment, animals and plankton *(Slokar et al., 1997)*. The release of degraded azo dye with sodium hypochlorite was acting as carcinogenic and toxic to our environment *(Banat et al., 1999)*.

1.6.2.5 Photochemical process

The method in which degradation of dye molecule into CO_2 and H_2O by free radical mechanism initiated by photochemical activation of H_2O_2 *(Peralto-Zamora et al., 1999; Yang et al., 1998)*. The mechanism for the degradation is the interaction of hydroxyl radical in very high concentration. The radicals are generated by the interaction of UV light with the different chemicals like hydrogen peroxide, ozone and Fenton's reagent. The rate of dye removal is affected by the pH, dye structure composition and UV light intensity *(Slokar et al., 1997; Gogate et al., 2004; Forgacs et al., 2004)*. To increase the capacity of dye removal, we have to set up the reaction in batch or continues mode *(Namboodri et al., 1996)*. During the photochemical treatment, many side products are formed, which depends on the composition of waste containing dye. Such by-products are inorganic acid and organic aldehyde halides etc. *(Yang et al., 1998)*. In this method, sludge is not produced and is non- toxic to the environment but a little bit expensive, resistant to the photo corrosion and biological immunity *(Gonzalez-Gutierrez et al., 2009; Guivarch et al., 2003)*.

$$H_2O_2 + h\nu \longrightarrow 2OH^*$$

This photocatalysis process can be performed by O_3 and Fenton's in which the improved bleaching and mineralization were found. To increase the efficacy of the process, solar energy can be used in the generation of radicals from TiO_2 *(Konstantinous et al., 2004)*.

1.6.2.6 Electrochemical destruction

Removal of dyes can also be done by electrochemical methods in which the dyes are degraded by the use of electric current. This method has a significant role in the dye degradation. In this method, there is no generation of sludge and no use of chemical during the process. The degraded metabolite generated after the treatment has no harm on others and can be directly released into the water body. We found that electrochemical destruction is very efficient method for dye removal with many advantages. But a major drawback of this method is the usage of continuous high power electric current, which increases the cost and make the process more expensive *(Pelegrini et al., 1999; Ogutveren et al., 1994)*.

1.6.2.7 Cucurbituril method

Cucurbiuril ($C_{36}H_{36}N_{24}O_{12}$) is the name because of its shape like pumpkin (a member of *the Cucurbitaceae* family plant). The name cucurbituril was first mentioned by Behehrend et al. *(Behehrend et al., 1905)*. The structure of cucurbituril was a cyclic polymer of formaldehyde and glycoluril *(Karcher et al.,1999a; Karcher et al.,1999b)*. It has good sorption capability for the water treatment; it helps in the removal of the aromatic compound from the contaminated water and also in the removal of various type of dye from the textile wastewater effluent *(Dantz et al., 1998)*; *Buschmann et al., 1994)*. The method for sorption of cucurbituril is still not cleared, but Karcher et al. proposed that sorption based on hydrophobic interaction *(Karcher et al., 1999b)*. This cucurbituril is the potential candidate for the removal of reactive dye-containing wastewater effluent.

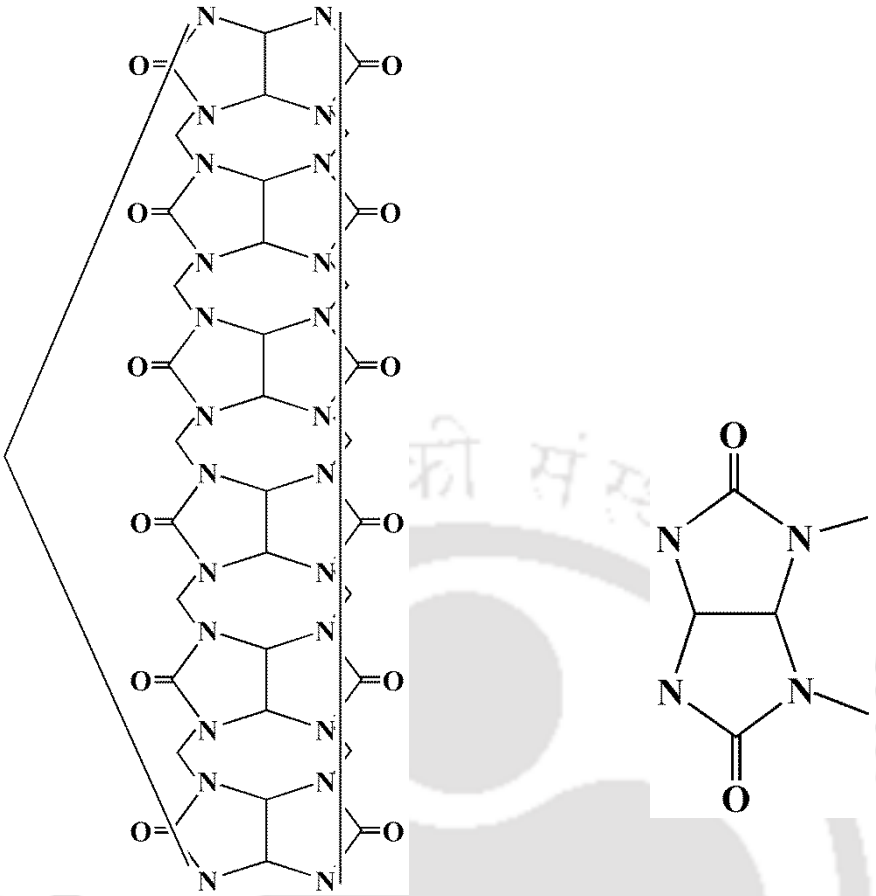


Figure 1.2: (A) Cucurbituril structure (B) Cucurbituril monomer structure

1.6.3 Biological methods

As we have discussed earlier that the azo group of the dye is the largest group among all. It comprises around 80% of all the dyes. These all high molecular weight azo dyes are continuously used in textile, food, medicines and others industries. These all dyes are converted into carcinogenic amines when released into the water bodies *(Chung et al., 1993).* So we have focused on the methods which completely remediate the dye pollutant. Bioremediation by the use of biological organisms is a key research area in environmental science and it attracts the attention of the scientific community. It is a very effective method and leads to the complete removal and decolourization of dye molecules from the waste generated from the industries. In the biological method, many organisms like yeast, fungi, bacteria, actinomycetes, algae and plant are involved *(Singh et al., 2015; Talarposhti et al., 2001).* Nowadays, the biological treatment methods are widely used because of their cost-effectiveness, ability to produce very less sludge and most importantly, due to the eco-friendly nature. In biological methods, an enzymatic method

is one of the important method for the degradation and decolourization of dye pollutant and it also help in the reduction of chemical oxygen demand (COD). The different available biological methods are described bellow in brief.

1.6.3.1 Decolourization by fungi

Fungi are such organisms which can grow almost everywhere and they can easily adapt the ecological niches of different organisms. From the last few years, fungi can be considered that it has the capability to degrade different types of dyes. Treatment of dye by using the fungi is economically cheaper than other biological methods. They rapidly adapt their metabolism rate at different carbon and nitrogen sources, which are important for their survival so that they can easily grow on the textile effluent containing dyes and reduces the dye pollutants. The fungal degradation and decolourization of dye have been described by many researchers *(McMullan et al., 2001; Hao et al., 2000; Fu et al., 2001).* There are much variety of fungi evolved in degradation and decolourization of dyes *(Fu et al., 2000).* From these all fungi, some of them involve degradation and some of them involve in decolourization. All these fungi can be categorized into two forms, living cells, which can degrade and biosorb dye and dead cells, which can absorb dyes but not able to degrade.

1.6.3.1.1 Mechanism for fungal biodegradation and biosorption

There are two mechanisms for biodegradation and biosorption, one is by using the leaving cell and another is by dead cells. In living cells, many enzymes have been reported which are manganese peroxidase (MnP), laccase and lignin peroxidase (LiP *(Raghukumar et al., 1996).* The function of all the enzyme is found to be different in different fungus-like lignin peroxidase (LiP) that are involves degradation of an azo dye, polymeric dye, heterocyclic and triphenylmethane dye in *P. Chrysosporium* (*Young et al.,1997*). The manganese peroxidase (MnP) enzyme released from many fungi and it plays a major role in the decolourization of effluent from cotton bleaching. Same way laccase enzyme is the extracellular enzyme from different fungus and playing in the decolourization of azo, indigo and anthraquinone dye. Apart from the degradation, biosorption also plays an important role in the decolourization of dye effluent by living fungi cells. Fungi (*Myrothecium verrucaria)* have the capability to remove 50% of the dissolved dye in 10 h so that by using *Myrothecium verrucaria* we can easily remove most of the dye in a very short period of time *(Knapp et al., 1995).* These biosorption

methods of dye removal by using living fungi are varied from 10% to 60% depending on species to species. In the dead cell, the mechanism of biosorption depends on the physicochemical interaction between the cells and dye molecules. These physicochemical interactions are adsorption, ion- exchange and deposition *(Zhou et al., 1991)*. Earlier the binding is not very clear between the dye molecules and cell wall in adsorption. But *Brahimi - Horn et al.*, reported that the dye was internalized into the cells, which is confirmed by sonicating the fungus cells after the adsorption of dyes *(Brahimi-Horn et al., 1992).*

Many researchers have compared the living cells and dead cells and they concluded that the dead cell are able to remove around 80% of dissolved dye and living removes 60% of dyes. With great removal capacity of dye, they also have serious disadvantage like the removal of dye by using the fungi depends on various factors (pH, temperature and concentration of effluent) and also have to maintain the fungal nutritional requirement for their growth *(Gadd et al., 1990)*.

1.6.3.2 Decolourization by white-rot fungi

White rot fungi are widely known for the degradation of many polymers like lignin, which is the structural polymer of plants *(Barr et al., 1994)*. White rot fungi have been mostly used for the degradation of xenobiotics, dioxins, chloro-organics and polychlorinated biphenyls (PCBs). The *P. chrysosporium* fungi have the ability to degrade the textile effluent upto 90% in 7 days. Lignin degrading white-rot fungi attracted scientific attention, as this organism was able to degrade a wide range of organic compounds. Lignin modifying enzyme is lignin peroxidase (LiP), manganese peroxidase (MnP) and laccases are directly and indirectly involved in the degradation of lignin, lignocelluloses compounds, xenobiotic compounds and various dyes *(Boer et al., 2004)*. Azo dyes are degraded by the use of white-rot fungi in different ways like adsorption and enzymatic process. The action of fungi is different for the different types of dyes. It means that the single fungi species are not able to degrade all dyes. We have seen the white-rot fungi are able to degrade the dyes in liquid fermentation and also able to produce the enzyme for dye decolourization. But fungi work more efficiently in their natural environment.

1.6.3.3 Decolorization of azo dyes by algae

Since the textile dyes effluent released into the environment, it seriously affect the aquatic life. In water body, photosynthetic organisms like cyanobacteria, algae and many others are widely distributed. Some of the azo dye is highly toxic to the fishes and inhibits the algal growth. Due to the presence of algae in both freshwater and saltwater, it can be used for the degradation of dyes effluents. The mechanism for the degradation depends on the molecular structure and types of algal species involve in it. Algae can also be used for the removal of nitrogen and phosphorus from the wastewater *(Tarlan et al., 2002)*. Several algae like *Chlorella* and *Oscillatoria* are capable of decolourization of azo dyes into the aromatic amines and further metabolized to the simpler organic forms like CO_2 *(Acuner et al., 2004)*. Many reports show that algae can be used in the decolourization**,** biosorption and bioconversion and also in the bio coagulation process**.** Several reports related to the degradation of textiles effluent by algae shows that different algal species are able to decolourize the different dyes depending upon the nature of the dye and algal species which are being used.

1.6.3.4 Decolourisation of azo dyes by plants

Phyto-remediation is one of the most important emerging area and ecofriendly methods, which can be used for the remediation of soil, groundwater, surface and air pollutants *(Patil et al., 2009)*. There are many methods that are available for the degradation of dye effluents, in which plant-based enzyme or plant-associated microbes can be used. The existing contaminant present into the environment is uptake by plant root and detoxifies them inside of the plant through various processes like degradation and elimination. These plants are also effective for the elimination of phenolic compounds, metals, colourants, azo dye and various organic and inorganic pollutants *(Sureshvarr et al., 2010)*. On the basis of plant-based detoxification process, the phytoremediation is devide in various categories as rhizofilterationfication, phytovolatilization, phytotransformation, rhizofilteration and phytoextraction *(Sureshvarr et al., 2010; Ali et al., 2013)*. Due to the revitalization of polluted soils, water body and air revealed that the use of the plant is the sustainable and ecofriendly remediating agents and its get much attention of the scientific community to explore the degradation potential and presence of dye degrading enzyme in plant species. The studies have shown that the plants *Brassica juncea, Sorghum vulgare, Phaseolus mungo, Thymus vulgaris, Rosmarinus officinalis* and cocoyam plant are

involved in the removal, detoxification and absorption of various dyes *(Patil et al., 2009; Torbati et al., 2015).*

1.6.3.5 Sorption of dyestuff by plants

The process of dye removal by sorption mechanism is already well known and many plants are involved in the decolourization and biosorption. Many plant-like *Eichhornia crassipes* exhibits the 95% of removal capacity for the black b and RB blue dyes by sorption. *Brassica alba* (mustard) show the maximum sorption for azo dye and ethidium bromide (EtBr) whereas *Solanum lycopersicum* (tomato) and *Vetivera zizanioides* (Vetiver grass) shows the average sorption of dye. Eucalyptus plant showed the sorption of azo dye from the soil, paper and pulp waste. These plants absorb dye molecules into their different body parts like leaf, root and stem, where they detoxify them within the plant cell and help in the reduction of COD, BOD, TDS content and nitrogenous compound from the waste *(Rizwana et al., 2014).* Hence we have seen that biosorption is very necessary for the accumulation, sequestration and subsequent metabolism of hazardous toxic dye by plants *(Muthunarayanan et al., 2011).*

1.6.3.6 The tolerance and stress avoidance mechanism with plant

In stress avoidance mechanism for dye removal by plants, the dye molecules are removed from the dye metal complex within the plant with the help of calcium and silicon. The precipitation of dye was mainly seen at plant root and leaf areas where the dyes break down into the smaller pieces. This smaller fragment gets easily absorbed by the plant through its semi-permeable membrane. The plant releases this compound into the surrounding to maintain gaseous composition through biochemical reaction and soil pH, where the toxic dye gets converted into the nontoxic molecules *(Nilratnisakorn et al., 2008).* Plant utilizes these degraded dye molecules for their growth, which results in decolourization of dyes molecules take place. In relation to the dye decolourization, a various enzymatic process are known in which the increase of glutathione activity lead to the conjugation of dye molecules, which protect the plant against the various chemical stress (*Carias et al., 2008).*

1.6.3.7 Degradation by the involvement of Plant-microbes

Many plants have the capacity to degrade the dye contaminant by forming a symbiotic association with the microbe. The fibrous root favours and gives support to provide shelter for pollutant degrading microbe because, after degradation, both plant and microbe can get the nutrient supply. Such type of dye metabolism using the plant-microbe interaction was studies in *P. Grandiflora (Glick, 2010; Khandare et al., 2013).*

1.6.3.8 Bioremoval of dyestuff by using enzymes from plant

As we already know, the plant contains multiple enzymes but the plant can able to degrade and decolourize the textile effluent containing dyes. To check the ability of plant for decolourization, the enzyme from the plant must be exposed to the dye contaminant. It is reported that the plant containing enzyme DCIP (2, 6-dichlorophenol-indophenol) reductase and tyrosinase, lignin peroxidase, riboflavin reductase and laccase, etc. These enzymes play important role in the degradation and decolourization of textile effluent containing dyes *(Khandare et al., 2011b)*. To study the plant based enzyme for dye decolourization, many cell-cultured plant and intact plant have been investigated. From these studies, cell cultured plant and wild type plant of *Portulaca grandiflora* is found to be more efficient and effective for azo dye degradation. When the plant was exposed to dye, lignin peroxidase and DCIP (2,6-dichlorophenol-indophenol) reductase and tyrosinase seem to be more efficient and more effective *(Khandare et al., 2011b).* The direct red, navy blue and sulfonated azo dye are more susceptible to the enzyme degradation and easily metabolized by the plant.

Among all the plants wild type plant found to be more efficient for the degradation of brilliant blue dye and remazol red (sulfonated azo dye). Enzymes laccases from *T. flagelliforme* and veratryl alcohol oxidase from *Aster amellus*, laccase, azoreductase, tyrosinase and others enzyme also play crucial role in the metabolism of their respective dye molecules *(Khandare et al., 2011a).* These enzymes from a plant not only help in decolorization and degradation, but also significantly reduce the BOD and COD from the effluents. Apart from these above enzymes peroxidase and polyphenol oxidase enzyme from banana also play a significant role in the metabolization of direct yellow, acid orange 7, aromatic amine, acid blue 92 dyes and basic blue 46 dyes in 120 h and direct blu in 90 h and direct red 5B in 48 h respectively. These all studies have confirmed that enzymes from the plant have crucial role in the degradation and decolourization of dye

molecules but the activity of enzymes also affected by the different environmental parameter (Concentration of dye, pH, Temperature and time).

Many plant roots also provide the degradation and transformation of dyesstuff by intracellular enzymes like lignin peroxidase, NADH reductase, laccase and tyrosinase *(Patil et al., 2009)*. After analyzing all the studies on plant enzyme, the hairy roots of the plants contain more enzymes for decolourization. These all enzyme play a significant role in the degradation, but if the enzyme is immobilized on a solid matrix, then it removed 78% more dye than non-immobilized enzyme *(Maddhinni et al., 2006).* The enzyme-based detoxification mechanism for metabolic pathway is well studies into the plant root hairs. All these studies suggested that plant root hair system can be considered a good source of enzymatic degradation and detoxification of dye effluents.

All these studies are very helpful in understanding the mechanism about decolourization and degradation of synthetic dye. But again we need to focus on the gene which is responsible for the metabolism of dye within the plant body so that we can use this enzyme in the remediation process in an eco-friendly manner. Apart from dye metabolism, the role of the enzyme is also important for us to elucidate the functional aspect and detoxification of colourant *(Khataee et al., 2013).*

1.6.3.9 Decolourisation of azo dyes by bacteria

Remediation is the process for the degradation of the complex substance into the simpler molecules. When complex compound degraded with the help of biological agents then its called as the bioremediation, biotransformation or biodegradation. The bioremediation involves the use of biological agents (microorganism) for the complete degradation or removal of contaminant from the environment, whereas the biodegradation and biotransformation involve some other chemical modification by living organism. Treatment of complex contaminant by using microorganism is getting much importance due to the capability of microorganisms to survive in such hostile environmental condition for more than thousands year. Microorganism plays a role in the restoration and reclamation of the contaminated environment through various process and has a role in the mineralization of xenobiotic compounds *(Singh et al., 2011).* The microbial degradation and decolourization are considered as the feasible environmental process because this process is relatively inexpensive, very low running cost is required and complete detoxification and mineralization of color-rich effluents. Numerous researcher

has worked on the microbiological process and concluded that wide verity of microorganism is there to decolorizes and degrade the dye effluent *(Singh et al., 2011; Tahir et al., 2016).* For degradation and decolorization of azo dyes using bacterial and their products are very common. This process of decolourization is more efficient, inexpensive and has maximum dye removal capacity from the textile effluent containing dyes. The microorganism can be used in various ways in the process of dye removal.

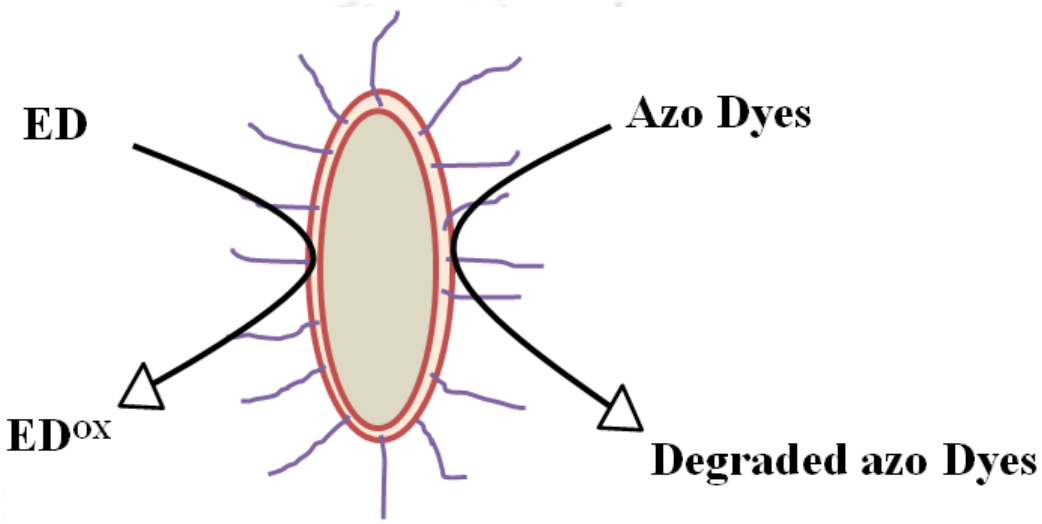


Figure 1.3: Showing the bacterial degradation of azo dyes and mediation of ED = Electron donor.

1.6.3.10 Use of microorganism in biosorption mechanism

Biosorption is the mechanism of uptake or accumulation of another substrate/chemical by microbial biomass *(Kumar et al., 1998).* In the process of biosorption, the dead cells of bacteria have been used for the absorption of dye effluent. The composition of textile effluent containing dyes is a mixture of the chemicals. The absorption of such dye effluent depends on the composition of bacterial cells. The rate of adsorption of dyes was different for various bacterial species. The adsorption was found to be much better if the mixed culture of bacterial cells were used. The single strain of the bacterial culture also has been used for absorption, but it is not enough to absorb most of the dyestuff *(Polman et al., 1996).* The microbial biomass is taking advantageous over other methods for the removal of very toxic effluents. The adsorption was found to be more quickly, maybe because of an increase of bacteria surface. The surface of bacteria was increased due to raptured bacterial cells*(Polman et al., 1996).*

1.6.3.11 Decolorization of azo dye in anaerobic conditions

The degradation of azo dyes under the anaerobic (methanogenic) condition ware studies and a wide range of bacteria involved in the degradation process. For the growth of bacteria in methanogenic condition various substrate is required and these substrates are starch, ethanol, acetate and glucose whereas for decolourization of synthetic blue wastewater containing dye effluent whey and tapioca are required *(Van der Zee et al., 2005; Chinwetkitvanich et al., 2000)*. Apart from methanogenic bacteria, acidogenic bacteria also plays a role in decolourization of dye effluents. In the decolourization of azo dyes, the reduction mechanism is followed with various time for decolourization and settling down the dye effluents. The reactive azo dye (Reactive red 411) was reduced into the aminonaphtalene- 1,5 disulfonic acid by microbial cells in anaerobic condition*(Carliell et al., 1995).* The anaerobic reduction process is the nonspecific process and various group of the azo compounds is decolourizes. The decolourization process depends on the various carbons sources we have used for the growth of culture *(Stolz., 2001).*

1.6.3.12 Decolorization of azo dye in aerobic conditions

There are several bacterial strains available in an environment which can degrade the azo dye effluents in aerobic conditions. In this condition, the bacteria requires organic carbon source as a growth substrate instead of using the dye as a growth substrate *(Stolz., 2001).* There are very fewer bacteria present into the environment which are able to use the azo dye as a carbon source. They reduced the azo bond and utilized the reductive amines as a source of carbon and energy for the growth. Some of the organisms like *Pigmentiphaga kullae* K24 and *Xenophilus azovorans* KF 46 are able to grow aerobically on the carboxy-orange II and carboxy-orange I, respectively *(Zimmermann et al., 1982).* However, *Sphingomonas sp.* reported as obligate aerobic bacteria which can utilize azo dye (AO7) as carbon, energy and nitrogen source for their growth. They utilize the reduced amines component (1-amino 2- naphthol), which formed after the degradation of AO7 azo dyes *(Coughlin et al., 1999)*. Several bacterial strains are isolated recently, which utilizes the methyl red as carbon source such bacteria are *Vibrio logei* and *P. nitroreductase*. In such condition, the trace of reduced amine is also found in the culture medium, which indicated that the aerobic growth of these bacteria is very helpful for the complete degradation of dye effluent from textiles wastewater *(Adedayo et al., 2004).*

1.6.3.13 Enzymatic degradation of azo dye

The degradation of dye effluent by using the enzymes has been extensively investigated. Use of enzyme shows considerable benefits over the other methods used for decolourization and degradation. The enzymatic process for degradation can easily standardize and provides accurate dosage. The process for the use of enzyme is very simple and easily modified according to the nature of dyes. The exact knowledge of dye degradation by enzymatic methods is very helpful in environmental protection. There are so many enzymes which can be used for dye degradation such as azoreductase, laccase, and peroxidase. This enzyme can be expressed either extracellular or intracellular depending upon various microorganism. Fungi are a very good source for enzyme production, but the bacterial production of enzymes has many advantages over others source because of the growth of bacteria is very fast, the medium used for the growth was very cheaper, low maintenance and downstream processing was also very easy and fast.

The primary step for the degradation mechanism of dyes by using enzymes is the reduction of azo bond (-N=N-). In this mechanism redox mediators are involved and the reaction is localized either in intracellular or extracellular. In this process of enzymatic degradation, mainly two enzyme family are involved, such enzyme families are azoreductase and laccases. Apart from this, various other enzymes are also involved in the degradation of azo dye such as lignin peroxidase (LiP), polyphenol oxidase (PPO) and manganese peroxidise (MnP)

1.6.3.13.1 Azo dye degradation with laccase enzyme

Laccase (EC 1.10.3.2) is commonly called as multicopper oxidase (MCO) because it belongs to the multicopper oxidase family. The most important feature of the laccase is the metal-containing oxidase. It was first isolated from the *Rhus vernifera*, a lacquer tree in Japan *(Giardina et al., 2010).* It has a significant role in the decolourization of textile wastewater containing dyes. The laccase enzyme performs the oxidation-reduction reaction with the absence of coenzyme. In the laccase reaction mechanism, the freely available oxygen was not utilized as an electron acceptor and also performs the non-specific oxidation reaction. Due to the distinct feature of laccase, it is mostly preferred in the bioremediation process *(Kalyani et al., 2012).* In laccase catalyzing reaction, low molecular weight compound act as a redox mediator in the electron transfer steps. The aromatic amines were oxidized by laccase with the mediation of Cu^{2+}. The enzyme was

mainly purified from the plant, fungal and bacterial sources. The specificity for the laccase enzyme was very low in non-specific free radical mechanism to form the phenolic compound instead of making the xenobiotics. In the presence of oxygen laccase also oxidize the aromatic compounds into phenol and aniline. In the oxidation of phenolic rings by the laccase, the carbonium ion formed on azo linkage after oxidation of phenoxy radical. It produces the 4-sulfophenyldiazene after nucleophilic attack by water and the dyes were degraded. Use of laccase has some disadvantage that decreases in the activity of laccase at a higher temperature and in alkaline condition. This is the reason the laccase can't be preferred by the majority of the industries.

1.6.3.13.2 Azo dye degradation and decolourization by peroxidase enzyme

The degradation of azo dye by using fungi is well known and investigated for a very long time. Fungi have a tremendous capacity to produce the enzyme which has an important role in the degradation of azo dyes. Such enzymes are phenyloxidase and peroxidase. Peroxidase (EC 1.11.1) is the enzyme which has a role in the degradation of dye effluent from industrial waste. It helps in catalyzing the dye degradation mechanism with the help of hydrogen peroxide. The peroxidase enzyme belongs to the ubiquitous enzyme family and produced by all organisms. This enzyme was further divided into the six others subfamily (1. Animal peroxidase,2 di-heme cytochrome-c peroxidases, 3- non animal peroxidase, 4. Haloperoxidase, 5. Catalase 6. DyP- type peroxidase) on the basis of their primary structure, their substrate and organism. The crystallographic study of peroxidase was done and the structure was elucidated. The crystallographic structure shows that it has three substrate binding sites namely the exposed tryptophan residue, c edges and the heme d *(Gumiero et al., 2010).* The textiles industries have a major concern about the degradation of wastewater containing the azo dye. In such case, the peroxidase enzyme has a very effective role in the oxidative degradation of orange G and sunset yellow with H_2O_2 *(Zhang et al., 2012).* Many enzymes like DCIP reductase, tyrosinase and lignin peroxidase from the plant origin also have a role in the degradation and detoxification of azo dye.

The mechanism of azo dye degradation using the lignin peroxidase is completed in two steps in which the oxidation of phenolic ring by H_2O_2 take place. The same way the mechanism for the manganese peroxidase involves the oxidative conversion of Mn^{2+} to Mn^{3+}, which involved in the chelation of organic acids. The Mn^{3+} found near to the active

site of enzyme oxidizes other substrates *(Mester et al., 1998).* This enzyme also shows the very high affinity to the manganese and dyes. The peroxidase enzyme was found to be very effective for the removal of phenolic compounds and aromatic amines from the textiles effluents. Based on the very versatile activity of peroxidase, many enzymes from the plant (*Phragmites australis , Typha angustifolia and Arundo donax)* are involved into the degradation of amido black, azo dye and amaranth dye *(Haddaji et al., 2015).*

1.6.3.13.3 Degradation of azo dye by azoreductase enzyme

The Azoreductase (EC 1.7.1.6) enzyme performs the reduction of azo dye into the colourless amine. In this process of reduction mechanism, many reducing agents involved and act as electron donor such reducing agents are FADH, NADH or NADPH *(Singh et al., 2015; Solis M et al., 2012).* Further azoreductase enzyme was divided into the two categories one is flavin-dependent azoreductase and other is flavin independent azoreductase *(Chen et al., 2004; Nakanishi et al., 2001; Blumel et al., 2002).* Based on different coenzyme the enzyme was further categorised as FAD-dependent azoreductase, NADH dependent azoreductase and NADPH dependent azoreductase. These coenzymes help in the reduction of azo bond (-N=N-) into their corresponding amines *(Nakanishi et al., 2000; Wang et al., 2007; Chen et al., 2006).* Many azo dyes are effectively degraded by azoreductase enzyme so it is important to explore the microorganism which can produce the azoreductase. The azoreductase enzyme in bacteria is cytoplasmic and membrane-bound. The mechanism of dye degradation for membrane-bound azoreductase is different from the cytoplasmic azoreductase. Membrane-bound azoreductase uses the redox mediator as an electron shuttle. The non-sulfonated azo dye is entered in the cell and mainly degraded by soluble cytoplasmic azoreductase *(Singh et al., 2015).* In some unfavourable environmental condition, other enzymes also behave as azoreductase and degrade azo dyes and they utilize the degraded azo dye as carbon and energy sources for the growth.

Azo dye degradation by the azoreductase enzymes from the obligate aerobic bacteria (*Pseudomonas* K22 and *Pseudomonas* KF46) was first isolated and characterized by the Zimmermann and his group *(Zimmermann et al., 1982).* These bacteria are mainly carboxy-orange degrading bacteria. The aerobic intracellular azoreductase was found to be very high specific for the carboxylated and sulfonated dye structural analogues. Azoreductase isolated from the two different strains are structurally different but

performs the same reduction. Many bacterial strains could not utilize the sulfonated azo dye for their carbon source and unable to degrade the sulfonated dye, but enzymes could do the same things. May azoreductase enzymes are fully characterized and very well able to degrade the series of reactive dyes from the *Bacillus sp. (Suzuki et al., 2001).*

The mechanism for the degradation of dye effluent with azoreductase enzyme are given bellow-

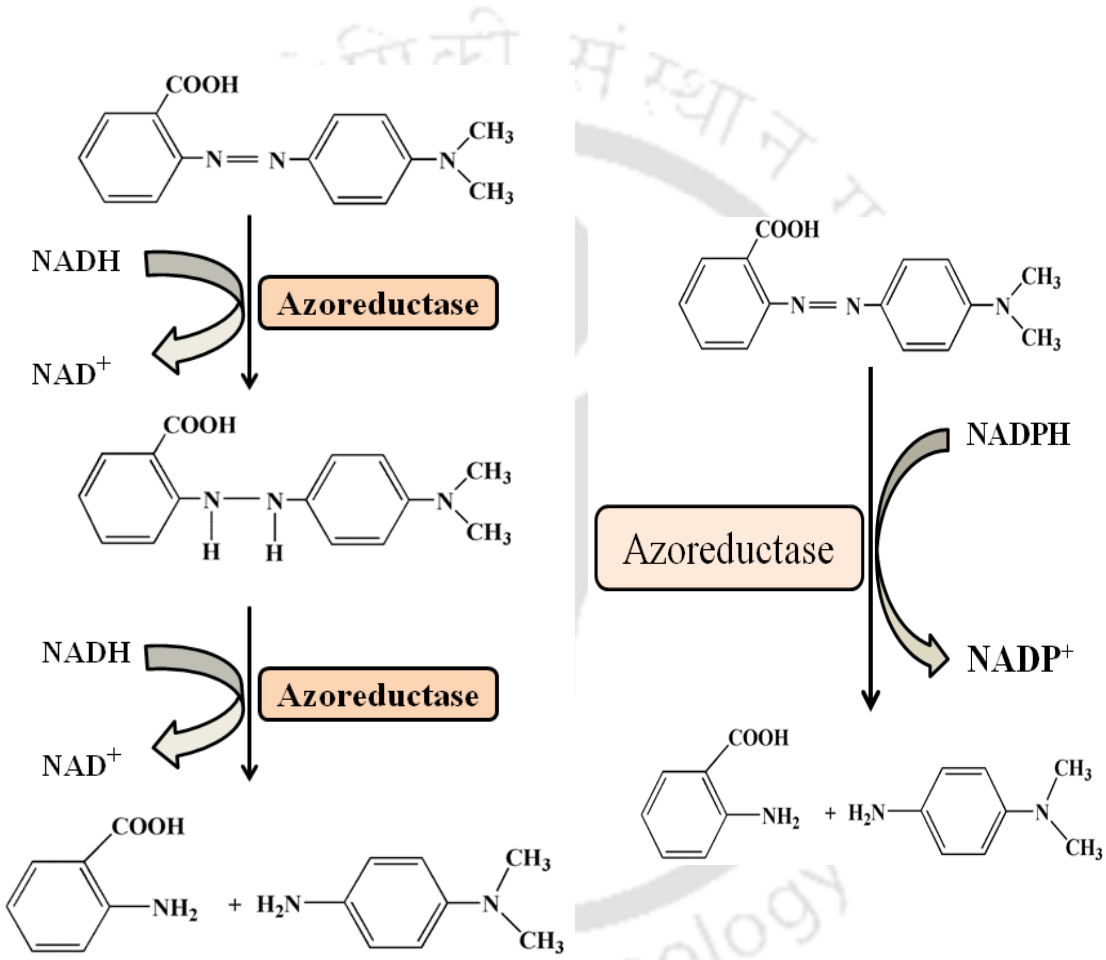


Figure 1.4: Pathway for degradation of azo dye (A) Methyl red converted into the dimethyl *p*-phenylenediamine and *o*-aminobenzoic acid by azoreductase **(B)** Reduction of methyl red by azoreductase enzyme with the help of NAD (P)H.

1.6.3.14 Degradation of dye by various immobilization methods

In recent years, many environmental engineers are trying to solve the problem of textile effluent. For the remediation of textile effluent, they are struggling to develop the new technology or to make a change into the existing technology *(Sathish kumar et al., 2014, dos Santos et al., 2007).* But none of the technology has been implemented in large scale to solve the problem, this is because of the many reasons like the toxicity of by-product, extreme operation condition, labour-intensive methods and also due to the high cost *(Hayat et al., 2015).* In order to develop new technology, additional effort can be required, which can solve the dye effluent problems from the environment. In order to solve the problem of dye effluent, enzymatic methods are found to be more effective. Due to the wider range of pH, temperature, different contaminant and saline range, the enzymatic methods attracted more and more people to solve the problem of dye effluent from the textiles waste *(Rao et al., 2014).* Due to the strong oxidative capabilities of enzymes and low substrate specificity into the contaminant, the enzymatic exploitability has seen into the environment. Due to the enzymatic exploitability, the enzymatic method is not preferred to be use for degradation of contaminants. To overcome the enzymatic exploitability into the environment, another method comes into existence such as enzymatic immobilization. Enzyme immobilization process has great applicability in the degradation of effluent. It offers a very effective approach to avoid the enzyme exploitability and have great stability against the salinity, denaturants, organic solvent and heavy metals *(Gardossi et al., 2010).* In the immobilization process, enzymes can be reused for several times and no wastage of enzymes was found.

Immobilization is the process of coupling of the solid substrate with biocatalyst (enzyme). There are many processes for immobilizing the enzyme on the solid matrix. Immobilized enzymes are very easy to use in a reaction. It provides a stable catalyst to use practically and economically, very cheap. Immobilization enhances the thermal stability of the catalyst and very easily degrades the effluent in the harsh condition of denaturation and aggregation *(Bilal M. et al., 2015).* In bellow fig. 1.5 process of different types of immobilization are explained.

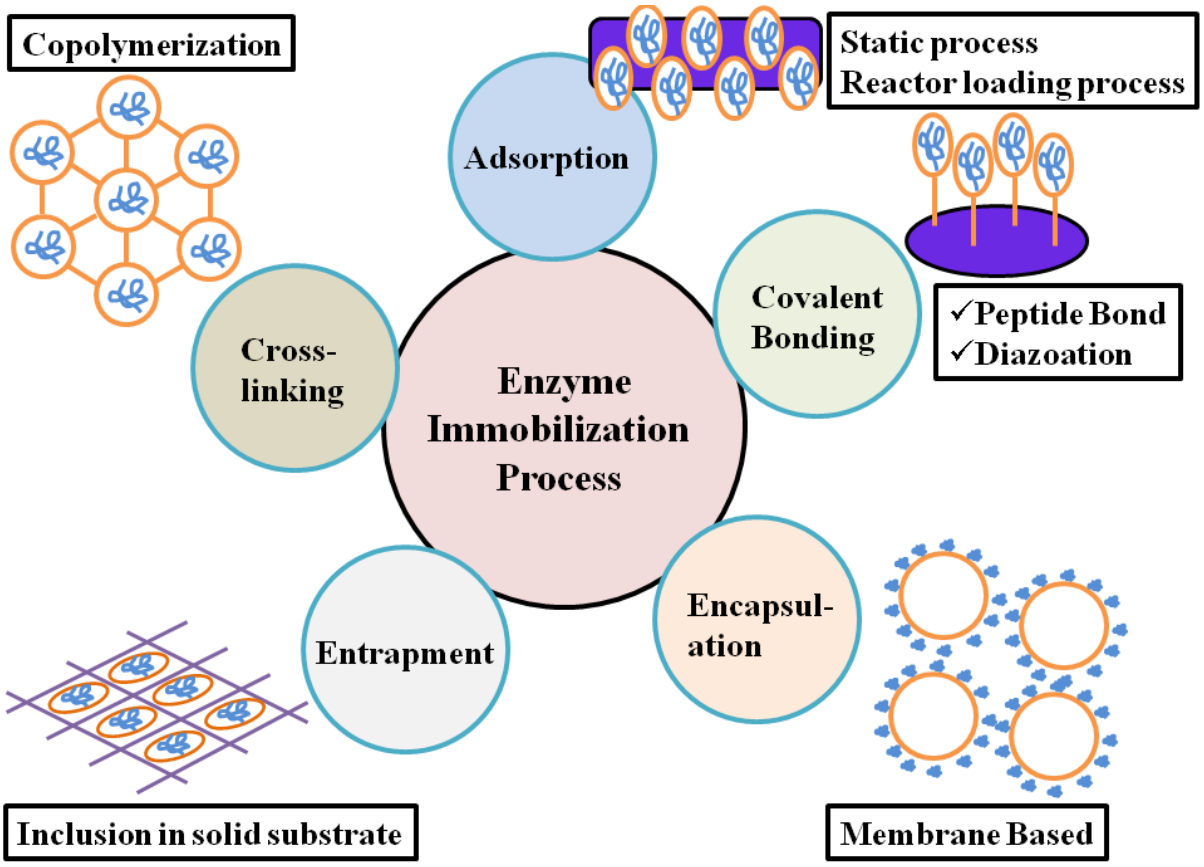


Figure 1.5: Representation of different types of available method for immobilization of the enzyme on various solid substrates (*Bilal et al., 2017).*

The use of synthetic dyes seems to be more common because of their long-lasting nature, resistant to degradation and also economically very cheap. They behaves like xenobiotic after releasing into the environment. So we need some more efficient technology for dye degradation *(Lucas et al., 2008).* To mitigate dyes pollution, mainly physical, chemical and biological methods are used. In physical methods, mostly adsorption, coagulation and biosorption are involved. But these methods produce a large quantity of sludge. Chemical methods are usually discouraged as they are quite expensive and a lot of chemicals are used in the process. All these methods have an advantage and disadvantage like they are expensive and produce a large amount of sludge after the treatment of dye effluent. Extensively from these methods, membrane filtration and adsorption generate a large amount of secondary waste, which needs further treatment before disposal. Flocculation or coagulation methods also generate a large amount of

sludge, which requires safe disposal. For the dye degradation and decolourization, many works have been done on physico-chemical based methods, But now biological methods seems to be the most widely used around the worlds. Nowadays most of the scientific community focused on it dues the cost-effective nature, effective degradation process, eco-friendly and also due to the very less sludge generation *(Song et al., 2003; Chen, 2006).* The bioremediation potential of microbes and enzyme produced from them is becoming an effective method for dye degradation and act as an alternative to the available conventional physiochemical based methods. In biological methods, enzymatic method is found to be the most effective for dye degradation. In such technology, immobilizing microbial enzymes can be a promising cost-effective field, as in this process repeated use of immobilized enzymes is possible. However, the enzymatic method is still an expensive one because of the high cost involved in isolation, purification and production of enzymes. Therefore, a holistic study in the enzymatic remediation of dyes is promising as it may bring up some efficient and cost-effective outcomes.

1.7 Motivation of the research and scope of the work

As day by day, increasing clean water crisis brings us to rethink about the issue. As a researcher, we need to make afforts to solve the problem of clean water. The increasing clean water crisis does not mean that water is not available; it means most parts the world water is wasted and polluted by many wastes. There is unavailability of proper cost effective method for water treatment for azo dye pollution. The textiles industries and paper industries are the prominent industries which can utilize maximum water and discharge into the river or another water body without proper treatment. This discharged water body can't be utilized directly for drinking purpose or in agricultural fields because they contains many toxic dyes and xenobiotic compounds. If we can degrade dye and other xenobiotics in this discharged water, we can utilize the water for drinking purpose and also for agricultural fields. The work reports a potential method for the treatment of discharged water containing dyes using immobilized enzyme.

CHAPTER 2

Biochemical characterization of a stable azoreductase enzyme from *Chromobacterium violaceum*: Application in industrial effluent dye degradation*

ABSTRACT

The presence of dyes, including azo functional group (-N=N-) containing dyes, in industrial wastewater is one of the major causes of water pollution. This report showcases the functional role of azoreductase from *Chromobacterium violaceum* (MTCC No: 2656) as a valuable enzyme for the degradation of azo dyes. The enzyme was cloned, expressed, purified and biochemically characterized and further tested for degradation efficiency of azo group containing dyes like methyl red, amaranth and methyl orange. The degraded azo dye products (metabolites) resulted by the action of azoreductase enzyme had reduced toxicity on fibroblast cell lines (L929) as compared to raw and intact dye. Further, good stability of the enzyme makes it more suitable for various applications related to the degradation and decolourisation of effluent dyes.

*Part of the work has been published in International Journal of Biological Macromolecules, 2019,121, 1011–1018.

2.1 Introduction

Azo dyes are characterized by the presence of one or more azo groups (-N=N-) in their chemical structure. Dyes currently in use can be classified as either natural or synthetic dyes. Most synthetic dyes contain azo group in their structure. Recently higher commercial productions of azo dyes have been observed owing to their wide applications in textile, pharmaceuticals, food and cosmetics sector *(Chung et al., 1992; Stolz., 2001; Chang et al., 2001)*. These dyes are relatively persistent in the environment because they are extremely stable even after being exposed to light and aerobic conditions. These dyes are highly toxic and their metabolic products have been shown to be of carcinogenic and mutagenic nature *(Banat et al., 2001; Dong et al., 2003; Pinheiro et al., 2004).* Several synthetic azo dyes have been used as tattoo colourants in which the derivative of 3, 3 dichlorobenzenes, derivative of 2 amino 4 nitro-toluene, phthalocyanine is mostly used *(Lehmann et al., 1998; Baumler et al., 2000).* The presence of toxic and carcinogenic components in dyes and their subsequent products prompted setting up of regulations regarding the release of industrial effluents only after proper treatment. The treatment processes of industrial wastewater containing dyes usually use biological methods of treatment and remediation by using microorganisms and plant extracts *(Mohan et al., 2005; Mohan et al., 2002).* Azo dye degradation by microorganisms is mostly observed in aerobic conditions *(Chung et al., 1992; Stolz., 2001);* however chances of degradation in anaerobic conditions cannot be ruled out. Different bacteria isolated from soil have shown tremendous capability in the degradation of dyes in aerobic conditions *(Stolz., 2001; Suzuki et al., 2001).* Microorganisms degrade azo dyes by reducing azo bonds present in all dyes and leads to the formation of amines (*Stolz., 2001; McMullan et al., 2001).* Azoreductase enzyme of bacteria has the catalyzing ability for the reductive cleavage of azo bond with the help of NADH/NADPH. These cofactors are considered as an electron donor *in vitro* conditions *(Ghosh et al., 1992).* Azoreductase enzymes are the key enzymes produced from the azo dye degrading bacteria. Recent years the characterization of few proteins displaying azoreductase activity. Several bacteria known to produce azoreductase include *Staphylococcus aureus, Escherichia coli, Enterococcus faecalis Bacillus spp*. and *Xenophilus azovorans* KF46F *(Suzuki et al., 2001; Chen et al., 2004; Liu et al., 2007; Blumel et al., 2002).* However, use of bacteria in industry involves various factors like cost-effectiveness and enzymatic efficiency. It is also noteworthy that the involvement of bacteria needs their rearing for a long period of time. In contrast, use

of enzymes extracted from bacteria can remove this laborious step of bacterial rearing and can also be developed as a cost-effective process.

Chromobacterium violaceum group of bacteria are usually found in the soil and water of tropical and sub-tropical regions across the globe *(Ribeiro de Vasconcelos et al., 2003). C. violaceum* azoreductase is quite dissimilar from its bacterial homologs in terms of nucleotide sequence similarity. *C. violaceum* was procured from Microbial type culture collection (MTCC) India. It has high potential in the degradation of various toxic azo dyes in aerobic conditions. The amount of native azoreductase enzyme produced by bacteria is very less, which makes the purification process very tedious. In the current study, we report the biochemical characterization of recombinant azoreductase enzyme from *C. violaceum* and also demonstrate the efficient dye degrading capability of *C. violaceum* azoreductase leading to the generation of degraded products with significantly reduced toxicity. The enzyme, in its immobilized form, can be used for the development of process for dye degradation in a bigger scale.

2.2 Materials and methods

2.2.1 Organism growth condition

Azo dye degrading bacteria *C. violaceum* (MTCC 2656) was commercially purchased from Microbial Type Culture Collection (MTCC) India. The *C. violaceum* was grown in media containing Beef extract, yeast extract, peptone and NaCl or in the nutrient agar plate. Cells ware grown at 37°C for overnight in a shaking incubator at the speed of 180 rpm *(Demoss et al., 1959).* All the chemicals used during the experiment was purchased from Sigma (USA) and HiMedia (Mumbai, India) in analytical grade.

2.2.2 Cloning of CV_RS09840 in pET28a(+) expression vector

Genomic DNA was isolated from *C. violaceum* using nucleotide sequence of azoreductase from *C. violaceum* (CV_RS09840) with accession number NC_005085.1. Azoreductase gene-specific primers were used for the amplification of full-length azoreductase gene. Forward primer CV Azo F: 5′-CGGGATCC**ATG**AAACTGCTCCACCTCG-3′ (underlined sequence is restriction site for *Bam*HI and highlighted sequences are for start codon) and reverse primer CV AzoR: 5′-CCGTCGAC**TTA**CAGCGCCAACTTGGC-3′ (underlined sequence is restriction site for *Sal*I and highlighted sequences for stop codon) specific for CV_RS09840 gene (NCBI

reference number: NC_005085.1). The *Bam*HI (NEB) and *Sal*I (NEB) restriction site were specifically incorporated in the primers. PCR reaction was performed in 40 µl reaction volume containing 20 µl of Dream Taq green PCR master mix (Fermentas), 0.1 µg of each primer, 0.1 µg of *C. violaceum* (CV_RS09840) genomic DNA which was mixed and final volume was adjusted by adding nuclease-free water. In PCR reaction, the conditions was optimized with an initial denaturation for 3 min at 95°C; 30 cycle of denaturation for 15 s at 95°C; annealing 30 s at 62°C; elongation 40 s at 72°C and final extension for 10 min at 72°C. The PCR reaction amplifies the 609 bp of amplicon shown on a 1% agarose gel stained with EtBr. The PCR product and pET28a (+) (Novagen) was restriction digested with *Bam*HI and *Sal*I, and ligated to the pET28a (+) vector by T4 DNA ligase. The recombinant pET28a (+) were transformed in *E. coli* DH5α cells. All the cells were screened for the presence of CV_RS09840. The initial confirmation was done by PCR amplification of azoreductase gene by using gene-specific primers. The clone was further confirmed by restriction digestion with *Bam*HI and *Sal*I showing the release of 609 bp and finally the presence of azoreductase (CV_RS09840) was verified by sequencing using T7 forward and reverse primer.

2.2.3 Expression and purification of recombinant azoreductase

Recombinant pET28a-CV_RS09840 (6x Histidine tag at the N terminal) was isolated from transformed *E. coli* DH5α cells and further transformed into *E. coli* BL21 (DE3) cells for soluble expression. The transformed cells of *E. coli* BL21 (DE3) were grown in 5 ml of LB(Luria-Bertani) media as a primary culture at 37°C in a shaking incubator at 180 rpm with 50 µg/µl of kanamycin (HiMedia) to avoid unnecessary growth. Secondary culture of transformed *E. coli* BL21 (DE3) cells after reaching an optical density of around 0.4 at A600 was shifted to shaking incubator maintained at 20°C, 180 rpm for 8 h.

2.2.4 Protein purification

For protein purification, secondary cultured cells were grown for 8 h at16°C and this cultured cells ware centrifuged at 5000 rpm for 10 min and sonicated with 6 s on and 10 s off-cycle sonicator (Sonics) with lysis buffer (20 mM Tris-HCl, 250 mM NaCl pH 7.5 with 2.5% Glycerol). The sonicated sample was centrifuged at 12,000 rpm for 40 min at 4°C. The clarified supernatant was collected and loaded on the pre-equilibrated Ni-Nitrilotriacetic acid column (with 10 mM imidazole in 20 mM Tris-HCl and 250 mM NaCl pH 7.5) for purification and protein was eluted with elution buffer (250 mM

imidazole in 20 mM Tris-HCl and 250 mM NaCl). The purified protein was dialyzed against potassium phosphate buffer (25 mM KH_2PO_4 and 25 mM K_2HPO_4 pH 7.2) overnight for the removal of residual imidazole. The size of purified azoreductase enzyme was estimated by running SDS-PAGE (12%). The concentration of purified protein was estimated by bradford assay using the bovine serum albumin (BSA) as a standard *(Bradford., 1976).*

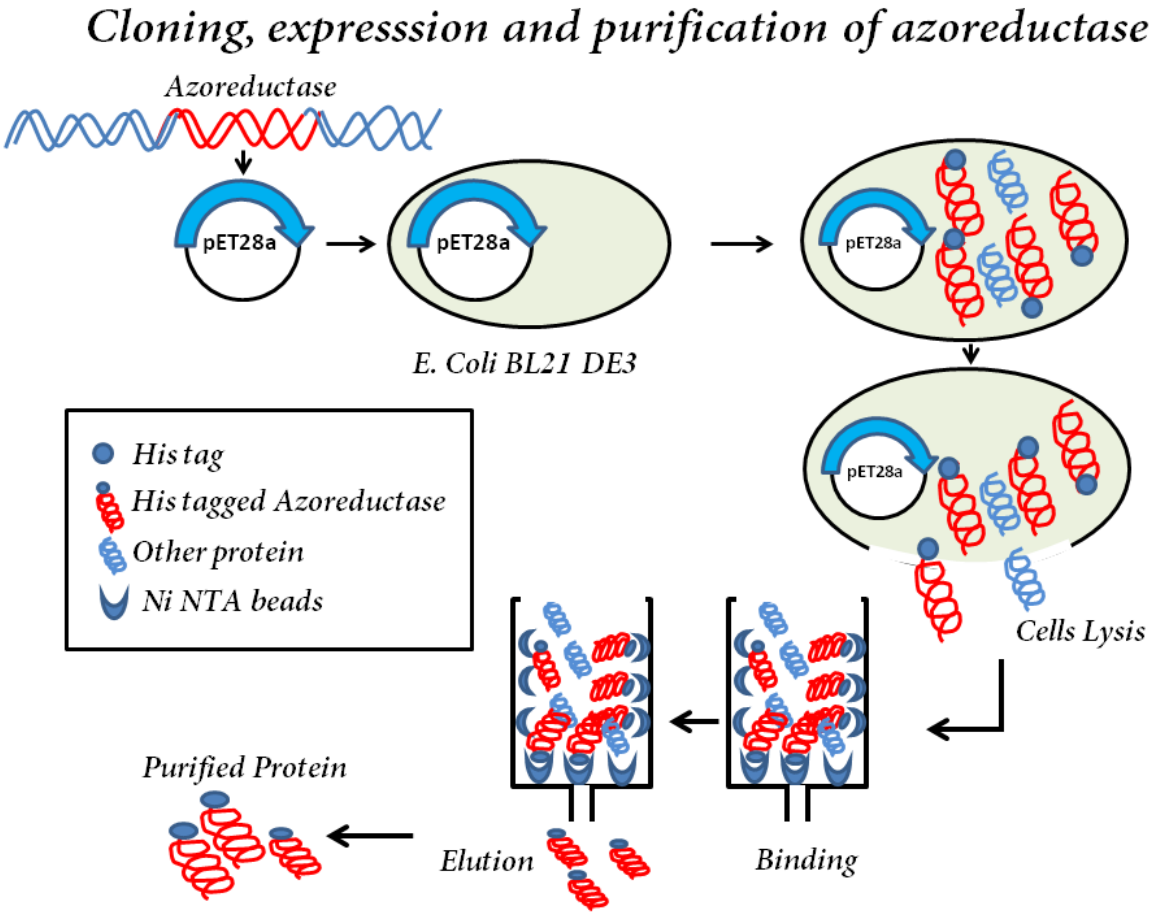


Figure 2.1: Schematic for cloning and expression of Azoreductase. Azoreductase gene was PCR amplified from the *Chromobacterium violeacum* genomic DNA and cloned in pET28a expression vector which was further transformed into *E. coli* BL 21(DE3) cells for the heterologous expression of recombinant His tagged dihydroorotase which was purified from the bacterial lysate using Ni-NTA metal affinity chromatography.

2.2.5. Kinetic characterization of enzymes

Enzyme activity of purified recombinant azoreductase was measured by decreasing the concentration of NADH using a earlier reported method *(Schoneck et al., 1997).* The azo bonds present in the dye are reduced when the reaction starts proceeding with the addition

of enzyme. The initial velocity of reaction was monitored by changing the amount of substrate in a glass cuvette of 1.0 cm path length and absorbance was taken at 340 nm. The optical density was measured in Agilent Technology Carry 100 UV–Vis spectrophotometer at room temperature. The standard reaction volume was 1 ml which contained 25 mM K_2HPO_4, 25 mM KH_2PO_4 pH 7.2 (HiMedia) varying concentration (1 nM–1000 nM) of methyl red, methyl orange or amaranth azo dye, 0.1 mM NADH, 10 µM FMN and 2 µg of enzyme. The reaction mixture was incubated in a water bath at 30°C for 1 min before taking the absorbance and the data obtained were fitted in Michaelis-Menten equation and double reciprocal plot through which K_m and V_{max} were estimated.

2.2.6 pH optimum

The pH optimum studies were carried out at a pH range of 4.0–10.0. The reaction mixture contains two buffers: 25 mM K_2HPO_4 (pH 4.0–11.0), 25 mM KH_2PO_4 (pH 4.0–11.0), 1 µM of an azo dye, 0.1 mM NADH, 10 µM FMN and 2 µg of enzyme. The reaction was initiated after the addition of enzyme followed by incubation in water bath at 30°C and absorbance was taken at 340 nm. The pH optimum was determined by plotting the relative activity at different pH values.

2.2.7 Temperature optimum

The temperature optimum studies were carried out at different temperatures ranging from 10°C to 60°C. The reaction mixture contains two buffers: 25 mM K_2PO_4 (pH 7.2), 25 mM KH_2PO_4 (pH 7.2), 1 µM of an azo dye, 0.1 mM NADH, 10 µM FMN and 2 µg of enzyme. The reaction was initiated after the addition of enzyme followed by incubation in water bath at different temperature range from 10°C to 60°C and absorbance was taken at 340 nm. Appropriate substrate blanks were included in all the reactions. The temperature optimum was determined by plotting the relative activity at different temperature values.

2.2.8 Prosthetic group identification of enzyme

The prosthetic group present within the enzyme flavin adenine dinucleotide (FAD) was confirmed by HPLC using earlier reported method after minor modification *(Chen et al., 2004; Lobenstein - Verbeek et al., 1984; Ooi et al., 2007).* High purity and analytical grade FAD purchased from Sigma Aldrich was used as a standard. In brief, 0.5 ml of recombinant azoreductase (0.4 $\mu g \mu l^{-1}$) present in 25 mM phosphate buffer (pH 7.2) was

taken and denatured in the water bath at 80°C for 10 min and centrifuged at 10,000 rpm for 10 min to remove the denatured protein. The supernatant was collected and used after filtering with 0.4 µm filter to measure the presence of FAD in Thermo Scientific HPLC ultimate 3000 system connected with UV–Visible detector and a reverse-phase C18 column (5 µm 120 Å 4.5 × 259 mm). The mobile phase was 25 mM phosphate buffer (pH 7.2) and acetonitrile (4:8, v/v) with a flow rate of 0.5 $mlmin^{-1}$. The presence of FAD was monitored at 260 nm. The standard FAD was also dissolved in 25 mM phosphate buffer and used as control.

2.2.9 Degradation kinetics study of dyes

The biodegradation product of methyl red, methyl orange and amaranth dyes was monitored by using the UV–Visible spectrophotometer (Agilent Technology Carry-100) and Fourier Transform Infrared Spectroscopy (Perkin Elmer Spectrum two) analysis. The reaction for UV–Visible spectroscopy contain 25 mM potassium phosphate buffer, 1 µM of azo dye, 0.1 mM NADH, 10 µM FMN and 2 µg of azoreductase enzyme. The reaction starts by adding the azoreductase enzyme in the sample and scanning the consumption of NADH used in this reaction at 340 nm. The biodegraded product was analysed after lyophilization (Labconco) in Fourier Transform Infrared Spectroscopy (Perkin Elmer Spectrum two) and compared with control dye. The FTIR analysis was done in a range of 400 to 4000 cm^{-1} with 20 scan speed.

2.2.10 Toxicity assay

The toxicity of methyl red, methyl orange and amaranth dye was checked on fibroblast cell line (L929), to find out the probable health impacts. In toxicity assay, the fibroblast cell line (L929) was procured from National Centre for Cell Sciences (NCCS) Pune India, and maintained in RPMI 1640 liquid media supplemented with 15% heat-inactivated fetal bovine serum (FBS). In the media, 100 U penicillin and 100 µg ml^{-1} streptomycin antibiotics was added to stop the unwanted growth. MTT [3 (4, 5 dimethylthiazol 2 yl) 2, 5 diphenyl tetrazolium bromide] assay was used to check the toxicity efficacy of the dyes. Healthy cells (5000) were seeded in 96 well plates and were allowed to adhere for 24 h at 37°C. After 24 h, the cells were treated with varying concentration (20 µg to 1000 µg) of dye and incubated again at 37°C for 24 h. After completion of 24 h, the media was removed from each well and 200 µl of MTT (0.5 mg ml^{-1}) was added in to them and again incubated for 4 h at 37°C. After completion of 4 h, all the MTT was discarded and

100 µl DMSO was added to each plate and mixed properly. Absorbance was taken at 570 nm in microplate reader by (BIOTEK Synergy HT) *(Tiwari et al., 2016).*

2.2.11 Stability of azoreductase with various denaturant

To analyse the capacity of the purified enzyme to retain its activity under denaturant conditions and organic solvents, 2 µg of enzyme was incubated with different concentration of urea, GdmCl (Guanidine hydrochloride) and SDS (Sodium Dodecyl Sulfate) for 12 h at room temperature and activity was measured in spectrophotometer. Stability of azoreductase was measured with respect to the percent activity of the enzyme. The reaction mixture contains amaranth dye, FAD, NADH and enzyme. The reaction was started by adding enzyme that was already incubated with different denaturant (urea, GdmCl and SDS) for 12 h.

2.3 Results

2.3.1 Cloning, expression and purification of C. violaceum azoreductase (CV_RS09840)

The open reading frame of CV_RS09840 gene is 609 bp. The azoreductase gene was amplified by using PCR amplification from the genomic DNA of *C. violaceus* shown in a 1% agarose gel stained with Etbr (fig. 2.2A). The amplified gene was cloned in the pET28a (+) plasmid. The pET28a CV_RS09840 construct was confirmed by double digestion, showing a release of 609 bp fragments on 1% agarose gel (fig. 2.2B). The clone was further confirmed by sequencing using T7 forward and T7 reverse primers. The pET28a-CV_RS09840 construct was transformed into the *E. coli* BL21 (DE3) cells for protein expression. The expression of protein was optimised at various temperatures and different concentrations of Isopropyl β D 1 thiogalactopyranoside (IPTG). Further, the protein was expressed in soluble fraction without induction with IPTG at 20°C temperature.

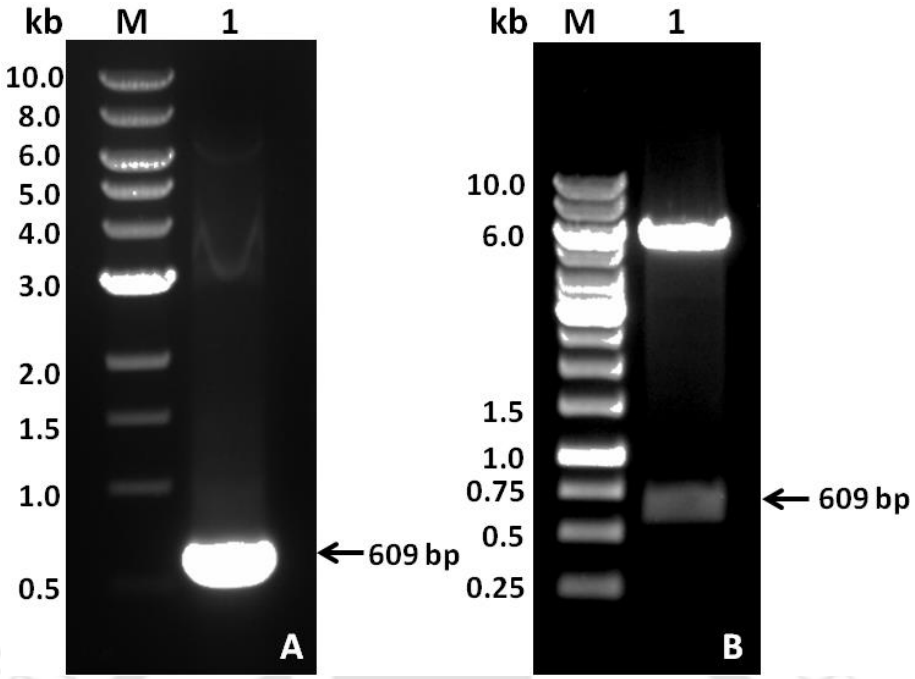


Figure 2.2: Cloning of azoreductase in pET-28a (+) (A) Lane M and Lane 1 shows the 10 kb ladder DNA marker and PCR amplification of azoreductase gene (609 bp) respectively (**B)** Lane M and shows the 1 kb ladder DNA marker and lane 1shows the fragmented release for clone confirmation by double digestion respectively . The 609bp of release is visible. Further the clone was also confirmed by sequencing.

2.3.1.1 Protein purification

The secondary cultured cells were centrifuged at 500 rpm for 10 min and sonicated with 6 s ON and 10 s off cycle sonicator (Sonics) using lysis buffer (20 mM Tris-HCl, 250 mM NaCl pH 7.5 with 2.5% Glycerol). The sonicated sample was centrifuged at 12,000 rpm for 40 min. The supernatant was directly used for binding to the pre-equilibrated Ni-NTA column for 2 h. After binding, the unbound fraction was collected and the column was washed with 50 ml of wash buffer (20 mM Tris-HCl, 250 mM NaCl and 25 mM imidazole). Protein was eluted with elution buffer (20 mM Tris-HCl, 250 mM NaCl and 250 mM imidazole) after washing. Further, the purified protein was dialyzed with 25 mM potassium phosphate buffer pH 7.5 for overnight. The purity of recombinant protein was checked on 12% SDS PAGE *(Bornhorst et al., 2000)*. His-tagged purified protein displayed a characteristic band of 24 kDa on SDS-PAGE (fig. 2.3A lane 4 and in fig. 2.3B).

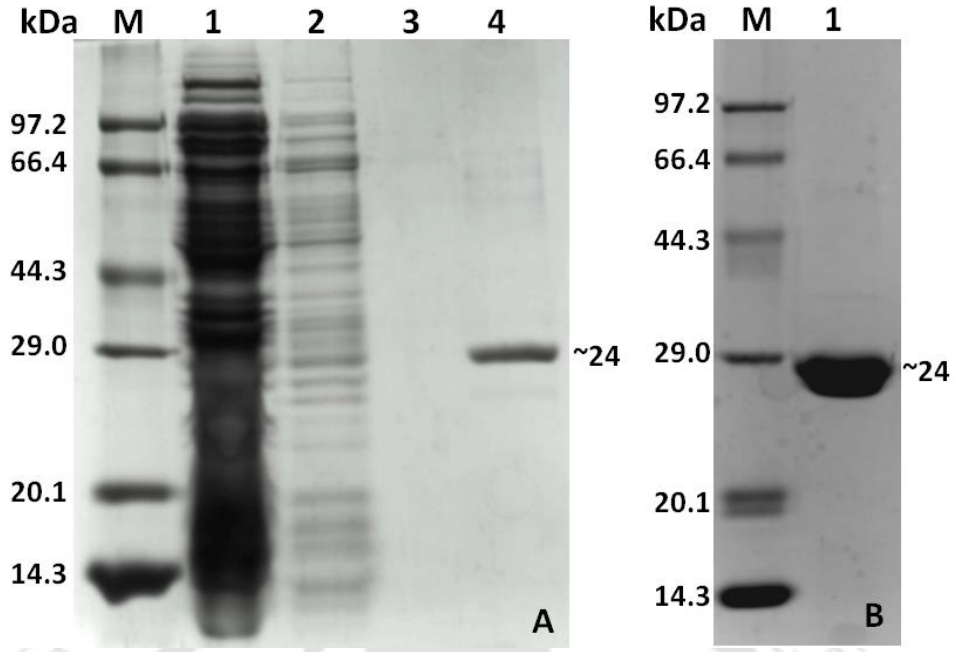


Figure 2.3: Expression of azoreductase enzyme from *E. coli* BL21(DE3) cells (A) Lane M showing low range (97.2 kDa to 14.3 kDa) marker with various cut off, Lane 1 shows the unbound fraction of protein, lane 2 shows the protein washed with 25 mM of imidazole, lane 3 shows the elution with 50 mM of imidazole and lane 4 shows the elution of protein with 250 mM concentration of imidazole, in which the purified azoreductase enzyme showing the 24 kDa molecular weight. (B) Purified azoreductase enzyme lane M showing low range marker and lane 1 showing the purified azoreductase enzyme with molecular weight 24 kDa.

2.3.2 Prosthetic group identification

The purified azoreductase enzyme with the prosthetic group is usually yellow in colour, which is non removable in dialysis or in the process of purification. The colour of the enzyme extracted and purified was also yellow, indicative of the presence of a flavin group with enzymes (protein binds to flavin). The colouration was found throughout the absorption spectrum at 262 nm with a retention time of 3.13 min in HPLC based analysis confirming the presence of FAD (fig. 2.4A), which also matched with the spectrum and retention time of standard FAD (fig. 2.4B) *(Zenno et al., 1996; Ooi et al., 2007; Chen et al., 2004).*

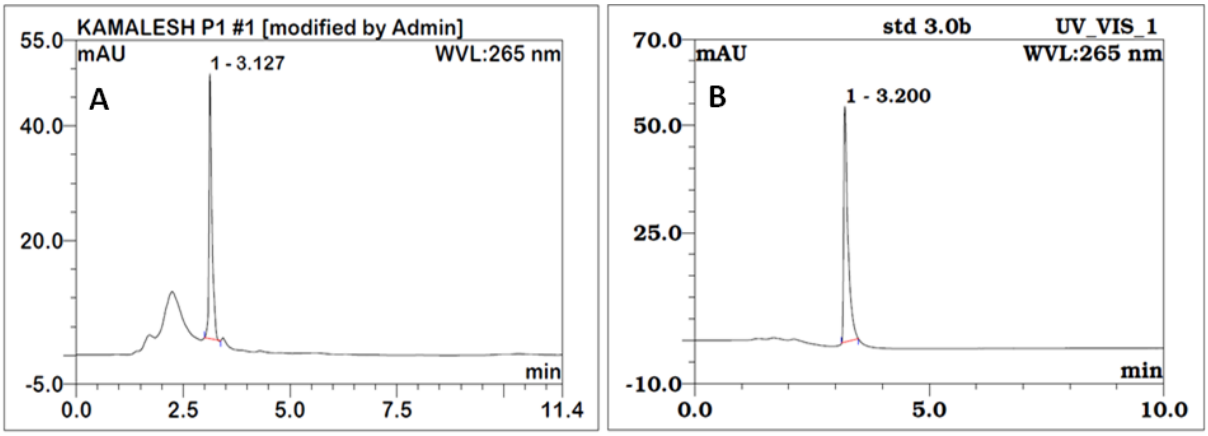


Figure 2.4: Studies on the prosthetic group by using HPLC. (A) Showing the peak of prosthetic group at 3.12 min with denatured enzyme (**B)** Standard FAD (control) eluted at very similar elution time (3.20 min).

2.3.3 Biochemical characterization of C. violaceum azoreductase

2.3.3.1 Effect of temperature and pH on the activity of azoreductase enzyme

The effect of temperature and pH on the activity of azoreductase is shown in fig. 2.5A and 2.5B. The effect of temperature on azoreductase activity was examined at a range from 10°C to 60°C and maximal activity occurred at 35°C. The effect of pH value was also examined at a range from pH 4.0 to 11.0 and optimum activity of azoreductase was found at pH 7.2, which is in agreement with the results of earlier report *(Bin et al., 2004).*

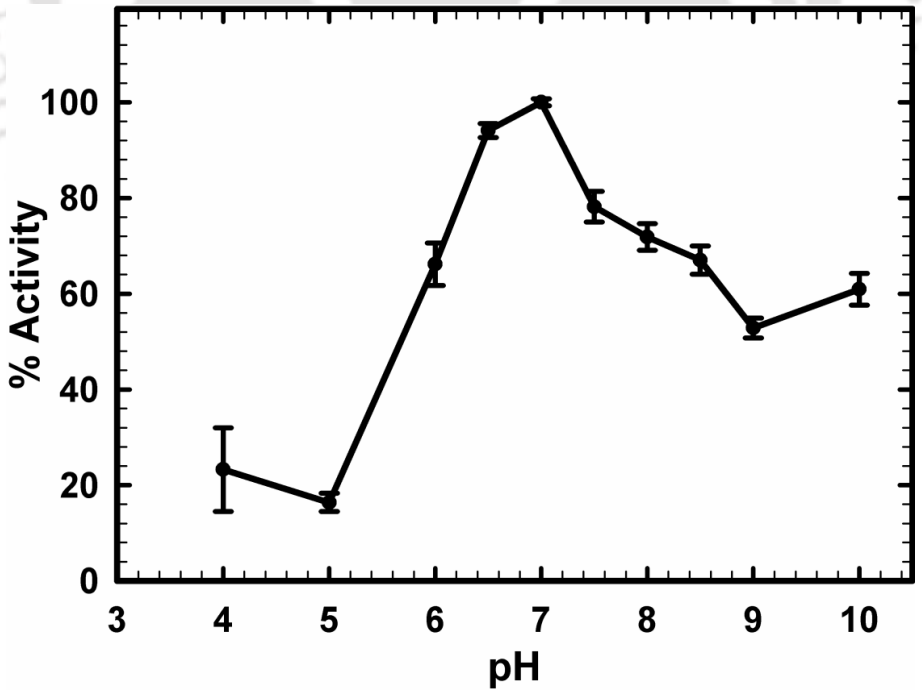


Figure 2.5: (A) pH optima: Optimum pH was analysed for azoreductase enzyme from the pH 4.0 to10.0. The optimum activity was found at pH 7.5 with a wide pH range of enzymatic activity.

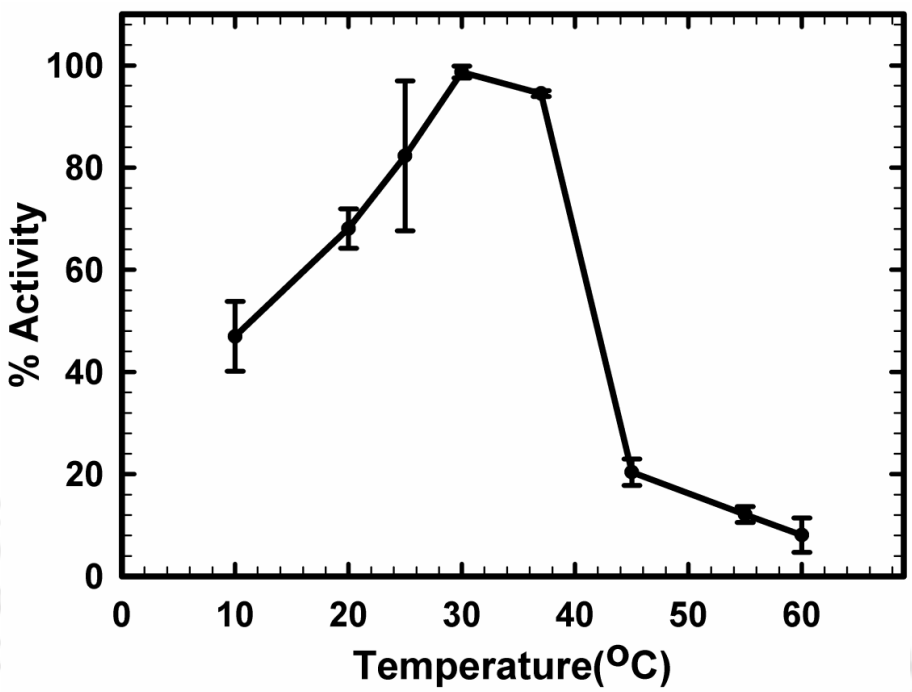


Figure 2.5: (B) Temperature optima for azoreductase enzyme: The optimum working temperature for azoreductase enzyme was 30°C to 37°C while the enzyme shows activity in the range of 10°C to 60°C.

2.3.3.2 Kinetic characterization of azoreductase

Azoreductase enzyme always catalyzes the oxidation-reduction reaction in the forward direction. In this reaction, three azo dyes were used as substrates with NADH as a co-substrate and K_m and V_{max} were estimated for both the substrates. The estimated K_m and V_{max} for methyl red were 24.06 ± 2.48 nM and 2.98 ± 0.23 nM s^{-1} (fig. 2.6A). K_m and V_{max} for NADH used with the methyl red reaction were estimated at 38.23 ± 1.57 µM and 2.30 ± 0.06 µM s^{-1} (fig. 2.6B), K_m and V_{max} for amaranth dye were 8.57 ± 1.08 nM and 3.82 ± 0.17 nM s^{-1} (fig. 2.6C), K_m and V_{max} for NADH used with amaranth dye were 59.94 ± 1.42 µM and 2.61± 0.10 µM s^{-1}(fig. 2.6D) and finally the K_m and V_{max} for methyl orange reaction were 25.22 ± 3.44 nM and 3.44 ± 0.20 nMs^{-1} (fig. 2.6E) and K_m and V_{max} for NADH used with methyl orange were 56.44 ± 9.33 µM and 1.63 ± 0.05 μMs^{-1} (fig. 2.6F).

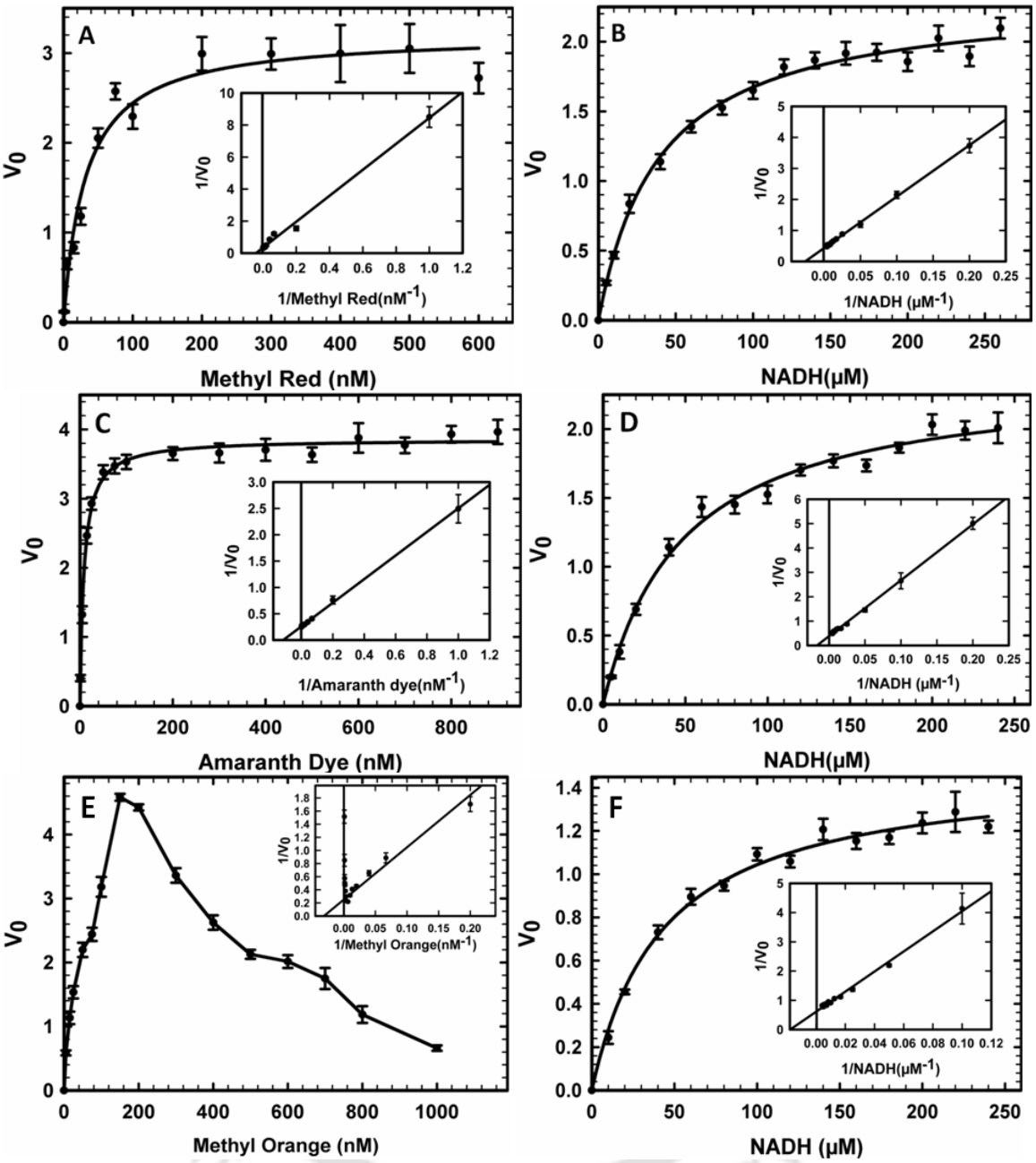


Figure 2.6: Kinetic characterization of azoreductase from *C. violaceum.* Michaelis-Menten plot for **(A)** methyl red taken as a varying concentration of substrate and concentration of NADH was fixed at 100 nM. The K_m and V_{max} for methyl red were calculated which is 24.06 ± 2.48 nM and 2.98 ± 0.23 nMs^{-1}. **(B)** The methyl red concentration was fixed at 100 nM with varying concentration of NADH. The K_m and V_{max} were calculated and found to be 38.23 ± 1.57 nM and 2.30 ± 0.06 μMs^{-1} respectively **(C)** The NADH concentration was fixed at 100 nM with varying concentration of amaranth dye in which K_m and V_{max} were calculated as 08.57 ± 1.08 nM and 3.82 ± 0.17 nMs^{-1} respectively **(D)** The amaranth dye concentration was fixed at 100 nM with varying concentrations of NADH. The K_m and V_{max} were calculated as 59.94 ± 1.42 nM and 2.61 ± 0.10 μMs^{-1} respectively. **(E)** The NADH concentration was fixed at 100nM with varying concentrations of methyl orange. The K_m and V_{max} were calculated as 25.22 ± 3.44nM and 3.44 ± 0.20 nMs^{-1} respectively. (**F)** The methyl orange concentration was fixed at 100 nM with varying concentrations of NADH. The K_m and V_{max} were calculated as 56.44 ± 9.33 nM and 1.63 ± 0.05 μMs^{-1} respectively.

Table 2.1: List of biochemical kinetic parameters calculated for the azoreductase.

S.No.	Substrate	K_m(nM)	V_{max}
1.	Methyl Red	24.06 ± 2.48	2.98 ± 0.23 nMs^{-1}
2.	NADH	38.23 ± 1.57	2.30 ± 0.06 μMs^{-1}
3.	Amaranth Dye	08.57 ± 1.08	3.82 ± 0.17 nMs^{-1}
4.	NADH	59.94 ± 1.42	2.61 ± 0.10 μMs^{-1}
5.	Methyl Orange	25.22 ± 3.44	3.44 ± 0.20 nMs^{-1}
6.	NADH	56.44 ± 9.33	1.63 ± 0.05 μMs^{-1}

2.3.4 Biodecolourization and biodegradation analysis

2.3.4.1 Analysis with UV – Visible spectroscopy

The degradation studies of methyl red, methyl orange and amaranth dye are clearly indicated in fig. 2.7. This UV–Visible data represents the degradation study of methyl red, amaranth dye and methyl orange, shown in fig. 2.7A, 2.7B and 2.7C respectively. The degradation of dye is measured as a decrease in absorbance of NADH. Methyl red dye was degraded very fast as compared to amaranth dye and methyl orange. The degraded results were compared with their undegraded counterpart.

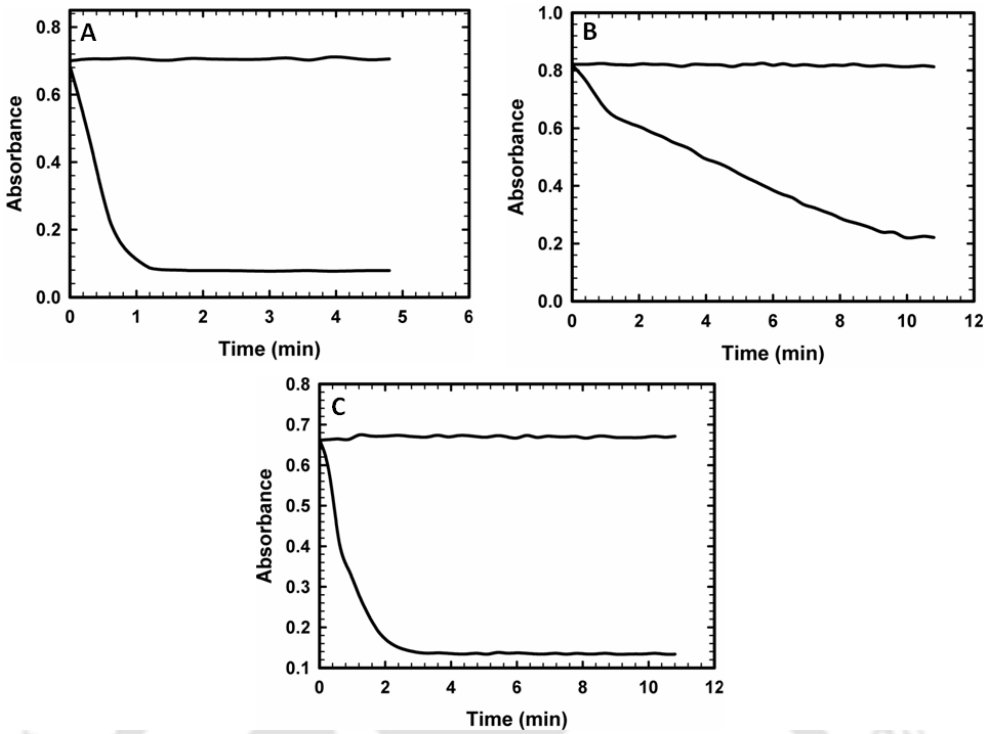


Figure 2.7: Degradation of dye using the enzyme **(A)** Absorption of NADH used in the reaction of methyl red degradation with control and treated sample. Absorption of both control and treated sample starts from the 0.70 OD value, but the optical density of treated sample goes down after 1 min scanning to the 0.1 OD value and for the control sample, OD value remains constant at 0.70. **(B)** Absorption of NADH (342 nm) used in the reaction of amaranth dye degradation with control and treated sample. Absorption of both control and treated sample starts from the 0.80 OD value, but the optical density of treated sample goes down after 10 min scanning to the 0.20 OD value and for control sample OD value remains constant at 0.80. **(C)** Absorption of NADH (342 nm) used in the reaction of methyl orange degradation with control and treated sample. Absorption of both control and treated sample start from the 0.65 OD value, but the optical density of treated sample goes down after 2 min scanning to the 0.13 OD value and for control sample OD value remains constant at 0.65.

2.3.4.2 Analysis with FTIR spectroscopy

Degradation of azo dye was confirmed by FTIR spectra. The FTIR spectra for the azoreductase-treated dyes (methyl red, amaranth and methyl orange) were compared to that of the untreated dyes. Fig. 2.8A shows the degradation of methyl red dye in which the peak at 3391 cm^{-1} represents the stretching of $-NH_2$ and the peak at 1656 cm^{-1}, bending of $-NH_2$ confirms the formation of $-NH_2$ group after reduction of the azo bond, thereby confirming the degradation of the dye upon treatment with the enzyme. Fig. 2.8B shows the FTIR analysis for the degradation of amaranth dye, which also shows stretching and bending modes of $-NH_2$ group at 3391 cm^{-1} and 1656 cm^{-1}, respectively, which were absent in the untreated dye. Similarly, in fig. 2.8C the FTIR spectra show the degradation of treated methyl orange, which shows peaks stretching of $-NH_2$ at 3391 cm^{-1} and bending of $-NH_2$ at 1656 cm^{-1} confirming the formation of $-NH_2$ group after the

reduction of azo bond. In case of all untreated dyes, azo peak is present at a range of 1429 cm^{-1} but in case of degraded dye the azo group was reduced to -NH_2 which can be characterized from the FTIR analysis.

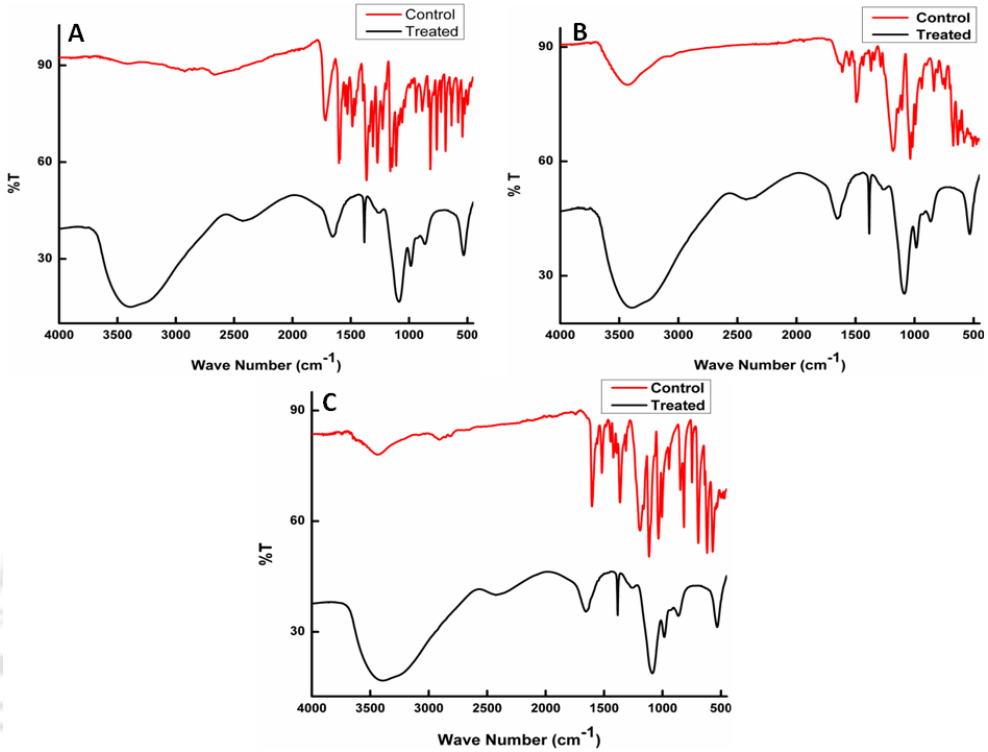


Figure 2.8: FTIR spectra of dye before degradation and after degradation, (A) IR spectra of methyl red dye before degradation and after degradation, in which peak showing the presence of stretching of –NH_2 at 3391 cm^{-1} in the degraded dye and also the presence of bending of –NH_2 at 1656.35 cm^{-1}, which confirms the formation of –NH_2 group after reduction of azo bond, **(B)** IR spectra of amaranth dye before degradation and after degradation, in which peak showing the presence of stretching of –NH_2 at 3391 cm^{-1} in the degraded dye and also the presence of bending of –NH_2 at 1656 cm^{-1}, which confirms the formation of –NH_2 group after reduction of azo bond and in **(C)** IR spectra of methyl orange before and after degradation, in which peak showing the presence of stretching of –NH_2 at 3391 cm^{-1} in the degraded dye and also the presence of bending of –NH_2 at 1656 cm^{-1}, which confirms the formation of –NH_2 group after reduction of azo bond.

2.3.5 Toxicity assay

The toxicity assay with dye showcases the significant cell death from very low concentration to higher concentration of dye. The concentration of dye was used from 20 $\mu g ml^{-1}$ to 1000 µg ml^{-1}. In this study we have taken L929 cells as control, study of all the three dyes was done before degradation and after degradation by the use of azoreductase enzyme. In case of methyl red dye, 80% cells were viable at 20 $\mu g ml^{-1}$ concentration and less than 10% cells were viable at 1000 $\mu g ml^{-1}$ concentration of methyl red (fig. 2.9A).

When degraded dye was used, 90% cells were viable at 20 μgml^{-1} concentration and 60% cells were viable at 1000 μgml^{-1} concentration. When amaranth dye was used, around 85% of cells were viable at 20 μgml^{-1} concentration and 10% of cells were viable at 1000 μgml^{-1} concentration. But in case of degraded dye, 90% of cells were viable and around 65% of cells were viable at 1000 μgml^{-1} concentration (fig. 2.9B). In case of methyl orange dye, 80% of cells were viable at 20 μgml^{-1} concentration and less than 10% cells were viable at 1000 μgml^{-1} concentration. When degraded methyl orange was used 80% of cells were viable at 20 μgml^{-1} concentration and 60% of cells were viable at 1000 μgml^{-1} concentration (fig. 2.9C) *(Kumar et al., 2017).*

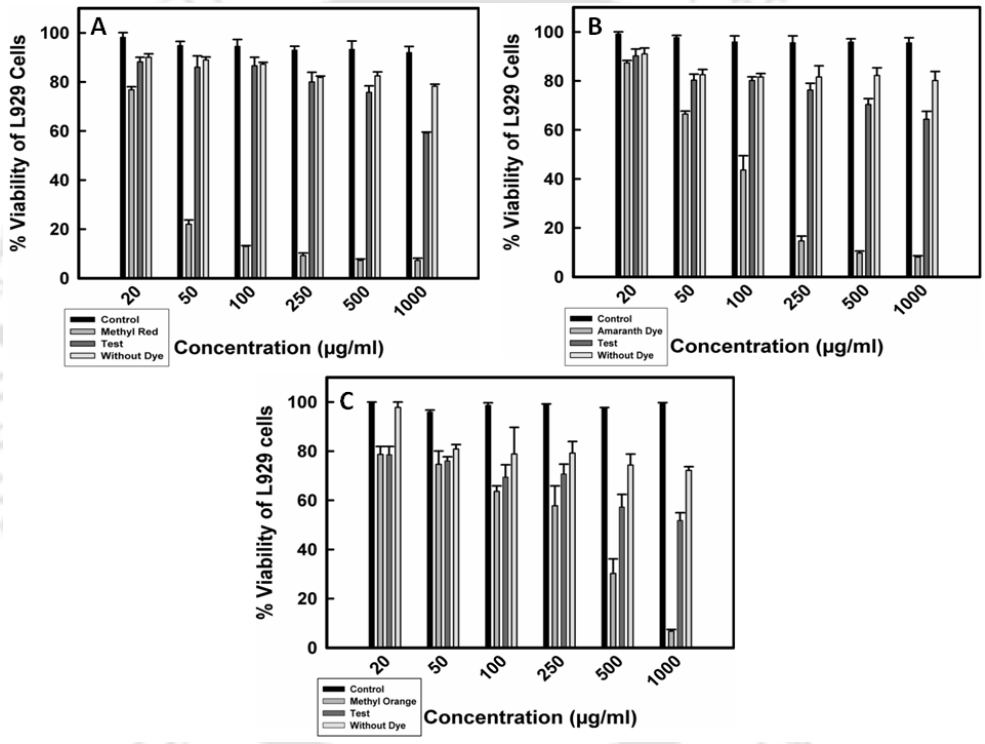


Figure 2.9: MTT assay showing the toxicity of dyes on fibroblast cell (L929 cells) in which normal healthy cells were taken as control, various concentrations of dyes without treated and treated with enzyme (degraded product, test) were used for the assay, from 20 μgml^{-1} to 1000 μgml^{-1}. **(A)** Toxicity of methyl red, at 20 μgml^{-1} concentration 75% of cells were viable and in 1000 μgml^{-1} concentration 10% cells were viable but after treatment of dye 80% of the cells were viable at 20 μgml^{-1} concentration and 60% of cells were viable at 1000 μg concentration. **(B)** Toxicity of amaranth dye in which 85% of cells were viable at 20μgml^{-1} concentration, 10% of cells was viable at 1000 μgml^{-1} concentration but in after-treatment of amaranth dye 90% of the cells were viable at 20 μgml^{-1} concentration and 70% of the cells were viable at 1000 μgml^{-1} concentration. **(C)** Toxicity of methyl orange dye in which 80% of cells were viable at 20 μgml^{-1} concentration and less than 10% of the cells were viable at 1000 μgml^{-1} concentration but after treatment of methyl orange dye percent viability of cells was increased which was 80% at 20 μgml^{-1} concentration and 55% at 1000 μgml^{-1} concentration.

2.3.6 Stability of the enzyme

Stability of azoreductase enzyme was measured with various denaturants. Azoreductase enzyme was found very stable with all the three denaturants. The percent activity of azoreductase was found to be 100% in the absence of urea, in contrast, to decrease in the activity when concentration of urea increases. 55% residual activity was reported with 2 M urea concentration while 10% activity was found at 4 M urea concentration (fig. 2.10). The percent activity of azoreductase was reported with varying concentration of GdmCl. 50% activity was reported at 2 M GdmCl while 7% residual activity was found at 4 M GdmCl concentration. The percent activity of azoreductase was reported with varying SDS concentration, where 60% residual activity was found at 0.1% SDS and 20% residual activity was found at 0.2% SDS which decreases to 5% activity at 0.4% residual activity. High stability of the enzyme is a decisive factor about the usefulness of an enzyme for industrial/commercial applications. The azoreductase enzyme shows very good stability under various conditions of urea, GdmCl and SDS (Table 1.2). The stability results for azoreductase enzyme under various harsh conditions make it an excellent protein for dye degradation applications.

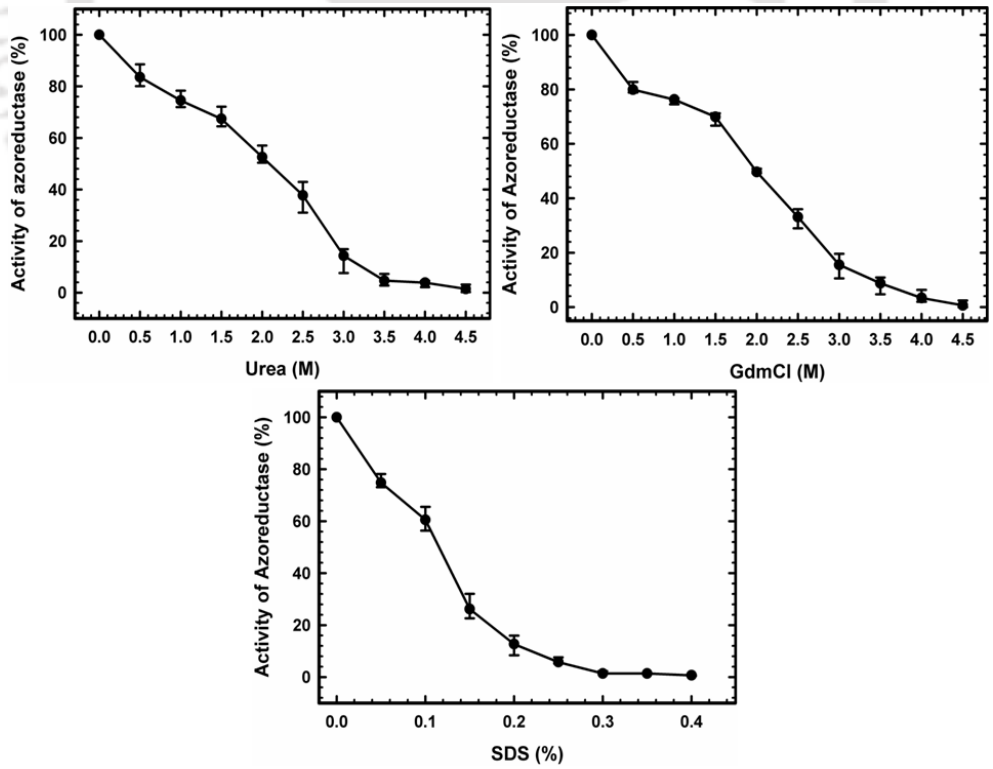


Figure 2.10: Stability of the azoreductase under different conditions. The residual activity was calculated considering activity without incubation with any denaturants under same conditions of the enzymatic reaction.

Table 2.2: Residual activity of azoreductase enzyme with various concentrations of different denaturants.

Conditions	Residual activity (%)
2M Urea	55
4M Urea	10
0.1% SDS	60
0.2% SDS	20
2M GdmCl	50
4M GdmCl	7

2.4 Discussion

More than 3000 azo dyes are currently available commercially which are widely utilized by a diverse group of industrial sectors including textiles, printing paper manufacturing, pharmaceutical, cosmetics and food industries. The remains and residues are discharged in the water bodies after the utilization of these dyes in the industries. These dyes are metabolized into aromatic amines after entering inside the living body *(Chung et al., 1992).* During manufacturing, 15% of total manufactured azo dyes are discharged which pollutes the environment *(Robinson et al., 2001).* Enzymatic degradation of azo dye has been found to be very efficient, useful and cost-effective method in present times *(Vander Zee et al., 2005).* It is also proved that azoreductase is involved in the metabolism of drug-containing azo groups *(Ryan., 2017).* In the present study, effectiveness of azoreductase extracted from *C. violaceum* was investigated. This bacterium has been used till now for its capability of producing violacein, a natural antibiotic which is helpful in the treatment of colon and other cancers *(Kodach et al., 2006).* The azoreductase enzymes of *C. violaceum* have not been discussed so far. Therefore this study aims to find the effectiveness of the azoreductase of *C. violaceum* as it can produce more enzyme than other bacteria and the gene sequence similarity of azoreductase is much different than other conventional bacteria. As it has the property to form antibiotics, it is also an old player of commercial world, which also makes it easy for quick and cheap availability of bacterial strain. This investigation was done in aerobic conditions by using the recombinant azoreductase from *C. violaceum*. This recombinant azoreductase was

purified from *E. coli* BL21 (DE3) shown in fig. 2.3A and 2.3B on SDS-PAGE. The size of the azoreductase was also confirmed to be 24 kDa.

The temperature range for enzymatic activity for azoreductase in other bacterial species was reported from 20°C to 55°C *(Cui et al., 2012; Ramalho et al., 2004; Misal et al., 2011),* but *C. violaceum* azoreductase is active in a wider temperature range with maximum enzyme activity at 30°C to 37°C (fig. 2.5B). Again the pH optimum for azoreductase enzyme also varies from species to species *(Ramalho et al., 2004).* In this case the enzyme is active over a wide range of pH with maximum activity at around pH 7.2 (fig. 2.5A). The wider temperature and pH range for enzyme activity make it an excellent enzyme for various applications related to the degradation and decolourisation of effluent dyes. Furthermore, the enzyme shows higher activity under very harsh conditions that may be useful for various applications.

The substrate specificity showed a variable efficiency rate with all the three tested dyes. Amaranth dye was found to be the best substrate for azoreductase. While the other two dyes, methyl red and methyl orange have shown significant activity with azoreductase, but less than amaranth dye. This observed difference is due to the difference in the chemical structure of the dye molecules. The nature of all the three dyes are aromatic in nature but the difference in the group attached to their aromatic ring changed the specificity for their substrate *(Moutaouakkil et al., 2003; Nachiyar et al., 2005; Liu et al., 2007).* Many azoreductases were known to be flavoenzymes as their activity depends on NADH/NADPH *(Chen et al., 2005; Punj et al., 2009).* Azoreductase activity depends upon NADH which in native condition always remains bound with FAD. So, FAD estimation data confirms the presence of coenzyme as FAD in native condition (fig. 2.4A) and also indicates that azoreductase enzyme from the *C. violaceum* was also a flavoenzyme.

The dye is usually very toxic to human health as well as to aquatic life. These dyes can convert into more dangerous forms when released in the environment. Apart from all these dyes, triphenylmethane is one of the most commonly used dyes in textiles, fish farming industries, paper, leather, food and medicines *(Azmi et al., 1998)* showing its carcinogenic property in biotic environment and is also mutagenic to some fishes. Precursors of many dyes are carcinogenic compounds like benzidine, accumulates in the environment and creates severe impacts *(Baughman et al., 1988).* Dyes analysed in the current investigation are among the most commonly used dyes both in domestic and industrial activities. Due to these reasons, determining the toxicity of these dyes becomes

a necessity which was checked on fibroblast cell line (L929). The obtained results are in agreement with the previous studies, which showed their severe toxic effects on living cells. In fig. 2.9A, it can be seen that in presence of methyl red, 75% of cells were viable at the concentration of 20 μgml^{-1}. This percentage viability of cells slightly goes down at 10% when concentration of methyl red was increased up to 1000 μgml^{-1}. When degraded dye was used, the percentage viability of cells increased from 75% to 80% at 20 μgml^{-1} concentration and 10% to 60% in 1000 μgml^{-1} concentration. Fig. 2.9B showed the toxicity of amaranth dye, in which 85% of cells were viable at the concentration of 20 μgml^{-1}. This percentage viability of cells slightly goes down at 10% when concentration of amaranth dye was increased up to 1000 μgml^{-1} concentration. But in the use of degraded dye, the percentage viability of cells increased from 85% to 90% at 20 μgml^{-1} concentration and 10% to 70% in 1000 μgml^{-1} concentration. A similar trend was also observed in the case of methyl orange dye, as shown in fig. 2.9C. 80% cells were viable at the concentration of 20 μgml^{-1} and less than 10% at 1000 μgml^{-1} while viable cells went up to 80% at 20 μgml^{-1} and 55% cells at 1000 μgml^{-1} concentration. After degradation of methyl red dye, amaranth dye and methyl orange dye, the percentage viability of cells increased and dyes became non-toxic to cells. So it can be inferred that azoreductase enzyme from *C. violaceum* can degrade the studied dyes and can reduce their toxic effects. So azoreductase of *C. violaceum* may become a suitable option for industries involved in the treatment of wastewater containing dyes. Degradation of dyes was also checked by using UV–Visible spectrophotometer and compared with control dye (fig. 2.7A, B and C). The degraded products of dyes were checked in FTIR analysis (fig. 2.8A, B and C) and compared with control dye. In control dye, the stretching vibration peak of N-N was present at the range of 1429 cm^{-1} which was reduced into the -NH_2 after degradation with azoreductase enzyme. The stretching vibration peak of NH_2 in all the three dye was present at the range of 3391 cm^{-1} and bending peak of NH_2 was also present at the range of 1656 cm^{-1} in all the three dye molecules. The reduction of the azo bond into NH_2 clearly indicates the degradation of dye molecule by the azoreductase enzyme. It was found in the present investigation that the activity of the enzyme depends on the presence of NADH and it also has a prosthetic group in bounded form and this prosthetic group was confirmed as FAD, a flavin molecule. The stability of enzyme was also checked at different temperature and pH range, which performed best in normal temperature range of 30°C to 37°C and neutral condition. The toxicity of the dye was also reduced up to 60% to 70% after degradation azoreductase enzyme and also the good

stability of azoreductase make it more efficient to use in various condition of environment. Efficient dye degrading capabilities of azoreductase in *C. violaceum* demonstrated in the present study will aid in the development of cheap microbial-based systems which will have the promising ability for efficient removal of azo dyes from the industrial effluents.

2.5 Conclusion

Methyl red, amaranth and methyl orange are widely used in industries and most common contaminant in the industrial wastewater with potential health hazards. In the present investigation, azoreductase enzyme from *C. violaceum* was made in recombinant form and characterized. A smaller value of K_m indicates very high affinity of azoreductase enzyme with all the three dyes. The enzyme is also found stable at very harsh conditions. This enzyme degrades dye product and shows much lower toxicity on various cell lines. The data indicate great potential of the enzyme for degrading dye in wastewater and can play a great role in cleaning the environment.

CHAPTER 3

Folding and stability of recombinant azoreductase enzyme from *Chromobacterium violaceum**

ABSTRACT

Azoreductase from *Chromobacterium violaceum* was characterized biophysically using experimental and computational tools. The azoreductase enzyme performs the oxidation-reduction reaction. The azoreductase enzyme perform the function with the help of cofactor FMN (Flavin mononucleotide). This FMN also helps in the proper folding and also provides space for substrate binding. The stability of azoreductase-cofactor complex is also depends on the binding of FMN. The stability-folding studies indicate that the cofactor, FMN is required for folding, stability and activity. The apoenzyme has significantly exposed tryptophan residue and sing type of tryptophan population are present as shown in quenching experiments. Overall, the data provides an interesting insight into stability, flexibility and biophysical parameters of the azoreductase protein.

*** Part of the work has been published in Enzyme and Microbial Technology 131 (2019) 109433.**

3.1 Introduction

A large number of proteins (around 30%) require a cofactor for their biological activity. The precise role of the cofactor in folding and stability of proteins is not fully understood *(Deu et al., 2007*). Proteins require co-factor for their activity and usually fold properly only with their respective co-factor. The cofactor not only helps in folding process but also dictate stability and function. However, the precise contribution for folding and stability needs more investigation. It is reported that the dissociation constant (K_d) for azoreductase for FMN binding correlates with stability and helps in measuring the stability of azoreductase enzyme *(Von Der Lehr et al., 2003; Havel et al., 1973; Wardell et al., 2005; Apiyo et al.,2002)*. We have recently reported detailed characterization of azoreductase from *C. violaceum (Verma et al., 2019).* The FMN dependent azoreductase (NC_005085.1) enzyme from *C. violaceum* also uses NADH as a co-substrate and converts azo dyes into respective amines *(Jingxian et al., 2016; Qi et al., 2017).* In this reaction, the NADH molecules are oxidised into NAD^+ and azo bond (-N=N-) reduces into single bond and in subsequent steps, the dye molecules get degraded *(Blumel et al., 2002; Zahran et al., 2019; Jagat et al, 2017)*. The azoreductase enzyme would have significant application in controlling azo dye pollution in industrial effluent *(Verma et al., 2019).* Few azoreductase enzymes have already been characterized. The activity of azoreductase enzymes is lost in absence of cofactor or when the dimeric structure of azoreductase is disrupted into monomer *(Prasanna et al., 1999).* However, recently reported azoreductase from *C. violaceum* is not yet characterized for similarities and differences with already reported azoreductase.

In this present study, the structural characterization of azoreductase holoenzyme and apoenzyme are highlighted by using fluorescence spectroscopy. Further, the structural components of azoreductase from *C. violaceum* were also investigated. The work was focused on the effect of FMN as a cofactor on protein activity, structure and stability. Chemical denaturants like urea and GdmCl (Guanidinium chloride) were used to study the enzyme's folding and stability. These investigations will help in understanding the azoreductase structure, mechanism of denaturation, folding and stability with cofactor molecules for better activity.

3.2 Materials and methods

3.2.1 Materials

All reagents used during the study were highly pure and purchased from Sigma-Aldrich chemical company, USA, HiMedia laboratories Pvt. Ltd and SRL Mumbai, India.

3.2.2 Over-expression and purification of azoreductase enzyme

The recombinant azoreductase enzyme was expressed and purified from *E. coli* by the method described by Verma et al *(Verma et al., 2019).* In brief, the azoreductase enzyme was expressed as apoenzyme and purified by Ni-NTA (Ni-Nitrilotriacetic acid) affinity chromatography column. The purified enzyme was extensively dialysed with 25 mM phosphate buffer (25 mM KH_2PO_4, 25 mM K_2HPO_4, pH 7.5) and concentrated with the concentrator. To perform the function of azoreductase apoenzyme, FMN was used as a cofactor to convert the apoenzyme into holoenzyme.

3.2.3 The azoreductase enzyme activity assay

The azoreductase enzyme activity was measured spectrophotometrically using Carry 100 UV-Vis (Agilent Technology) spectrophotometer. The azoreductase activity was measured by decreasing the absorbance of NADH after adding the azoreductase enzyme into the reaction mixture. The standard reaction mixture contained 25 mM K_2HPO_4, 25 mM KH_2PO_4 pH 7.2 (HiMedia) with varying concentration of methyl red, methyl orange or amaranth azo dye (Himedia) (1 to 1000 nM), 0.1 mM NADH and 10 µM FMN, respectively. The reaction started immediately after addition of 2 µg of an enzyme into the reaction mixture. The standard volume of reaction was 1 ml *(Verma et al, 2019; Suzuki et al, 2001).*

3.2.4 Spectroscopy measurement

All the UV-Visible absorption spectra were measured in Carry 100 UV-Visible spectrophotometer (Agilent Technology). The absorption spectra were taken from 230 nm to 500 nm at normal room temperature. The tryptophan intensity spectra for recombinant azoreductase apoenzyme and holoenzyme were recorded from 310 nm to 400 nm after excitation at 295 nm with 5 nm slit width. The fluorescence spectra of the enzyme in the presence of FMN was recorded from 370 nm to 600 nm with excitation wavelength at 355 nm. In ANS (8-Anilino-1-naphthalene sulfonic acid) (Sigma, USA)

binding studies, 100 µM of ANS ware added to each sample and ware incubated for 1 h at room temperature. After incubation all the sample ware properly mixed and fluorescence spectra was recorded from 400 nm to 600 nm with excitation wavelength at 365 nm and slit width of 5 nm *(Parray et al., 2019).*

3.2.5 Determination of K_d for FMN - azoreductase complex by fluorescence spectroscopy

The azoreductase enzyme was used for the titration with coenzyme (FMN) in potassium phosphate buffer (25 mM K_2HPO_4 and 25 mM KH_2PO_4) pH 7.3. The binding of FMN with apoenzyme was measured by quenching of tryptophan fluorescence emission. This quenching of tryptophan fluorescence emission was already used to study the binding affinity of the cofactor (FMN) with apoenzyme previously (*Verma et al., 2016).* This binding of FMN to the apoenzyme was monitored by measuring the tryptophan fluorescence emission at 340 nm with the excitation wavelength at 295 nm. The dissociation constant for FMN- azoreductase was calculated from equation 1.

$$1/(1-a) = 1/ K_d [\{(L_0)/a\} - (E_0)] \quad \text{Eq. 1}$$

Whereas 'a' is the fractional saturation of binding sites, (which is also equal to the $\Delta F/\Delta F_{max}$ in which ΔF is the difference in the FMN fluorescence intensity after addition to the enzyme solution and ΔF_{max} is the change in the fluorescence intensity when all the protein converted to the holoenzyme); K_d is the dissociation constant; L_0 is the total FMN concentration and E_0 is the total enzyme concentration *(Verma et al., 2016; Grove et al., 1976).*

3.2.6 Fluorescence quenching study with acrylamide and potassium iodide (KI)

The quenching of intrinsic tryptophan fluorescence spectra for apoenzyme and holoenzyme for azoreductase were performed with acrylamide and potassium iodide (KI) as a quencher. The concentrations of both quenchers were varied from zero to 1 M. The Stern–Volmer quenching constant (Ksv) was calculated from the F_0/F plot at maximum emission against the concentration of both quencher with equation 2 *(Balasera et al.,2003).*

$$F_0/F = Kvs\ (Q) + 1 \quad \text{Eq. 2}$$

In this equation, F_0 is the fluorescence intensity at zero quencher concentration (absence of quencher) and F is fluorescence intensity at the given quencher concentration (Q), whereas K is the Stern-Volmer quenching constant for the accessible tryptophan. The Stern–Volmer equation was used to study the population of tryptophan (W), tyrosine (Y) and phenylalanine (F). Apart from these amino acids, a few were accessible whereas rests were buried inside of the core of enzyme and thus were not accessible by the quencher *(Suthar et al., 2013).*

3.2.7 Equilibrium unfolding of apoenzyme and holoenzyme of azoreductase

The unfolding of azoreductase apoenzyme and holoenzyme was carried out by incubating the enzyme with various concentrations of different denaturants like urea (0 to 8M) and GdmCl (0 to 5M) before the activity or spectroscopic measurements as reported in the literature *(Bertoldi et al., 2005; Venktesha et al.,1998).* The fluorescence or activity was measured until no further change in fluorescent intensity or enzyme activity was observed *(Singh et al., 2010; Verma et al., 2019).*

3.3 Results and discussion

3.3.1 The spectroscopic property of apoenzyme and holoenzyme

The UV-Vis absorption of apoenzyme showed the maximum absorption at 274 nm whereas holoenzyme showed the maximum absorption at 257 nm. The slight hypochromic shift in the absorbance of holoenzyme was seen due to the presence of FMN. The absorption for the binding of the enzyme with NADH (cosubstrate) was also measured but no significant conformational changes was found (fig. 3.1A). The difference in the absorption of apoenzyme and holoenzyme was probably due to the conformational changes of protein after binding with co-factor. The Fluorescence absorption spectra of azoreductase apoenzyme and holoenzyme were measured with the excitation at 295 nm. The emission maxima for apoenzyme and holoenzyme were found at 325 nm and 330 nm respectively (fig. 3.1B). These absorption was found due to the presence of tryptophan residue, which indicated that the tryptophan molecule might be buried in the hydrophobic core of protein or very less exposed to the solvent molecule *(Gorga et al., 1982).*

The increase in fluorescence intensity of azoreductase holoenzyme and redshift indicated the exposure of tryptophan residue side chain to a more polar environment. The

holoenzyme emission spectra of tryptophan was affected after binding with FMN. The binding of FMN quenched the azoreductase fluorescence and also masked the tryptophan emission spectra. The decrease in fluorescence emission spectra after binding to FMN exposed tryptophan residue to the surface of the enzyme. The change in fluorescence emission spectra of protein after binding to the cofactor is directly related to the quenching of protein fluorescence.

The ANS treatment of azoreductase apoenzyme and holoenzyme (fig. 3.1C) indicated that hydrophobic site is completely exposed in apoenzyme but after binding to FMN molecules the same showed a slightly decrease. Again, when NADH was bonded to the FMN-azoreductase complex, the spectrum showed a fully dominated hydrophobic binding site. From fig. 3.1C, we can understand that after binding to the cofactor the conformational changes occurred in apoenzyme azoreductase and hydrophobic sites are not affected by FMN binding but NADH binding would affect the hydrophobic sites.

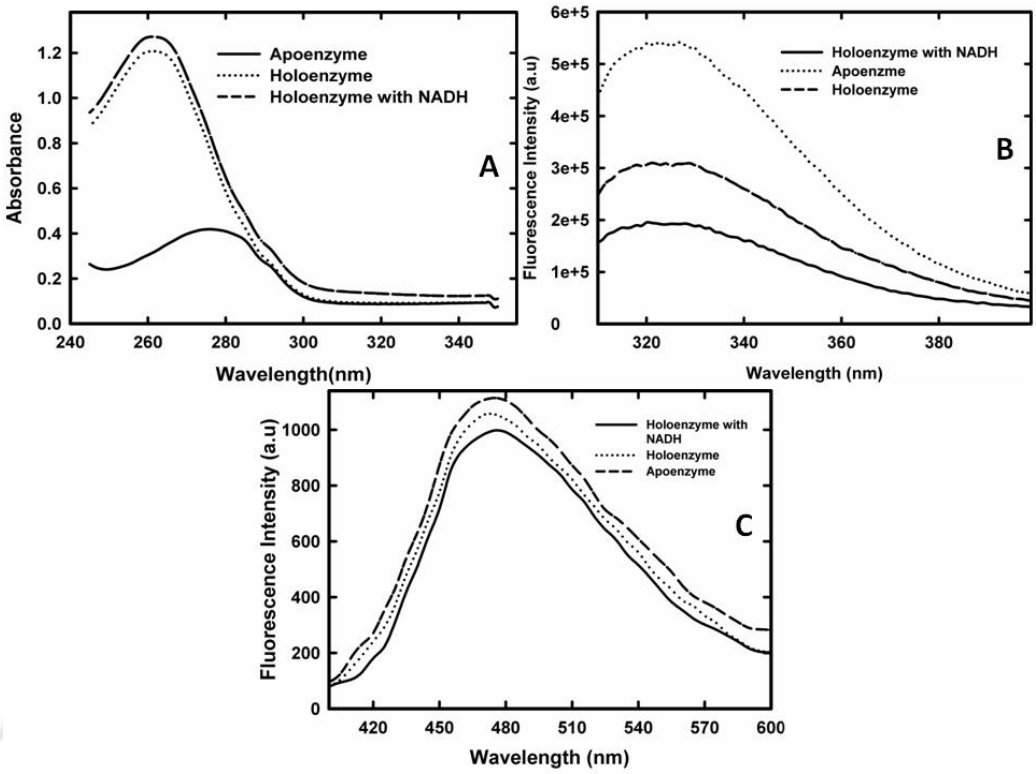


Figure 3.1: Biophysical characterization studies **(A)** The UV-Vis absorption spectra of azoreductase. Solid line, dotted line and dashed line represents apoenzyme, Holoenzyme and azoreductase holoenzyme with NADH, respectively **(B)** The fluorescence emission spectra for azoreductase enzyme. The dotted line, dashed line and solid line represent the intrinsic fluorescence spectra of apoenzyme, Holoenzyme and azoreductase holoenzyme with NADH, respectively. The fluorescence measurements were done after excitation at 295 nm. **(C)** ANS fluorescence spectra. The dashed line, dotted line and the solid line represents apoenzyme, Holoenzyme and azoreductase holoenzyme with NADH, respectively.

3.3.2 Determination of K_d for FMN and azoreductase complex

The dissociation constant (K_d) for FMN was determined by using the fluorescence enhancement method of FMN binding with azoreductase enzyme and tryptophan quenching experiment respectively. The dissociation constant for the cofactor-azoreductase was calculated by taking the assumption of 1:1 and 1:2 equilibrium *(Zahran et al., 2019; Guex et al., 1997; Morris et al., 2009).* The dissociation constant for the cofactor (FMN) and azoreductase was determined by monitoring the ligand-induced fluorescence spectra of azoreductase apoenzyme. In fig. 3.2A, the titration for the fluorescence of azoreductase was started from 0.1 mM concentration of FMN and quenching of fluorescence spectra for protein binding with FMN was also started from the 0.1 mM concentration. The complete quenching of fluorescence spectra for FMN was stopped with the concentration of 15 mM. In fig. 3.2B, the change in fluorescence

intensity (ΔF) versus the concentration of FMN was shown, where it gave the binding curve for the FMN-azoreductase. In this plot, the fluorescence emission intensity decreased with respect to the maximum fluorescence intensity. As shown in fig. 3.2C, the binding site was gradually saturated with increasing concentration of FMN. After analysing all the fluorescence data as found in fig 3.2, the dissociation constant was calculated as 6.859 ± 0.145 x 10^{-5} M. From this analysis we can conclude that a part of FMN binds to the azoreductase active site while others help in folding and maintaining the native state of azoreductase.

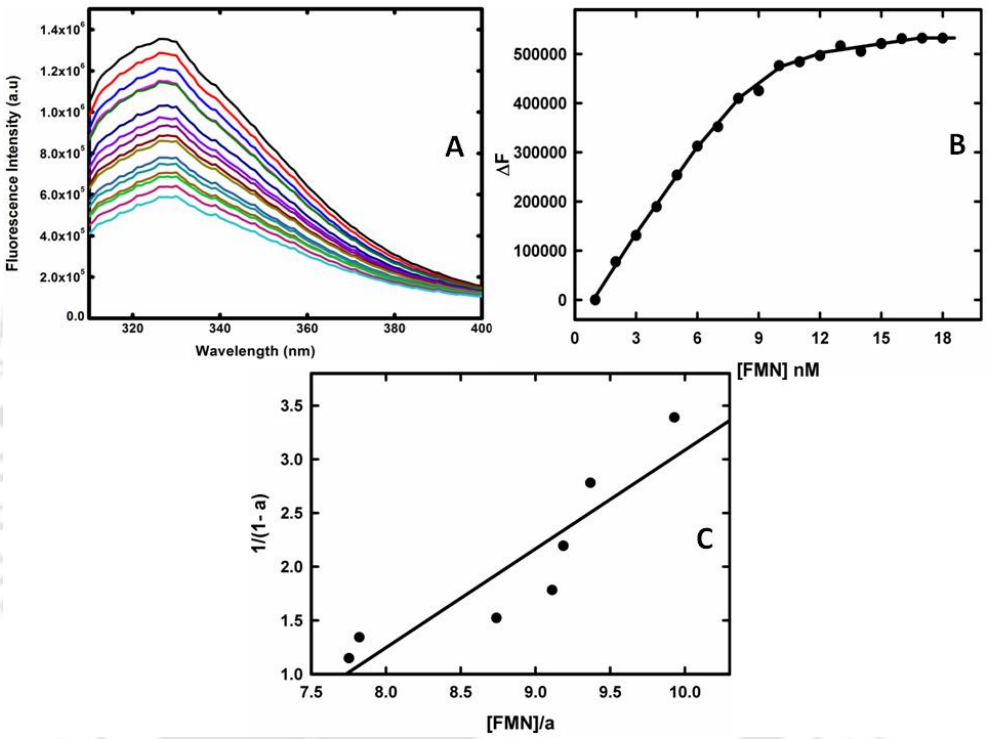


Figure 3.2: Biophysical characterization studies **(A)** Determination of the dissociation constant by the intrinsic fluorescence emission spectra. For the FMN- Azoreductase complex and the graph was plotted against fluorescent intensity versus the wavelength. **(B)** The graph was plotted against the ΔF verses FMN concentration, in which ΔF is the changes in fluorescent **(C)** The dissociation constant was Determined from the slop of graph.

3.3.3. Fluorescence quenching of azoreductase apoenzyme and holoenzyme

In fluorescence quenching study, the relative solvent exposure of fluorophores (Tryptophan and Tyrosine) was analysed. Due to the uncharged nature of acrylamide and potassium iodide, it is used as the intrinsic fluorescence quencher. In this study, the concentration of acrylamide and potassium iodide was used up to the concentration of 0.8 M. The gradual decrease in the fluorescence emission intensity was observed due to the

presence of quencher. The tryptophan residues present in azoreductase were accessible to the acrylamide and potassium iodide was partially exposed to the solvent. In fig. 3.3A, the Stern - Volmer plot for acrylamide with apoenzyme and holoenzyme showing the linear plot, Stern – Volmer quenching constant was also calculated from the slops of graph and found to be Ksv = 3.5 M^{-1} and Ksv = 2.32 M^{-1} respectively. It clearly indicates that tryptophan residue was interacting with the quencher in apoenzyme but the decrease of Stern – Volmer quenching constant with holoenzyme indicated a decrease in tryptophan population as well as interaction to quencher. In fig. 3.3C, the upwards curving Stern – Volmer plot for potassium iodide (KI) with apoenzyme and holoenzyme indicates the static quenching mechanism where potassium iodide (KI) form non-fluorescent complex or quencher is very close to the tryptophan residue. The Stern-Vomer quenching constant was also calculated from the slope of curve for fig. 3.3C, which was Ksv = 1.3 M^{-1} and Ksv = 3.5 M^{-1} respectively. In fig. 3.3C, Stern-Volmer quenching constant was 1.3 M^{-1}, which means the quencher was not fully interacting with the tryptophan residue on the surface but in holoenzyme case the Stern – Volmer quenching constant was 3.5 M^{-1}, indicating that all the tryptophan population were fully accessible to the quencher.

The linear graph was found in fluorescence quenching of azoreductase apoenzyme and holoenzyme with both the quenchers. The fraction of tryptophan accessible to quencher (Fa) was calculated from the modified graph (fig. 3.3B), which was plotted by $F_0/\Delta F$ verses 1/[Q]. The fraction of tryptophan accessible to acrylamide in both apoenzyme and holoenzyme was found to be one which clearly indicates that the single types of population of tryptophan were present on enzyme. The Stern - Volmer quenching constant indicates that all the tryptophan residues is fully involved with the quencher molecules and accessible to the acrylamide in both azoreductase apoenzyme and holoenzyme. This might be because of the binding of the cofactor (FMN) with azoreductase enzymes.

In fig. 3.3D when potassium iodide was used as a quencher, the fraction of tryptophan accessible to the quencher (fa) was found as more than one for apoenzyme (filled circle), and one for holoenzymes (open circle). This results clearly indicates that the tryptophan population found in azoreductase apoenzyme was not fully accessible to the quencher molecule. But in case of the holoenzyme, the entire tryptophan residue was fully accessible to the quencher molecule. These same results were also found in the above

paragraph. In which, the Stern – Volmer quenching constant for apoenzyme and holoenzyme was 1.3 M^{-1} and 3.5 M^{-1} respectively. This fraction of tryptophan accessible to quencher (Fa) and stern – Volmer quenching constant confirm that the entire tryptophan residues were involved with the surrounding quencher in holoenzymes. This is because of cofactor (FMN) binding with azoreductase apoenzymes.

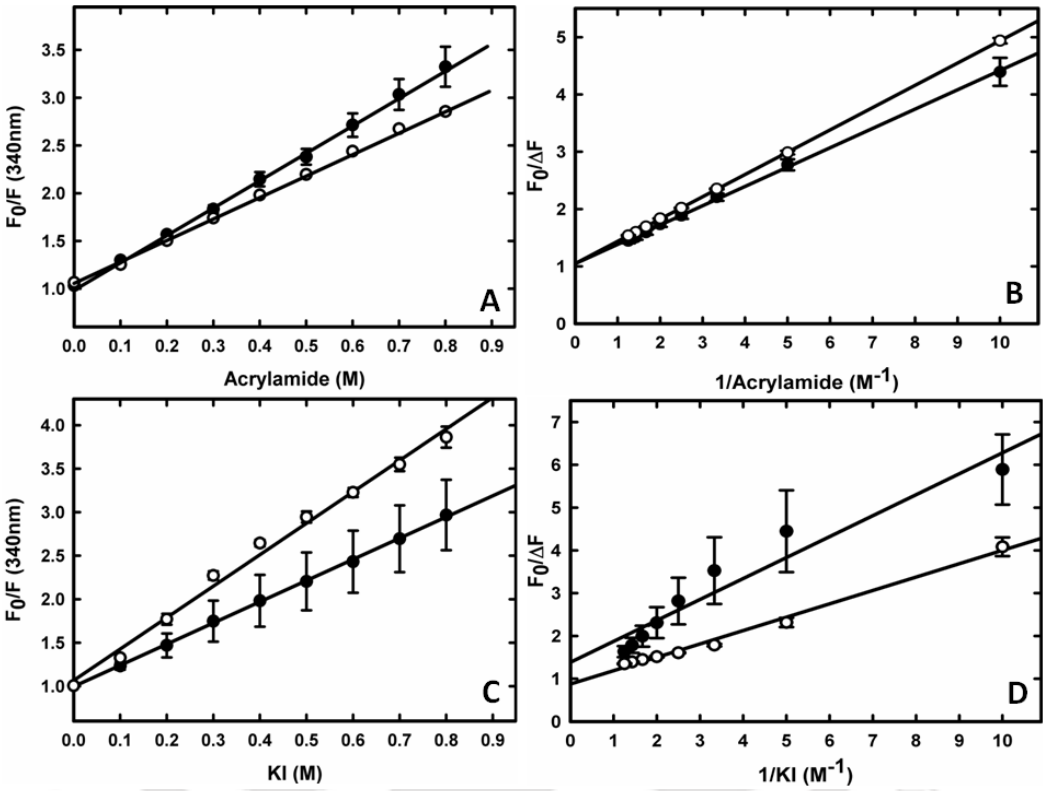


Figure 3.3: Quenching of tryptophan of azoreductase apoenzyme (●) and holoenzyme (○) with acrylamide and potassium iodide (KI). **(A)** The Fluorescence intensities at 340 nm **(B)** The fluorescence intensities were indicated as modified Stern-Volmer plot. **(C)** The fluorescence intensities with different concentration of potassium iodide (KI) **(D)** The fluorescence intensities with potassium iodide (KI) were indicated by the modified Stern-Volmer plot.

3.3.4 The equilibrium unfolding of apoenzyme and holoenzyme of azoreductase

Enzyme activity and tryptophan fluorescence emission spectra of the enzyme can be regarded as the most sensitive probe to study the protein conformation. The oligomeric structure and tryptophan fluorescence emission spectra of the enzyme are a very important marker for unfolding of azoreductase from *C. violaceum* as tryptophan is also present in its structure. The effect of urea and GdmCl on folding and unfolding of

azoreductase has been shown in fig. 3.4. The folded structure of azoreductase is very important to perform its function. The UV-Visible spectroscopy of urea/GdmCl on activity of azoreductase provides information about the folding and unfolding of structure of azoreductase enzyme. The effect of urea and GdmCl on activity of enzyme summarised in fig. 3.4A and 3.4B. The activity of azoreductase start decreasing while increasing the concentration of urea in fig. 3.4A. The 50% activity was lost up to 2M urea concentration and complete loss of activity was observed at 4M urea condition. These observations clearly indicate 50% of azoreductase was unfolded up to 2M urea and complete denaturation stage was up to 4M urea concentration respectively. The activity of azoreductase enzyme was also decreased with respect to increasing concentration of GdmCl. The 55% activity of azoreductase decreased at up to 2 M GdmCl concentration while complete loss of activity was found at 3.5 M GdmCl concentration, which showed that 55% of azoreductase was unfolded up to 2 M GdmCl condition.

The equilibrium unfolding study of protein using denaturant, provides information about the conformational stability of the protein. The common denaturant like urea and GdmCl made changes in the tertiary structure of azoreductase apoenzyme and holoenzyme. These changes were monitored by studying the tryptophan fluorescence emission spectra in fig. 3.4C and 3.4D, in which changes of tryptophan fluorescence emission of apoenzyme and holoenzyme was plotted against urea and GdmCl concentration. The urea and GdmCl induced three state transitions in apoenzyme of azoreductase while in case of holoenzyme, it showed two-state transition of azoreductase. In the first stage of transition of tryptophan, it started with unfolding of azoreductase but in second stage of transition corresponded with final stage of unfolding state. In case of holoenzyme, up to 2 M urea concentration, the cofactor FMN stabilized holoenzyme, implying, FMN binding site becomes folded up to 2 M urea concentration. Subsequently, FMN binding site becomes unfolded while increasing the concentration of urea. In case of apoenzyme, transition stage was found from 1 M to 2 M urea concentration while in holoenzyme, the transition stage was from 2 M to 3 M urea concentration. The folded state of azoreductase was also measured by increasing the GdmCl concentration for both apoenzyme and holoenzyme. The FMN binding site became unfolded with increasing GdmCl concentration of holoenzyme. The transition state was observed from 0.75 M to 2 M GdmCl for apoenzyme while in case of the holoenzyme, transition state was observed from 1 M to 3 M GdmCl concentration. The similar profile was observed for both

apoenzyme and holoenzyme, from 3.5 M to 8 M urea concentration while with GdmCl, similar profile was observed from 4 M to 8 M GdmCl concentration. This biphasic unfolding plot was found with holoenzyme in both graphs due to the presence of FMN with azoreductase. The presence of cofactor stabilized structure of azoreductase in dimeric condition. The apoenzyme and holoenzyme did not unfold up to 1M and 2M urea condition respectively; unfolding of holoenzyme started with 2M urea and completely unfolded at 4M urea concentration. It clearly indicates conformational change due to binding of FMN with apoenzyme, where these changes stabilize the azoreductase up to 2 M urea and 1M GdmCl condition respectively.

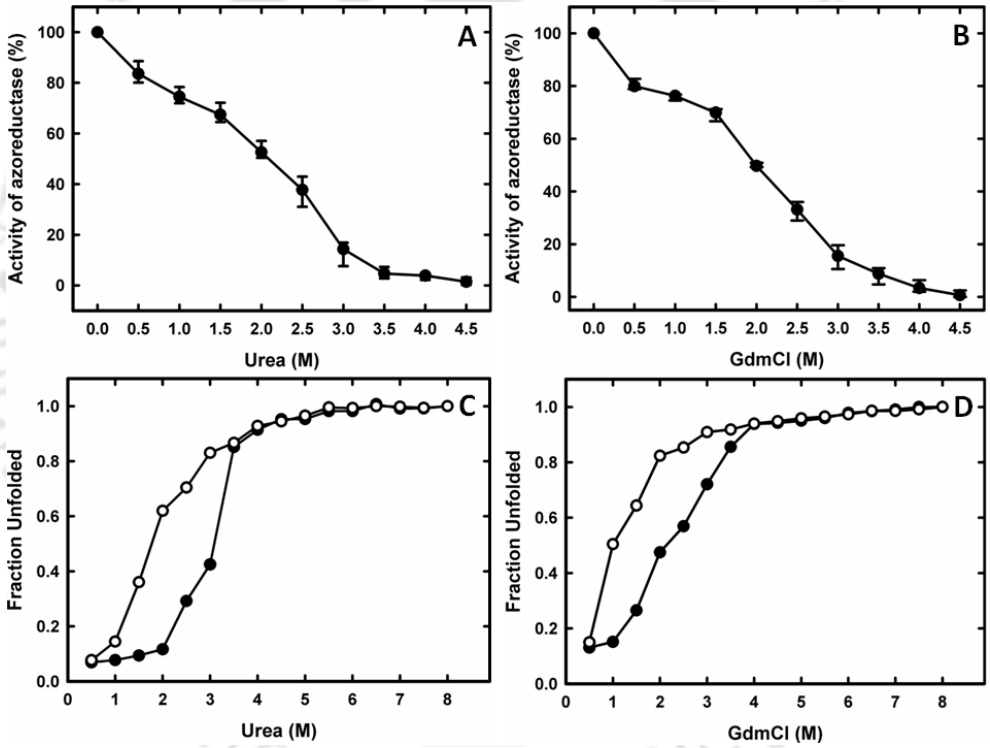


Figure 3.4: Folding study of azoreductase with urea and GdmCl. (A) Loss of azoreductase activity in percentage with respect to increasing urea concentration. (B) Loss of azoreductase activity in percentage with respect to increasing GdmCl concentration. (C) The folding study of tryptophan fluorescence emission spectra of apoenzyme (open circle) and holoenzyme (filled circle) with increasing urea concentration. (D) The folding study of tryptophan fluorescence emission spectra of apoenzyme (open circle) and holoenzyme (filled circle) with increasing GdmCl concentration. The data are shown in fig. (C) and (D) are the average of three experiments.

3.4 Conclusion

The accessibility of tryptophan, stability and folding in azoreductase from *C. violaceum* was affected by cofactor binding. The cofactor (FMN) binding, results in more than 70% quenching of intrinsic fluorescence of azoreductase enzyme. In this study, we have observed that the cofactor molecules are tightly associated with the azoreductase enzyme. Quenching experiments with acrylamide or potassium iodide (KI) also showed that single type of tryptophan population residues were associated with azoreductase. The azoreductase enzyme was treated with denaturants like urea and GdmCl to check structural flexibility of protein and it was found that azoreductase activity decreased with increase of both denaturant Urea and GdmCl. The change in azoreductase activity and its folding-unfolding nature confirms the flexibility of the structure. The FMN was bound very strongly with cofactor binding sites of azoreductase enzyme and stabilize the structure.

CHAPTER 4

Binding study of cofactor with oligomeric state of azoreductase enzyme from *Chromobacterium violaceum**

ABSTRACT

The azoreductase enzyme from *Chromobacterium violaceum* was characterized biophysically using experimental and computational tools. The *in silico* study, suggests that the azoreductase enzyme from C. *violaceum* is very unique and do not show very sigh similarity with other azoreductase enzyme. The modelling and cross-linking experiments using glutaraldehyde suggest that the enzyme is dimeric in nature. The modelled secondary structure of azoreductase and circular dichroism (CD) spectroscopy studies suggest 40% α-helix, 30% β-sheet and 30% random coils. In docking experiments, the cofactor (FMN) binds strongly with enzyme with -3.8 kJ/mol binding energy. The binding of FMN stabilizes the enzyme structure.

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4.1 Introduction

Around all available enzymes, more than 30% requires various cofactors for their biological activity. The precise role of cofactor with the associated enzyme was still not very clear *(Deu et al., 2007*). Stability, folding and the activity of enzyme depends on the cofactor binding. So that, it is very important to understand the binding property of cofactor with the enzyme. All these knowledge about the binding of cofactor may be important for the enhancement of the activity, modification of activity and also in the diversification of enzyme activity *(Von Der Lehr et al., 2003; Havel et al., 1973; Wardell et al., 2005; Apiyo et al., 2002).* The proteins molecule fold into their natural environments with the specific cofactor. It also helps in the folding of misfolded or partially folded protein to convert into its native or properly folded state. Most often the role of the cofactor is to stabilize the native state of their respective proteins *(Caldinelli et al., 2008; Apiyo et al., 2002).*

Recently, the gene encoding the azoreductase enzyme from *Chromobacterum violaceum* was cloned and characterized by Kamalesh et al *(Verma et al., 2019).* This azoreductase (EC1.7.1.6) enzyme belongs to the oxidoreductase family and catalyses the oxidation reaction. The main function of azoreductase was the conversion of the azo bond into the respective amines with the help of co-substrate NADH/NADPH *(Ooi et al., 2007).* This azo bond was mainly found in azo dye which is utilized by textiles industries. So, the azoreductase enzyme can be used for the degradation of dyes pollution from the environments *(Chung et al., 1992; Stolz., 2001; Chang et al., 2001)*. The coding sequence of azoreductase encodes 202 amino acid of protein sequence. The structure of FMN dependent azoreductase was dimeric in nature. One monomer was engaged in binding with azo dye and another monomer binds with cofactor FMN molecules, while catalysing reaction *(Jingxian et al., 2016; Qi et al., 2017*). While degrading the azo dye with help of azoreductase, the NADH molecules were oxidized into the NAD^+ and the electron was transferred to the azo bond and degraded it in the subsequent steps *(Blumel et al., 2002; Zahran et al., 2019; Jagat et al, 2017).* The activity of azoreductase enzymes depends on the presence and absence of cofactor. *(Prasanna et al., 1999).* However, there is not much information about the similarities and differences of azoreductase from *C. violaceum* with already reported azoreductase enzymes.

In this chapter, we are reporting about the similarity and dissimilarity of azoreductase enzyme with well-characterized azoreductase enzyme from *E.coli* and the secondary structure of the azoreductase enzyme after modelling the structure. The modelled secondary structure was confirmed by Circular-dichroism (CD) spectropolarimeter. The confirmation of modelled oligomeric structure with glutaraldehyde cross-linking experiments and docking of modelled azoreductase enzyme with their cofactor (FMN) helps in the better understanding of the azoreductase binding with FMN.

4.2 Materials and methods

4.2.1 In silico studies on azoreductase C. violaceum

The amino acid sequence of azoreductase enzyme was obtained from the NCBI (NC_005085.1). The sequence alignment between the azoreductase sequence and the sequence of another azoreductase *(E. coli)* was done by using the ClustalW *(Chenna et al., 2003).* This sequence was used to predict the model structure of azoreductase by Phyre 2 software *(Kelley et al., 2015).* The modelled structure was verified by procheck and patch dock by generating the Ramachandran plot *(Laskowski et al.,1996; Duhovny et al., 2002).* The secondary structure analysis was done in PDB sum after modelling the structure of azoreductase *(Laskowaski., 2009).* The energy minimization was done by using the Swiss PDB viewer *(Guex et al., 1997).* The visualisation for the analysis of three-dimensional structures was done in PyMOL (The PyMOL Molecular Graphics System, Version 1.2r3pre). The energy minimized structure of azoreductase was used in Autodock program to study the binding of the cofactor (FMN) to its respective residue present on azoreductase protein *(Morris et al., 2009).* The three-dimensional structure of FMN used for autodock study, was chosen from the Zinc database *(Irwin et al., 2005).*

4.2.2 Azoreductase secondary structure prediction in CD spectropolarimeter

Circular-dichroism (CD) measurement was made on JASCO J-1500 spectropolarimeter (JASCO, Inc., MD, USA). The calibration of instrument was done with 25 mM potassium phosphate buffer (25 mM K_2HPO_4 and 25 mM KH_2PO_4) pH 7.5. The concentration of enzyme was found to be 0.5 μgml^{-1}, and 25µg of enzyme used for the study of secondary structure. The sample contained purified enzyme and the volume was madeup with phosphate buffer. The cuvette used for the reaction was found to be 1 mm

thick and the path length is 1 cm. the sample was scanned from 190 nm to 250 nm. The results have collected an average of three scans per sample. The obtained values were normalised by subtracting the baseline recorded for the buffer in a similar condition. The recorded spectrum was analysed *in-silico* using the K2D2 online software and secondary structure of azoreductase was determined *(Verma et a., 2016).*

4.2.3 Conformation of the oligomeric structure of azoreductase with glutaraldehyde cross-linking

The cross-linking studies of azoreductase were performed using glutaraldehyde (Sigma-Aldrich) as described by Singh et al *(Singh et al., 2010).* The reaction mixture contained 1% of freshly prepared glutaraldehyde solution (Sigma-Aldrich) and 2 µg of protein (0.6 $\mu g \mu l^{-1}$) was also added to it. The total volume of the reaction became 30µl. The reaction was optimised with various concentrations of glutaraldehyde solution and incubation time was also optimized with various time intervals. The reaction was stopped by adding 10 µl of the tris-HCl buffer, pH 8.0. The cross-linked protein was dissolved in the Laemmli buffer and ran on 12% SDS-PAGE *(Wu et al., 1996; Duddempudi et al., 2013).*

4.3 Results and Discussion

4.3.1 Sequence similarity of azoreductase

The amino acid sequence similarity alignment of azoreductase (*C. violaceum*) with other azoreductases (*E. coli*) was found to be 42% (fig. 4.1). The residue found in red text is similar in both the azoreductase enzymes while residue highlighted in the red box is the conserved/ identical residue found in both organisms.

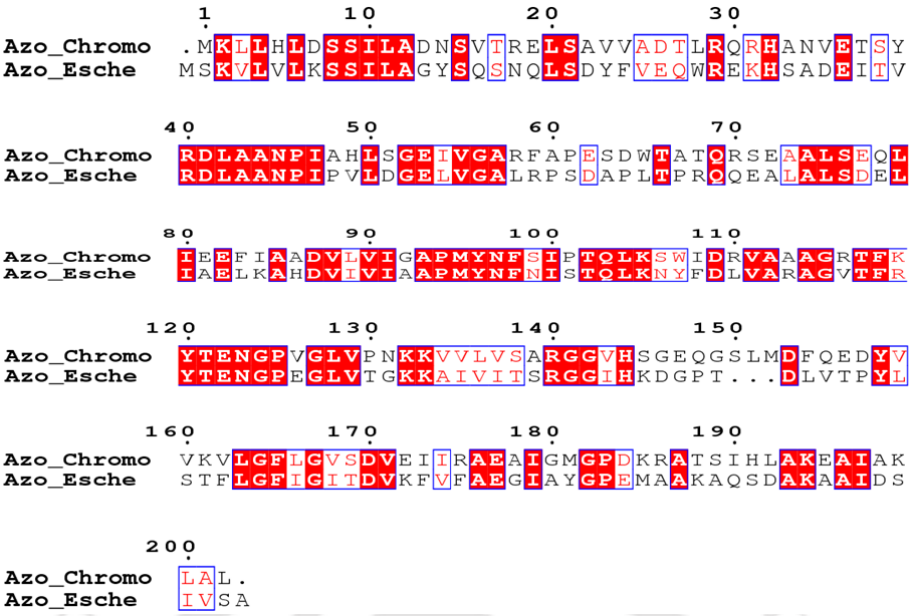

Figure 4.1: The sequence similarity alignment using ClastalW: The residue found in red text is similar in both proteins while residue highlighted in the red box is the conserved/ identical residue.

4.3.2 Homology modelling of azoreductase

Three-dimensional models for azoreductase were produced by using the software Phyre2 *(Kelley et al., 2015).* The modelled azoreductase and active site residue of the structure were shown in fig. 4.2. The conserved active site residues involved in the binding of FMN were shown as Met95, Asn97, Phe98, Gly141, Gly142, Arg186 and His144 of chain A (fig. 4.2).

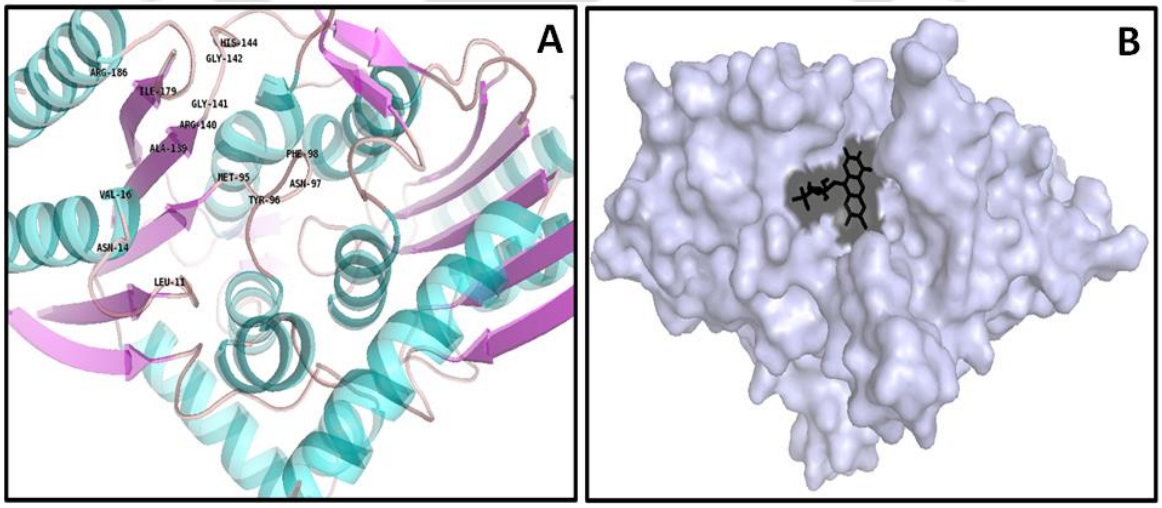

Figure 4.2: *In silico* studies on azoreductase enzyme: (A) The homology modelled structure of azoreductase enzyme (B) The cofactor FMN buried inside of azoreductase enzyme binding site.

4.3.3 Validation of model structure with Ramachandran plot

In Ramachandran plot, different regions shown with different colour cote in which dark yellow shows the most favoured regions, pale yellow shows the additional allowed region and faded yellow shows the residue found in the generously allowed region (fig. 4.3). The amino acid from modelled azoreductase found in the most favoured is 89.5%, the amino acid found in additional allowed reason is 9.6% and 0.8% in the generously allowed region.

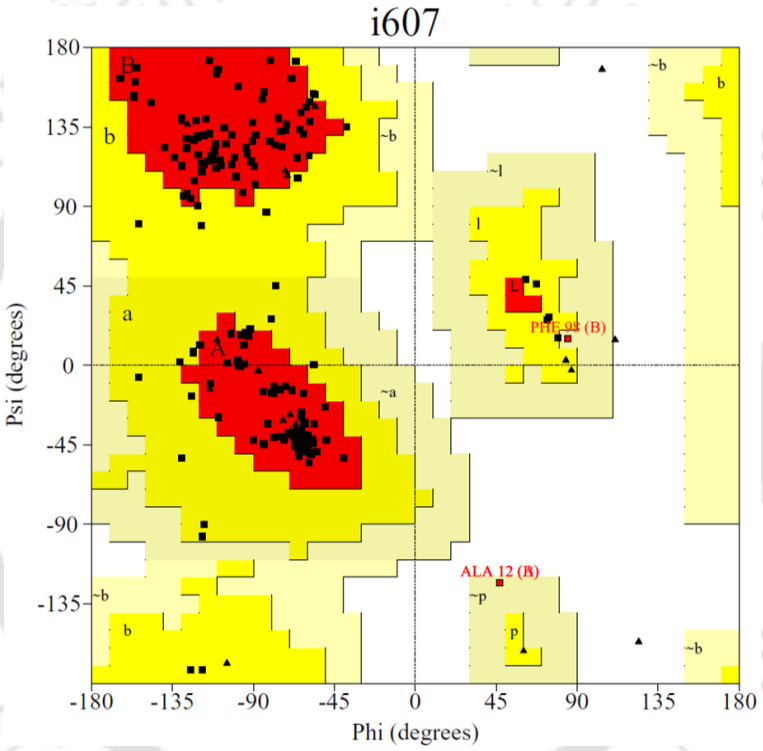

Figure 4.2: The Ramachandran plot for modelled azoreductase structure: this plot showing different colour areas indicated by white (Disallowed regions), light yellow (generously allowed regions), red (Mostly favoured regions) and yellow (Additional allowed) regions respectively.

4.3.4 Azoreductase secondary structure prediction

The secondary structure compositions of the predicted structure showed 40% α-helix, 30% β-sheets, two β- α - β motifs, one β hairpin, 19 β turns and 2 γ-turns respectively.

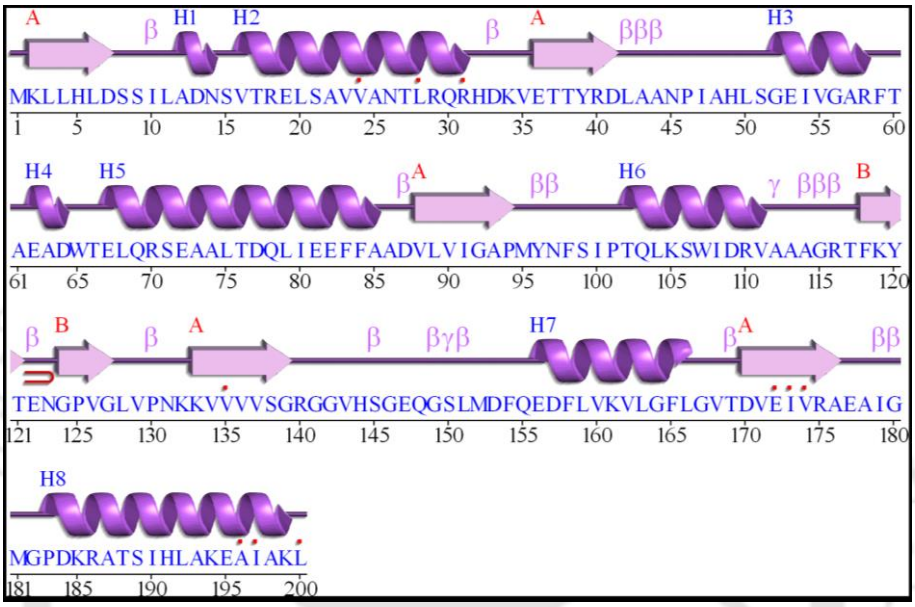

Figure 4.4: Schematic diagram showing modelled azoreductase enzyme. The secondary structure elements (α-helices and β-sheets) were indicated by wiring diagram and all structural motifs β- turns, γ-turn, and β-hairpins present.

4.3.5 Secondary structure determination of the purified, recombinant azoreductase by circular dichroism (CD)

The secondary structure composition of recombinant azoreductase from *C. violaceum* was determined by circular dichroism (CD). This secondary structure is important parameter for protein to study the presence of α helix, β sheet and random coil. Study of secondary structure for azoreductase enzyme was performed in (Jasco CD - Circular Dichroism J-1500). The spectra of enzyme were dependent on structural conformation. Thus, CD can be used for the detection of conformational change in protein and for the estimation of the structure of unknown protein like azoreductase *(Bhoir et al., 2018)*. The spectra for azoreductase enzyme were taken in far-UV region. The data was analyzed in

K2D2 program and it showed the presence of 87.59% α-helix, 0.48% β-sheet and remaining 11.93% was coiled structure *(Verma et al., 2016; Sun et al., 2019).*

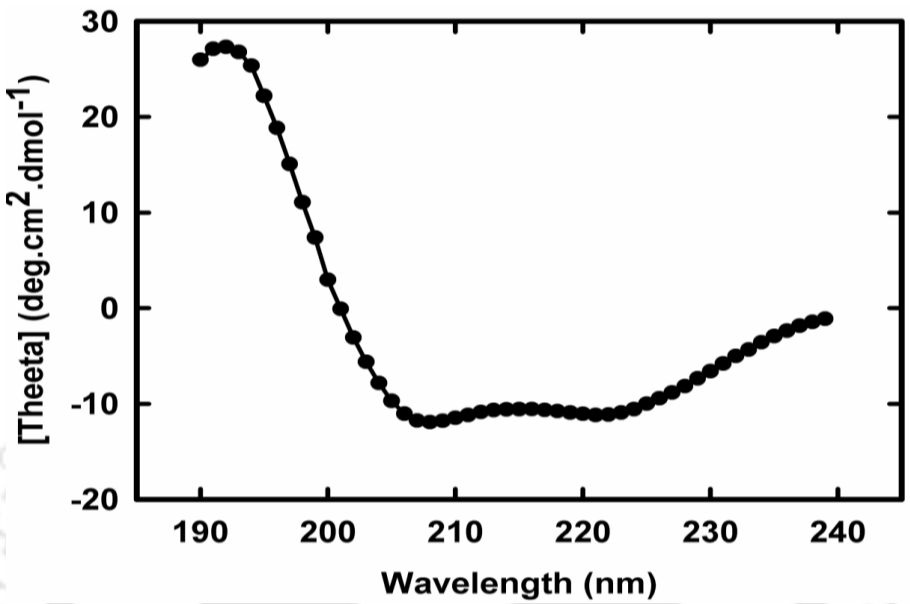

Figure 4.5: The far UV CD spectra of azoreductase enzyme, The obtained CD spectrum of azoreductase after analysis in K2D2 software, obtained data was plotted from 190 nm to 240 nm.

4.3.6 FMN docked with azoreductase enzyme

The cofactor (FMN) was docked with modelled azoreductase structure to find the residue involved in binding with cofactor. The FMN showed complete binding with azoreductase cofactor binding site. The cofactor binds with surrounding residues present only on azoreductase chain A but chain B is not involved in hydrogen bonding with FMN molecule. The residues Met95, Asn97, Phe98, Gly141, Gly142, Arg186 and His144 present on chain A, are involved in binding with modelled azoreductase protein; having binding energy - 3.8 kJ/mol (fig. 4.6D). The FMN binds into the cleft of azoreductase formed by α-helix and β-sheet (fig. 4.2B). The hydrogen bonding between the Met95, Asn97, Phe98, Gly141, Gly142, Arg186 and His144 with FMN, to stabilise the structure. If azoreductase docked with FMN structure, binds with NADH, it may destabilise the enzyme cofactor binding. Further, the potential binding of cofactor with modelled structure can be seen in fig. 4.6C and 4.6D.

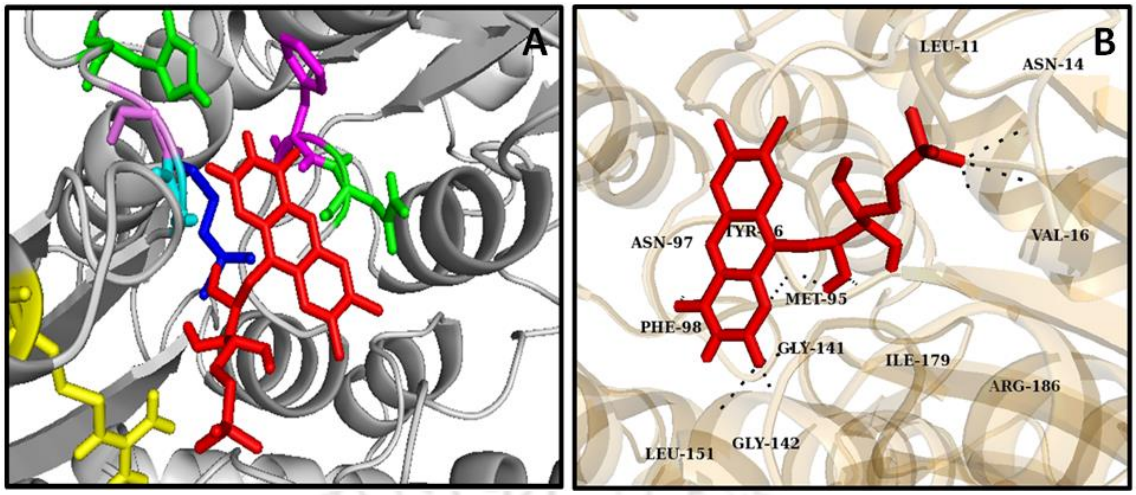

Figure 4.6: Cofactor (red) with surrounding amino acid residues (Met 95- Blue, Asn 97-Green, Phe 98-Magenta, Gly 141-Cyan, Gly 142-Orange, His 144- Green and Arg 186-Yellow) present on azoreductase enzyme (D) The cofactor showing hydrogen bond with respective amino acid residues on azoreductase with binding energy -3.8 kJ/mol.

4.3.7 Study of azoreductase after cross-linking with glutaraldehyde

The oligomeric state of azoreductase enzyme was confirmed by modelling the azoreductase enzyme and further confirmed by glutaraldehyde cross-linking. In a cross-linked experiment with glutaraldehyde, dimeric form of the structure was shown and results were compared with the control one (monomeric form). In fig. 4.7A, the dimeric form of the enzyme was clearly shown in lane 2 and monomeric form was shown in lane 1 and lane M showing the marker on SDS-PAGE. This increase in molecular weight of cross-linked azoreductase indicates the homodimeric state of azoreductase (as molecular weight gets doubled i.e. ~ 48 kDa). In fig. 4.7B, the modelled structure of azoreductase also revealed its homodimeric nature of azoreductase.

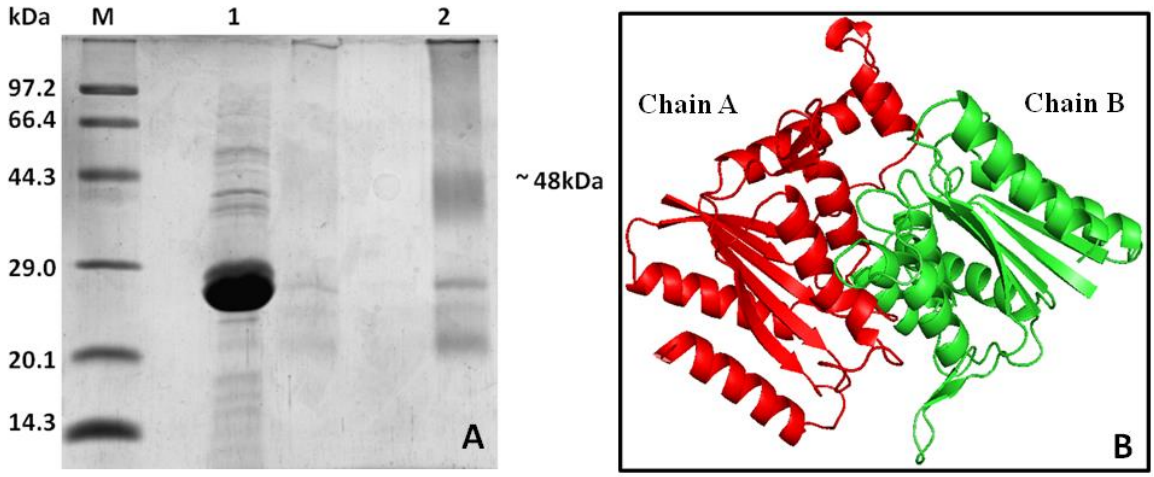

Figure 4.7: The cross-linking study of azoreductase. **(A)** The cross-linked azoreductase observed on SDS-PAGE, which indicates Lane M protein marker, Lane 1 control azoreductase showing molecular weight ~24 kDa as subunits get dissociated; Lane 2 shows cross-linked azoreductase in which protein band is around ~ 48 kDa of homodimeric azoreductase. **(B)** The modelled structure of azoreductase by using Phyre 2, which is showing the homodimeric structure.

4.4 Conclusion

The azoreductase enzyme from *C. violaceum* was found to be unique with respect to other reported azoreductases from various organisms. The oligomeric state of azoreductase enzyme was confirmed by modelling and further confirmed by cross-linking with glutaraldehyde, and it was found to be homodimeric nature. The predicted secondary structure of the enzyme was different from the structure predicted in CD spectroscopy; this may be because of the modelled structure. The azoreductase enzyme performs its function with the help of cofactor as FMN. The binding of FMN helps in the stabilization of azoreductase structure.

CHAPTER 5

Degradation of dye-waste using immobilized azoreductase enzyme from *Chromobacterium violaceum* in a continuous bead pack reactor*

ABSTRACT

Various industries utilize several types of azo dyes (containing azo group -N=N-) every day. After utilization, a majority of it cames out as industrial waste and discharged into the water without proper treatment, which causes severe pollution to the environment. In this report, we are reporting a new method with the capability for complete-degradation of azo dyes from industrial waste. This report showcases the functional role of immobilized azoreductase enzyme from *Chromobacterium violaceum* (MTCC No: 2656) as an important enzyme for the degradation of azo dyes. The purified azoreductase enzyme was immobilized on amberlite beads and the degradation efficiency of various azo dyes (methyl red, methyl orange, and amaranth dye) have been studied. The toxicity and phytotoxicity of degraded azo dyes were verified in fibroblast cell lines (L929) and *Cicer arietinum*, respectively. Further, the reusability of the immobilized azoreductase enzyme makes the process cheaper and can be utilized by various industries for the degradation of dye waste before releasing into the environment.

*** Part of the work is submitted for publications.**

5.1 Introduction

A very small amount of dye contaminant in the water affects the water transparency, gas solubility, and light penetration into the water bodies *(Banat et al., 1996; Robinson et al., 2001).* We have seen that the industrial effluents, containing textile wastewater is very difficult to treat because of the complexity and diverse chemical nature of dye in wastewater. All these parameters make the process very difficult to treat by using conventional procedures *(Alinsafi et al., 2006).* Nowadays, more than 700,000 tons of the commercial dyes are being produced annually, and more than 100,000 dyes are being utilized only in textile industries *(Lukas et al., 2007).* Around 70% of the produced dye belongs to the azo group and is released in the environment *(Zollinger et al., 2004).* Among all the synthetic dyes used during the dyeing process, azo group of dyes are significant. Azo dyes are mostly used in the textiles, plastics, pharmaceuticals, food, and cosmetics products *(Chung., 1992; Stolz., 2001).* More than 10% of total dyes, used during the dyeing process, are not bound to the fabrics and directly released into the effluent *(McMullan et al., 2001).* These dyes are very stable and persistent into the environment, which leads to environmental pollution like bioaccumulation into the aquatic life, hampering human health *(Golka et al., 2004; Saratle et al., 2011).* More than thousand types of organic azo dyes are daily used by various industries. The biodegradability and persistence nature of dye into the environment are still unexplored. These compounds are cytotoxic, carcinogenic, and might be the invisible threat to the world *(Wackett et al., 1999; Hsueh et al., 2009).* All the azo dyes produced by industries are very toxic, carcinogenic, and also mutagenic in nature *(Verma et al., 2019; Lukas et al., 2007; Zollinger., 2004; Chung et al., 1992).* It is also very difficult to degrade azo dyes using a conventional sewage treatment plant *(Stolz., 2001).* To ensure a pollution-free environment, we have to ensure the treatment of the dye-effluents before releasing into the environment. The degradation and decolorization of an azo dye is a reductive process, in which the dye is converted into an aromatic amine under anaerobic condition. The degraded aromatic amines are more dangerous than the parental ones *(Wong et al., 1996).* For complete mineralization of the azo dyes, aerobic condition is required *(Ekici et al., 2001; McMullan et al., 2001).* The complete degradation of azo dyes, under aerobic condition, have been studied using microbial, fungal, and enzymatic methods. Among these, enzymatic methods were found to be highly effective for the complete-degradation of dye effluent *(Verma et al., 2019; Stolz., 2001; Spadro et al., 1992).* In this case, single-

step enzymatic degradation in aerobic condition is enough for complete decolorization of the azo dyes. In recent years, many enzymes from different sources, have been explored to study the degradation of azo dyes. Among the various enzymes studied, azoreductase from *Chromobacterium violaceum*, was found to be a very effective enzyme for the degradation *(Verma et al., 2019).*

Recently, many environmental engineers are trying to solve the problem of textile effluent. To remediate the textile effluent, they are struggling to develop the new technology or to make a change into the existing technology *(Sathishkumar et al., 2014; Santos et al., 2007).* But none of the technologies has been implemented in large scale to solve the problem, primarily due to the toxicity of the by-products, extreme operation condition, labor-intensive and high cost process *(Hayat et al., 2015).*

In the present study, a new method is explored for the degradation of effluent azo dyes. The immobilization of the azoreductase enzyme would be preferred because of its high efficiency, easy separation of by-products, cost-effectiveness, and prevention in the consumption of enzyme during the process. The immobilization is a process of physical localization of an enzyme on the specific surface. In this process, the enzyme property was improved and enhancement of operational performance without disturbing the catalytic property *(Mateo et al., 2007; Krajewska., 2004)*. Most importantly, the reusability of the immobilized azoreductase enzyme is expected to make the process economically feasible.

5.2 Materials and methods:

5.2.1 Materials

The entire chemicals used, during the process was highly pure and of analytical grade. The azoreductase enzyme was cloned into the pET28a (+) vector to make it recombinant and further purified from the *Escherichia coli* BL21(DE3) cells by methods described by *Kamalesh et al., 2019 (Verma et al., 2019).* Amberlite MB-150 beads, glutaraldehyde, and bradford reagent were purchased from the Sigma Chemicals Co. (St. Louise, MO). Potassium phosphate buffer (K_2HPO_4 and KH_2PO_4), sodium chloride (NaCl), sodium dodecyl sulfate (SDS), sodium hydroxide, and dialysis bag were purchased from the Himedia. All the buffers were prepared in the Milli Q (Millipore, USA) water.

5.2.2 Overexpression and purification of recombinant azoreductase enzyme

The recombinant azoreductase enzyme was purified using a method described by *Kamalesh et al., 2019 (Verma et al., 2019).* The concentration of the enzyme required for immobilization was optimized and measured by bradford method where BSA was taken as a standard *(Bradford., 1976).*

5.2.3 Immobilization methods

The purified recombinant azoreductase enzyme was immobilized on amberlite bead (500 mg to 1000 mg) of size 0.5 mm diameter. The immobilization was optimized under different conditions like pH, glutaraldehyde concentration, activation time of bead with glutaraldehyde and also with enzyme concentration. The azoreductase enzyme was immobilized on glutaraldehyde activated beads. Before activation, the bead was incubated overnight with different range of pH from pH 4.0 to 10 with 25 mM phosphate buffer (K_2HPO_4 + KH_2PHO_4). The equilibrated beads were activated with various concentration of glutaraldehyde at room temperature. The different concentrations of glutaraldehyde (1% to 5% v/v) was used at various time intervals. Excess glutaraldehyde was washed extensively after the activation of amberlite bead. The complete removal of unutilized glutaraldehyde was confirmed by measuring the absorbance spectrophotometrically. The activated bead was incubated with different concentrations of enzyme for various time intervals to determine the appropriate time of coupling reaction, which correspond to the maximum immobilization of the enzyme. The beads with maximum absorbed enzyme are used for further experiments *(Singh et al., 2013).*

5.2.4 Immobilization confirmation by Field Scanning Electron Microscopy (FESEM) and Energy Dispersive X-ray (EDX)

The immobilization of azoreductase on amberlite bead (MB-150) was confirmed by FESEM analysis. For this, non-activated beads, beads activated with gluteraldehyde and beads immobilized with azoreductase were studied. Before analysis, the surface of the bead was coated with gold to make the surface conducting for better quality of an image. For analysis, sample was placed on the small siliconized surface and dried it at room temperature and directly used for analysis. EDX spectra were also recorded to quantify the elemental difference among the normal, activated, and immobilized amberlite MB-150 beads *(Singh et al., 2013; najar et al., 2018).*

5.2.5 Reactor design and setup

The laboratory-scale fixed bed reactor was setup with the main component of the reactor containing the glass column of 50 ml volume was packed with immobilized beads. One side of the column is connected to the sample container through the peristaltic pump and considered as inlet while other side is considered as an outlet. The layer of cotton (3 mm) was present on both sides of the column to support the bead and also to assist the distribution and uniformity of the effluents. The sample container contains non-degraded azo dyes which pass through column and comes out as degraded products through outlet. The rate of the process can be controlled with a peristaltic pump. The model of the reactor is shown in fig. 5.1.

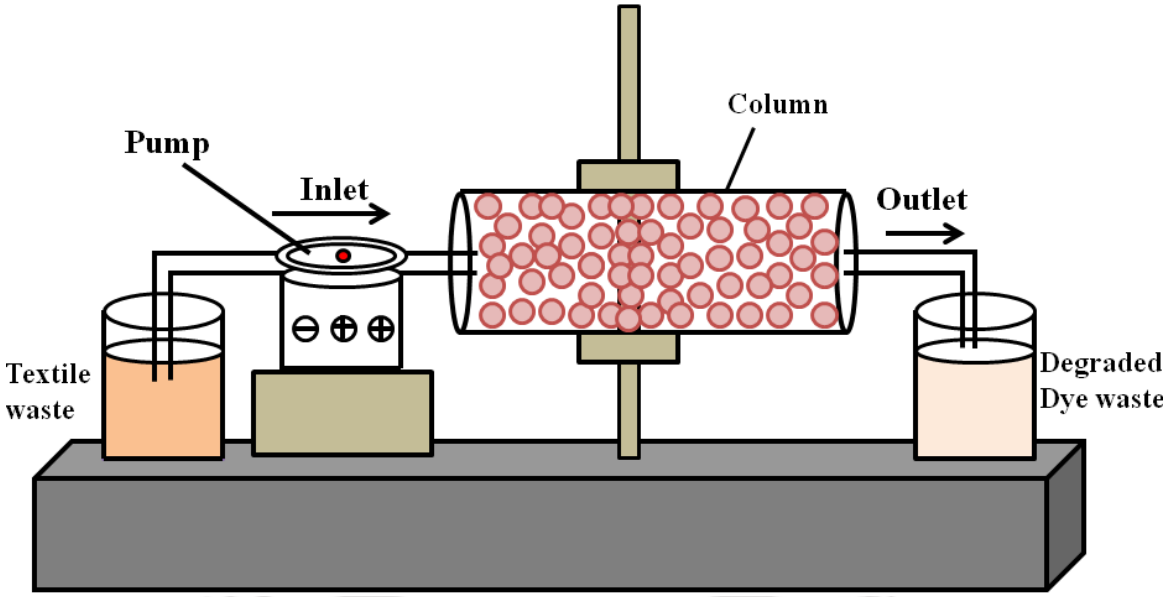

Figure 5.1: The schematic representation of fix bed pack reactor setup for the degradation of dyes.

5.2.6 Effect of pH and temperature on the activity of immobilized azoreductase enzyme

The immobilization process for azoreductase enzyme on amberlite beads, was optimized with respect to the pH. The immobilization of the azoreductase was measured at different pH range from pH 4.0 to 11. To analyze the immobilization, the entire reaction component was incubated at different pH. The reaction starts immediately after coming to the contact with immobilized enzyme. The control experiment was also performed at each pH range. All the reaction were performed in triplicate order, and the best pH was optimized for the maximum immobilization of the azoreductase enzyme *(Chen et al., 2005; Verma et al., 2019).*

The activity of immobilized azoreductase enzyme on amberlite beads was optimized with respect to the various temperatures. The activity of the azoreductase was measured with a different temperature range from 10°C to 70°C. The immobilized amberlite bead was incubated at various temperatures for 10 minutes with 25 mM phosphate buffer (25 mM K_2HPO_4 and 25 mM KH_2PO_4) at 7.2 pH. The reaction mixture contains 0.1 mM NADH, 25 mM of phosphate buffer, and 1 µM of azo dyes. The entire reaction component is incubated at different temperature range, and reaction starts immediately after the addition of immobilized enzyme to the reaction mixture. The control reaction was also performed with each reaction at different temperatures. All the experiments were done in triplicate, and the optimum temperature was analyzed by plotting results of different temperatures *(Chen et al., 2005).*

5.2.7 Biodegradation kinetic study of immobilized azoreductase enzyme

The biodegradation study of azo dye (methyl red, methyl orange, and amaranth dye) by using immobilized azoreductase enzyme was monitored by using the UV-Visible absorption spectrophotometer (Carry 100, Agilent Technology) and Fourier Transform Infrared spectroscopy (FTIR) (Perkin Elmer Spectrum two). In UV-Visible absorption spectra, the reaction was monitored by measuring absorbance of co-substrate, i.e., NADH at 340 nm, which is being used in reaction. The reaction mixture contains the continuous supply of 1 µM of azo dyes, 0.1 mM NADH and 25 mM of phosphate buffer *(Verma et al., 2019; 3 Eslam et al., 2016).* The degraded waste coming out from the reactor outlet was used for the FTIR analysis and sample was prepared by the method described in section 2.4*(Verma et al., 2019; Rosu et al., 2019).*

5.2.8 Toxicity analysis of degraded azo dyes

The assessment of the degradation efficiency of azoreductase enzyme on azo dyes (methyl orange, methyl red, and amaranth dye) was analyzed by measuring the toxicity of the degraded products. The toxicity and phytotoxicity of dyes were checked on the Hela cells and on chickpeas (*Cicer arietinum* L.) respectively. For the toxicity assay, HeLa cells were procured from the National Centre for cells science (NCCS) India. DMEM (Invitrogen) liquid media supplemented with 10% fetal bovine serum (FBS), 100 $\mu g ml^{-1}$ streptomycin, and 100 U of penicillin was used to maintain the culture. Approximately 5000 cells/well were seeded on 96 well micro-titer plates and kept it on 37°C for 24 h for adherence. After 24 h of adherence, the cells were treated with all the three degraded dyes in varying concentrations. After the treatment, again the cells were kept in a CO_2 incubator for 24 h at 37°C. After the incubation, media was removed and 200 µl of 3- (4, 5-dimethylthiazol-2-yl)- 2, 5-diphenyltetrazolium bromide (MTT) (0.5 $mg ml^{-1}$) was added into each well and again incubated for 4 h, then MTT was removed and 100 µl DMSO was added and mixed properly. The absorbance was taken at 570 nm in a micro plate reader (BIOTEK Synergy HT) *(Verma et al., 2019; Tiwari et al., 2016; Stocker et al., 2018).*

The phytotoxicity assay was performed with respect to the germination index. In phytotoxicity assay transfer of permeate (treated sample) through the membrane was tested in the seed germination process. The experiment was set up in four different types, and the results were compared with the control (distilled water), raw (untreated), and tap water. In phytotoxicity test, the 50 number of chickpeas seed (*Cicer arietinum* L.) taken in petri plates and distilled water (Contol), tap water, untreated water (raw dyes) and treated water (permeate) added into each plate separately. The petri plates were kept in the dark for 72 h. The germination index (GI%) was calculated with given equation no. 1 *(Divyapriya et al., 2014; Paul et al., 2019).*

$$\text{Germination index (GI \%)} = \frac{\text{Seed germination (\%) x Root elongation (\%)}}{100} \quad \text{Eq. 1}$$

5.2.9 Reusability of immobilized azoreductase enzyme

The reusability assay was performed by repeated use of the same bead immobilized with azoreductase until the degradation capability of the enzyme was decreased. The reusability assay for degradation studies, was analyzed by UV-Visible spectra at different time intervals. After each usage the bead was washed with phosphate buffer pH 7.5 and then used for the next batch of reaction.

5.3 Results

5.3.1 Optimization of azoreductase immobilization on amberlite beads

The azoreductase enzyme was immobilized on glutaraldehyde activated amberlite beads. The immobilization was optimized at various glutaraldehyde concentrations from 1% to 4%, activation times, coupling time and various enzyme concentrations. The best immobilization results were shown as 70.62 ± 0.71%, at 3% glutaraldehyde concentration (table 5.1), the activation time was optimized at 3% glutaraldehyde concentration, and it was found that 69.63 ± 0.56% at 4 h activation time (table 5.1). The coupling time was optimized at 3% glutaraldehyde concentration and 4 h activation time. The immobilization was found to be 67.36 ± 0.37% at 12 h coupling time (table 5.1). Further enzyme was optimized at 3% glutaraldehyde concentration, 4 h activation time and 12 h coupling time. The maximum immobilization of enzyme was found on this condition was 67.37 ± 0.61% and enzyme concentration was found to be 0.5 $mgml^{-1}$ (table 5.1).

Table 5.1: The optimization of the different parameters for the immobilization of azoreductase enzyme on amberlite bead

	The concentration of Glutaraldehyde (%)	Activation Time (h)	Coupling time (h)	Enzyme concentration ($mgml^{-1}$)	Immobilization (%)
Variation in Glut. concentration	1	5	12	0.4	55.82 ± 0.63
	2	5	12	0.4	65.46 ± 0.92
	3	5	12	0.4	**70.62 ± 0.71**
	4	5	12	0.4	67.43 ± 0.55
Variation of activation time	3	2	12	0.4	61.28 ± 0.63
	3	4	12	0.4	**69.63 ± 0.56**
	3	6	12	0.4	60.32 ± 0.72
	3	8	12	0.4	56.32 ± 0.23
Variation in coupling time	3	4	4	0.4	53.28 ± 0.73
	3	4	8	0.4	62.38 ± 0.73
	3	4	12	0.4	**67.36 ± 0.37**
	3	4	16	0.4	56.47 ± 0.82
Variation in enzyme concentration	3	4	12	0.2	59.27 ± 0.46
	3	4	12	0.3	63.37 ± 0.92
	3	4	12	0.4	64.56 ± 0.81
	3	4	12	0.5	**67.37 ± 0.61**

5.3.2 Analysis of enzyme immobilized amerlite beads by FESEM and EDX

The FESEM analysis showed change in amberlite bead surface topology after activation and immobilization as in fig. 5.2D, fig. 5.2G. Fig. 5.2A shows the control beads fig. 5.2C shows the Energy Dispersive X-ray (EDX) analysis, in which the elemental composition for control beads was analyzed. Fig. 5.2D and 5.2E shows, 100 µm and 2 µm size of activated amberlite beads, while 5.2F was shows EDX for activated beads. The fig. 5.2G and 5.2H shows the 100 µm and 2 µm size of enzyme immobilized beads and 5.2I showed EDX graph, which shows that elemental composition for enzyme immobilized on beads.The activation of bead and immobilized enzymes on the surface was clearly confirmed by the FESEM analysis.

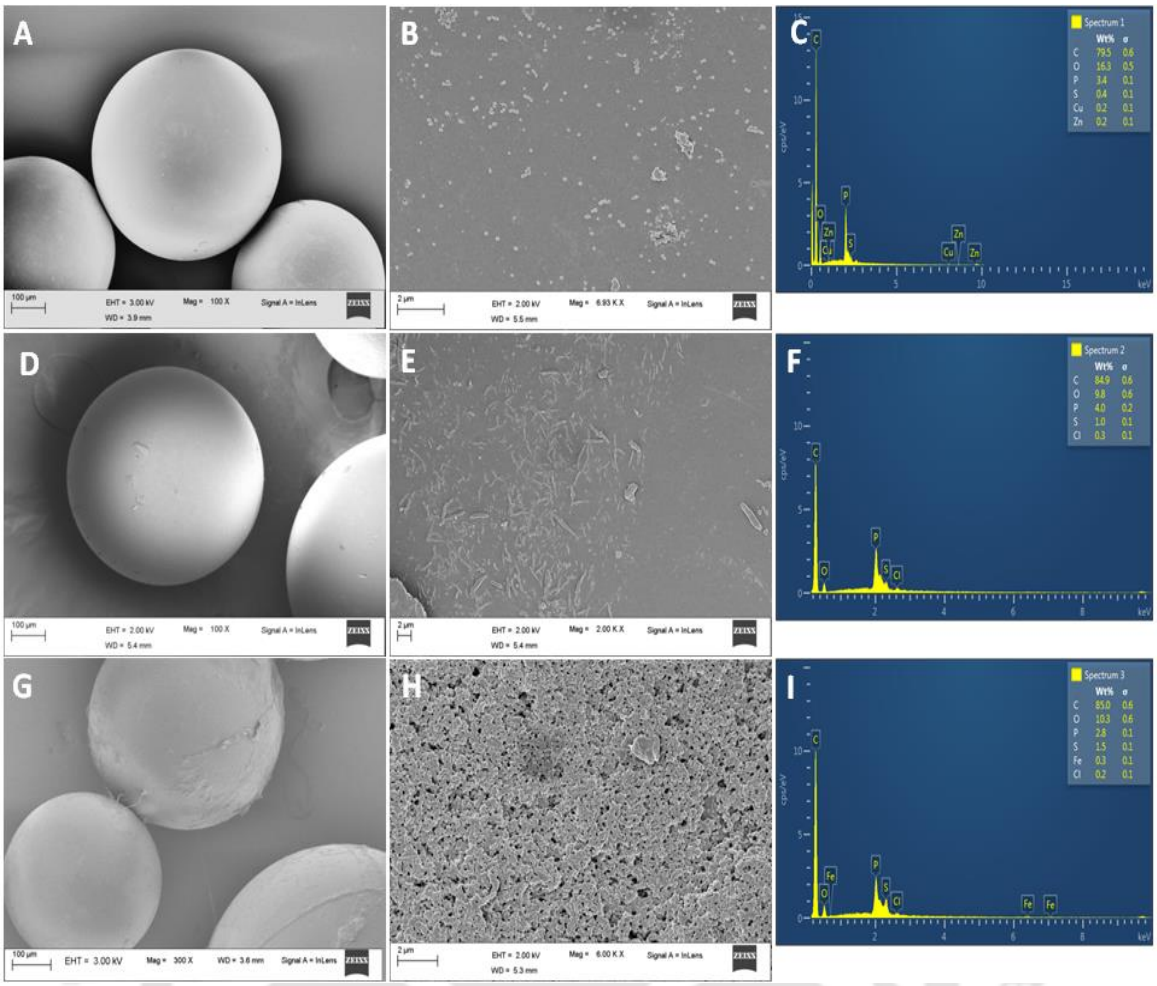

Figure 5.2: Immobilization of enzyme on amberlite bead by FeSEM analysis: (A) and (B) showing the normal amberlite bead with 100 µm and 2 µm size. (C) And (D) showing the glutaraldehyde activated amberlite bead, with 100 µm and 2 µm size and fig. (G) and (H) showing the azoreductase immobilized on amberlite beads. Fig. C, F and I were showing EDX analysis in which elemental composition varies with the respective image and composition.

5.3.3 Effect of pH on immobilization of azoreductase enzymes and the effect of temperature on its activity

Since we know that the amberlite beads contain the mixture of cationic and anionic resins, It is the important parameter to find out the suitable pH for the immobilization of azoreductase enzyme on amberlite beads. For this, the beads were equilibrated at different pH for overnight and activated with glutaraldehyde (3% v/v) for 6 h. The activated beads were washed and incubated for 12 h with azoreductase enzyme for immobilization. The maximum immobilization of azoreductase was found at pH 7.0 (fig. 5.3A). The maximum immobilization occurs at pH 7.0 may be because of the proper ionization of bead surface. Further, the temperature was optimized for the activity of immobilized

azoreductase enzyme and optimum activity was measured in the temperature range 30°C to 40°C (fig.5.3B)

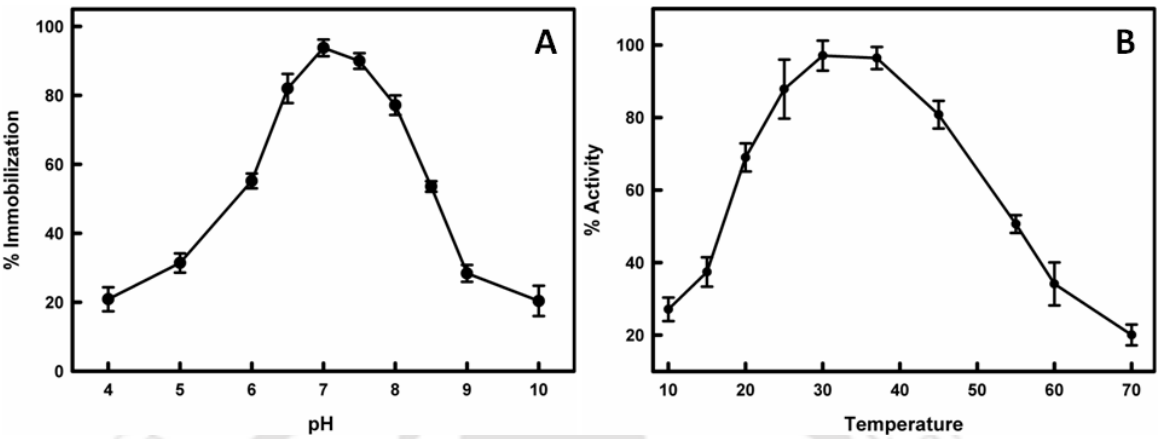

Figure 5.3: Effect of pH on immobilization of azoreductase enzyme: (A) showing the pH optima in which maximum immobilization was found at the ~7 pH and in (B) showing the temperature optima in which 30°C to 40°C temperature is most suitable for the activity of immobilized azoreductase enzymes.

5.3.4 Comparative study for the degradation of dyes with UV-Visible spectroscopy

The complete degradation of methyl orange, methyl red, and amaranth dyes was studied by using the UV-Visible spectroscopy shown in the fig. 5.4. The degradation of dyes was studied by decreasing the absorption spectra of NADH with different time intervals. The degradation of various azo dyes was compared with control, which gives a clear understanding of the degradation of various azo dyes. In UV- Visible degradation, the complete degradation of methyl red was found to be very fast and easily degraded within minutes but the degradation of methyl orange and amaranth dye was little slow as compaired to methyl red dye (fig. 5.4).

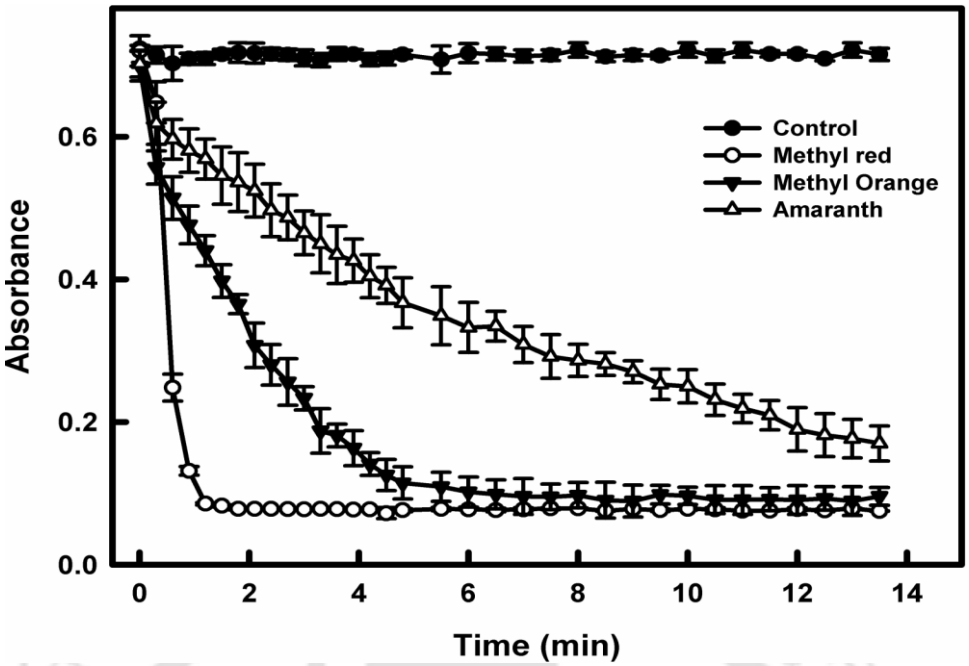

Figure 5.4: Degraded effluent dyes by absorption in UV-Visible: The methyl red dye was degraded very fast, the degradation of methyl orange was a little bit slow as compared to the methyl red and the degradation of amaranth dye very slow as compared to the methyl red and methyl orange.

5.3.5 Comparative study for the degradation of dyes FTIR analysis

In FTIR spectra, degraded azo dyes (methyl red, methyl orange, and amaranth dye) was analysed and result was compared with non-degraded dye. The fig. 5.5A and 5.5B shows the control and degraded amaranth azo dye which depict the N-H bond stretching and conversion of the azo bond into the primary amine. The primary amine peak was shown at 3436 and 1645 cm^{-1} respectively and peak shown at 764 cm^{-1} represents the N-H wagging for primary and secondary amine. In fig. 5.5C and 5.5D shows control and degraded methyl red dye in which peak at 3436 and 1645 cm^{-1} depict the N-H bond stretching and peak at 764 cm^{-1} shows the N-H bond wagging for primary and secondary amine. Moreover, fig. 5.5E and 5.5F shows the control and degraded methyl orange dye. The peak found at 3436 and 1645 cm^{-1} in fig. 5.5F shows N-H stretching and peak at 764 cm^{-1} shows the N-H wagging for primary and secondary amine respectively.

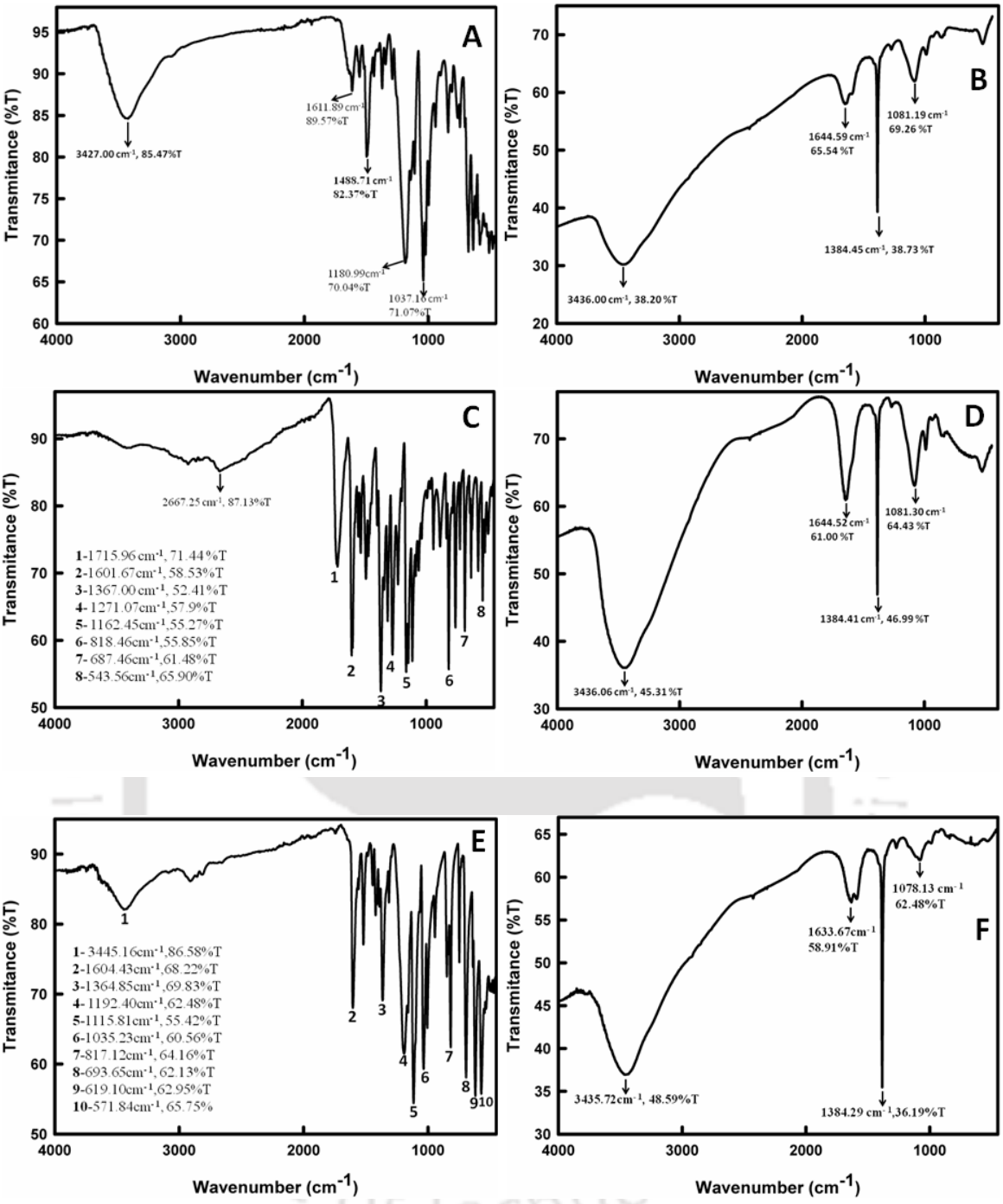

Figure 5.5: Analysis of degraded azo dye by FTIR spectra, (A) and (B) showing the control and degraded amaranth azo dye in which the peak at 3436 and 1645 cm^{-1} showing N-H stretching and conversion of azo bond into the primary amine and peak found at 764 cm^{-1} showing the N-H wagging for primary and secondary amine respectively (C) and (D) showing the control and degraded methyl red dye, in which the same results was found as amaranth dye after degradation, in which peak showing at 3436 and 1645 cm^{-1} represented as N-H stretching and conversion of azo bond into the primary amine and peak at 764 cm^{-1} showing the N-H wagging for primary and secondary amine while in (E) and (F) showing the control and degraded methyl orange dyes in which the peak showing at 3436 and 1645 cm^{-1} represented as N-H stretching and conversion of azo bond into the primary amine and peak found at 764 cm^{-1} showing the N-H wagging for primary and secondary amine respectively.

5.3.6 Toxicity study of degraded azo dyes on L929 cells

The toxicity assay for azo dye shows significant cell death at various concentration of azo dyes. The concentration of non-degraded and degraded azo dye was varied from the 20 μgml^{-1} to 1000 μgml^{-1}. Fig. 5.6A shows 78% to 10% of cells was viable from the 20 μgml^{-1} to 1000 μgml^{-1} concentration of non-degraded methyl red dye while after degradation the percent viability of the cells increased from 90% to 60% at same range of concentration. Moreover, fig. 5.6B shows 80% to 7% of cells was viable at 20 μgml^{-1} to 1000 μgml^{-1} concentration of non-degraded methyl orange dye while after degradation the percent viability of the cells increased from 90% to 66% at same range of degraded methyl orange concentration. The same pattern was observed with amaranth dyes in which 77% to 8% of cells was viable from the 20 μgml^{-1} to 1000 μgml^{-1} concentration of non-degraded amaranth dye while after degradation the percent viability of the cells increased from 85% to 63% at same range of concentration for amaranth dyes (fig. 5.6C). The increase in percent viability of cell after degradation of dye clearly indicate that the decrease in toxicity of methyl red dyes and azoreductase enzyme play a significant role in the reduction of toxicity of all azo dyes.

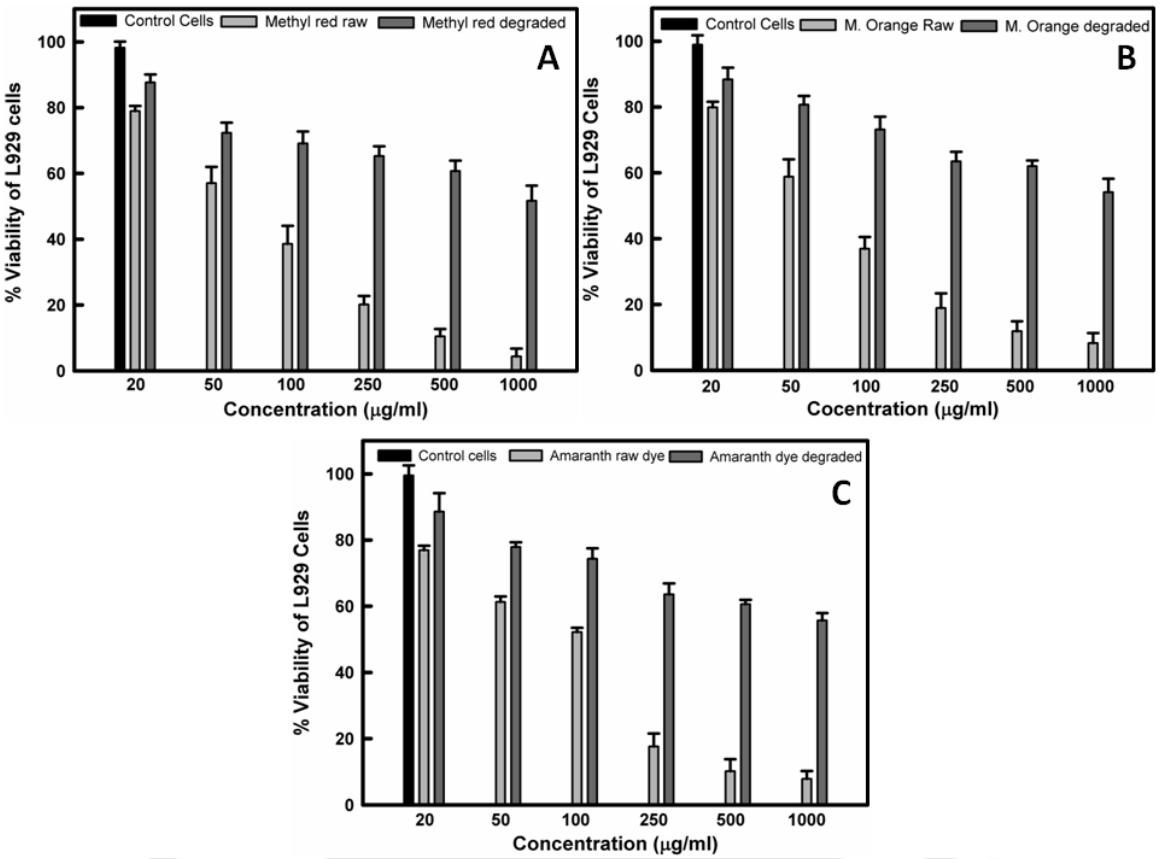

Figure 5.6: Toxicity assay for various azo dyes by MTT assay: this assay was performed on fibroblast cells (L929 cells) in which the normal healthy cells ware taken as control and various concentration of degraded and un-degraded azo dye used for the assay. Fig 5.6A showing the toxicity of methyl red in which 78% to 10% of cells was viable from the 20 μgml^{-1} concentration to 1000 µg/ml concentration in un-degraded dye while after degradation the percent viability of the cells increased from 90% to 60% at 20 μgml^{-1} concentration to 1000 μgml^{-1} concentration. Fig 5.6B showing the toxicity of methyl orange in which 80% to 7% of cells was viable at 20 μgml^{-1} to 1000 μgml^{-1} concentration of un-degraded methyl orange dye while after degradation the percent viability of the cells increased from 90% to 66% at 20 μgml^{-1} to 1000 μgml^{-1} concentration while in fig. 5.6C showing the toxicity of amaranth dye in which 77% to 8% of cells was viable from the 20 μgml^{-1} to 1000 μgml^{-1} concentration of un-degraded amaranth dye while after degradation the percent viability of the cells increased from 85% to 63% at 20 μgml^{-1} to 1000 μgml^{-1} concentration of degraded amaranth dyes.

5.3.7 Phyto-toxicity study of degraded azo dye on chickpea (Cicer arietinum)

The germination index (GI) for *Cicer arietinum* was used to study the phytotoxicity of untreated (raw) and treated dyes collected from continuous bead pack reactor. The GI is a very important parameter for the assessment of toxicity. It is used to monitor the change in phytotoxicity of non-degraded and degraded azodyes. The germination index was calculated as per equation 1,. For analysis, raw dye (without degradation), degraded dyes, milli Q water, and tap water was used as control. The GI for the raw dye (undegraded) was calculated as 28% (fig. 5.7A), the GI for degraded dye 83% (fig.5.7B), GI for milli Q

water was 65% (fig. 5.7D) and GI for the tap water system (Control) was 96% (fig. 5.7C) which is the highest among all. The increase in GI after degradation clearly indicates the removal of the toxic compound from the dye waste.

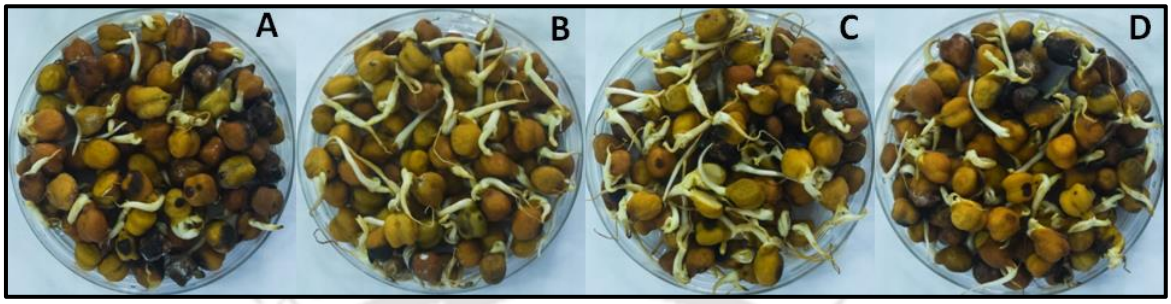

Figure 5.7: Germinated *Cicer arietinum* seed in various condition: (A) showing the seed grown under the raw dye condition (mixture of methyl orange, methyl red and amaranth dye) (B) showing the seed grown under the degraded dye condition with enzyme (C) showing the seed grown under tap water (control) and (D) showing the seed grown under the Milli Q water condition.

5.3.8 Reusability of immobilized azoreductase enzyme

The reusability assay of immobilized amberlite beads is the most important parameter to study the degradation of various azo dyes with immobilized azoreductase enzymes. This reusability assay enhance the importance of methods and it makes the immobilized enzyme a preferred choice as compare to the soluble forms. The azoreductase enzyme immobilized on amberlite beads showed 25% activity till the 15^{th} batch of reaction cycle (fig. 5.8).

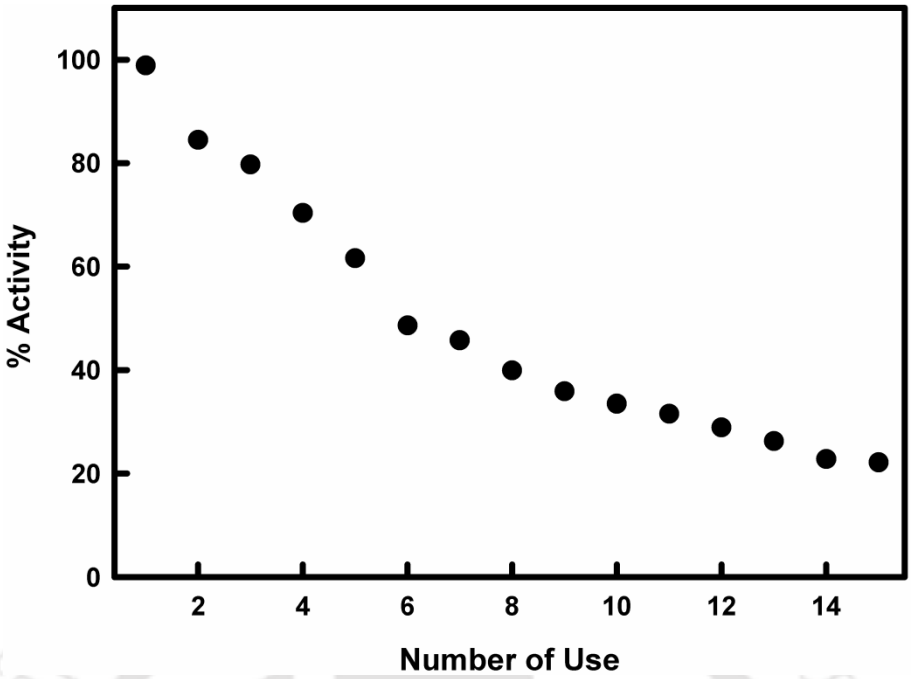

Figure 5.8: Reusability of azoreductase enzyme immobilized on amberlite beads, the reusability assay was performed by the repeated use of same beads, and before next round of use the beads were washed with phosphate buffer (pH 7.5).

5.4 Discussions

More than 700,000 tons of the commercial dyes are being produced annually, and more than 100,000 dyes are being utilized only in textiles industries *(Lukas et al., 2007).* Apart from the total production, around 70% of them belongs to the azo group and is released into the environment after utilization *(Zollinger., 2004).* Immobilization is also preferred because of the reusability of enzymes after completion of the process. For immobilization, the azoreductase enzyme from the *C. violaceum* was found to be the most effective enzyme because of their better degradation capability. The reason for choosing the azoreductase from *C. violaceum* was already explained in my previous report *(Verma et al., 2019).* In this report the various parameters to immobilize the azoreductase enzyme on amberlite beads have been explored in detail, and each condition is available in table 1. The beads were activated with different concentration of glutaraldehyde, activation times, coupling time, and various enzyme concentrations. The best-suited condition for immobilization was 3% glutaraldehyde, 4 h activation time, 12 h coupling time, and 0.5% enzyme concentration. The maximum immobilization, under each condition, was found to be 70.62 ± 0.71%, 69.63 ± 0.56%, 67.36 ± 0.37% and 67.37 ± 0.61% respectively.

Immobilization of enzyme on beads were also confirmed by FESEM analysis shown in fig. 5.2. The normal amberlite beads was shown in fig. 5.2A and 5.2B whereas, fig. 5.2D and 5.2E showing the beads after activation with glutaraldehyde. The clear patches of azoreductase enzyme found on the surface of beads in fig. 5.2G and 5.2H also confirm the immobilization of the enzyme on the surface. The respective EDX elemental analysis also confirms the activation and enzyme immobilization. There is another parameter such as pH, which also play role in immobilization of enzymes. The immobilization was performed with various pH ranges from 4.0 to 10, but the maximum immobilization was found at pH 7.0 (fig. 5.3A). This is because of the proper ionization of beads at pH 7.0 *(Beynon et al., 1989)*. After immobilization of enzyme, the temperature was also optimized from the 10°C to 70°C temperature. The maximum activity of immobilized azoreductase was found to be in the range 30°C to 40°C (fig. 5.3B) The temperature range for the azoreductase enzyme was varied from species to species. But in this case, a wider range of temperature makes the enzyme more perfect for the degradation and decolorization of azo dye effluents. Since we know that the dye is very toxic to human health as well as to the environment, the degradation of such dyes are very important. The immobilized azoreductase enzyme on amberlite beads, packed into column, help in the degradation of commonly used azo dyes (methyl red, methyl orange and amaranth dyes) in very large scale and in a very easy way. The complete setup of the reactor is shown in fig. 5.1.

The degradation of the dyes was studied using UV- Visible spectroscopy and FTIR analysis. The rate of degradation of methyl red was found to be faster as compared to methyl orange and amaranth dyes, and methyl orange degraded more easily as compared to the amaranth dyes. The degradation results was compared with control dye. Complete degradation of methyl red, methyl orange, and amaranth dye were calculated to be within 1 minute, 1.5 minutes and 10 minutes, respectively (fig. 5.4). The degraded products coming out as effluents were also analysed by FTIR for the conformation of degradation. In FTIR analysis, the azo bond (N=N) in a raw dye was reduced to $-NH_2$. The fig. 5.5A shows the FTIR for pure amaranth dye in which 1488 cm^{-1} peak was due to the azo bond. In fig. 5.5B, the 1488 cm^{-1} peak completely disappeared, and new peaks at 3436 and 1645 cm^{-1} appeared, showing N-H stretching, thereby suggesting conversion of the azo bond into the primary amine. Another peak at 764 cm^{-1} showing the N-H wagging for primary and secondary amine suggesting presence of amine group. The same pattern

was also found in fig. 5.5C and 5.5D for methyl red and in fig. 5.5E and 5.5F for methyl orange dyes. After analyzing all the data, it is very clear that azo bonds reduced into the primary or secondary amine and complete degradation of azo dyes took place with the presence of immobilized azoreductase enzyme.

The dyes used in different industries are very toxic to human health and also for aquatic life because, most of the unutilized water containing dye effluents are being utilized for agricultural fields or discharged into the water body. Hence, determining the toxicity of these dye effluents is a necessity before releasing into the environment. The toxicity of the degraded dye effluent was monitored in fibroblast cell (L929) (fig. 5.6) and chickpea seed (*Cicer arietinum*) (fig. 5.7). The toxicity of dye was verified in respect to the percent viability of cells and germination index of the chickpea seeds. Fig. 6.6A shows the toxicity of methyl red dye in which 78% to 10% of cell is viable at 20 μgml^{-1} to 1000 µg/ml concentration of raw dyes but after degradation of dye the percent viability of cell increased from 90% to 60% at 20 μgml^{-1} to 1000 μgml^{-1} concentration of degraded methyl red dye. In fig. 6.6B the toxicity of the methyl orange is demonstrated in which the percent viability of cells was found to be the 80% to 7% at 20 μgml^{-1} to 1000 μgml^{-1} concentration of raw methyl orange dye. However, after degradation of dye the percent viability of the cells increased up to 90% to 66% at same range of dye concentration. The toxicity for the amaranth dye was explained in fig. 5.6C in which the percent viability of the cells was found to be 77% to 8% at 20 μgml^{-1} to 1000 μgml^{-1} concentration. This percent viability of the cells increased up to 85% to 63% at the same concentration of degraded amaranth dyes. The increase in percent viability of the cells after the degradation of all dye confirms that toxicity of dye was reduced. Concerning the toxicity assay, fig. 5.7 showed that the toxicity of azo dye was measured on chickpea seed with respect to the germination index of chickpea seed. The seed was grown at various conditions, and GI was measured. The GI for seed grown under raw dye was calculated as 28% (fig. 5.7A), the GI for seed grown under degraded dye condition was 83% (fig. 5.7B), again the GI for seed grown under Milli-Q water was measured as 65% (fig. 5.7D) and GI for seed grown under tap water (control) was calculated as 96% (fig. 5.7C). The germination index for the dye was increased from 28% to 83% after degradation of dye with azoreductase enzyme. This further varifies the importance of the present method and role of immobilized azoreductase for the removal of the toxic compound from the dye effluents. The decrease in toxicity of the wastewater containing dye effluents helps the

farmer to utilize the wastewater for the agricultural lands. By using this method we can use such water for irrigation. The reduction in the toxicity of dye after treatment with azoreductase enzyme assured that there is no toxicity in the effluent. Therefore, such methods can be utilized in industries for the degradation of dye effluents. The reusability assay for the immobilized enzyme on beads also make the methods more useful, cheaper and cost-effective for the continuous degradation of azo dyes effluents (fig. 5.8).

5.6 Conclusion

There are many azo dyes which are used by various industries. These dye are very toxic to human health, plant, and aquatic life because of the dye that are being discharged into the water body without proper degradation. In this study, we are trying to solve the pollution caused by various azo dye effluents with the development of a new method. In this method, the immobilized azoreductase enzyme from *C. violaceum* on amberlite beads was used for continuous degradation of the dye effluents. All the parameter regarding immobilization and degradation were optimized. To test the reusability, the bead pack reactor was run several times in continuous mode which showed efficient degradation of the dyes. The degraded dye showed very low toxicity in fibroblast L929 cell line and very high germination index on chickpea seeds**.** All the data indicates that this bead pack reactor has great potential for degrading dyes from wastewater and can play a great role in cleaning of the environment. The future objective of this works is to validate the methods for degradation of dye effluent with immobilized azoreductse enzyme at pilot scale and industrial scale.

Bibliography

Acuner A, Dilek F. B. (2004) Treatment of tectilon yellow 2G by *Chlorella vulgaris* Process Biochemistry, 39, 623-631.

Adedayo O, Javadpour S, Taylor C, Anderson W. A, Moo-Young M. (2004) Decolorization and detoxification of methyl red by aerobic bacteria from a wastewater treatment plant. World Journal of Microbiology and Biotechnology. 20, 545-550.

Aksu Z. (2003) Reactive dye bioaccumulation by *Saccharomyces cerevisiae*, Process Biochemistry, 38(10), 1437-1444.

Ali H, Khan E, Sajad M. A. (2013) Phytoremediation of heavy metals: concepts and applications, Chemosphere, 91, 869-881.

Alinsafi A, Motta M. da, Bonte S. Le, Pons M. N, Benhammou A. (2006) Effect of variability on the treatment of textile dyeing wastewater by activated sludge, Dyes and Pigments. 69, 31- 39.

Allegre C, Maisseu M, Charbit F, Moulin P. (2004) Coagulation-flocculation-decantation of dye house effluents: concentrated effluents. J Hazard Mater.116(1-2), 57-64.

Anliker R. (1979) Ecotoxicology of dyestuffs – a joint effort by industry. Ecotox. Environ. Safe. 3, 59-74.

Apiyo D, Wittung-Stafshede P. (2002) Presence of the cofactor speeds up folding of Desulfovibrio desulfuricans flavodoxin. Protein Sci. 5, 1129-35.

Azmi W, Sani R. K, Banerjee U. C. (1998) Biodegradation of triphenylmethane dyes. Enzyme Microb Technol. 22 (3), 185-91.

Bafana A, Devi S. S, Chakrabarti T. (2011) Azo dyes: past, present and the future. Environmental Reviews 19, 350–370.

Balsera M, Arellano J. B, Pazos F, Devos D, Valencia A, De Las Rivas J. (2003) The single tryptophan of the PsbQ protein of photosystem II is at the end of a 4-alpha-helical bundle domain. Eur J Biochem. 270, 3916-27.

Banat I. M, McMullan G, Meehan C, Kirby N, Nigam P, Smyth W. F, Marchant R. (1999) Microbial decolorization of textile dyes present in textile industries effluent. In: Proceedings of the Industrial Waste Technical Conference, Indianapolis, USA. 1–16.

Banat I. M, Nigam P, Singh D, Marchant R. (1996) Microbial decolorization of textile dye-containing effluents: a review Bioresour. Technol. 58, 217-227.

Barr D. P, Aust S. D. (1994) Mechanisms white rot fungi use to degrade pollutants, Environ. Sci. Technol. 28, 320-328.

Baughman G. L, Perenich T.A. (1988) Fate of dyes in aquatic systems: I. Solubility and partitioning of some hydrophobic dyes and related compound. Environ Toxicol Chem. 7, 183-199.

Baumler W, Eibler E. T, Hohenleutner U, Sens B, Sauer J, Landthaler M. (2000) Q-switch laser and tattoo pigments: first results of the chemical and photophysical analysis of 41 compounds. Lasers Surg Med. 26, 13-21.

Behrend R, Meyer E, Rusche F. (1905) About condensation products of glycoluril and formaldehyde (Ueber Condensationsprocucte aus Glycoluril und Formaldehyd) Justus Liebig's Ann. Chem. 339,1-137.

Bertoldi M, Cellini B, Laurents D. V, Borri Voltattorni C. (2005) Folding pathway of the pyridoxal 5'-phosphate C-S lyase MalY from *Escherichia coli.* Biochem J. 389, 885-98.

Beynon R. J, Bond J. S. (1989) Proteolytic Enzymes-A Practical Approach, Oxford: IRL Press. 18 (1989) 25–55.

Bhoir S, Shaik A, Thiruvenkatam V, Kirubakaran S. (2018) High yield bacterial expression, purification and characterisation of bioactive Human Tousled-like Kinase 1B involved in cancer. Sci Rep. 8, 4796-4804.

Bilal M, Asgher M. (2015) Dye decolorization and detoxification potential of Ca-alginate beads immobilized manganese peroxidase. BMC Biotechnol. 15 (1), 1.

Bin Y, Jiti Z, Jing W, Cuihong D, Hongman H, Zhiyong S, Yongming B. (2004) Expression and characteristics of the gene encoding azoreductase from *Rhodobacter sphaeroides* AS1.1737, FEMS Microbiol Lett. 236, 129-36.

Black J. J, Holmes M, Dymerski P. P, Zapisek W. F. (1980) Fish tumor pathology and aromatic hydrocarbon pollution in a Great lakes estuary. In: *Hydrocarbons and Halogenated Hydrocarbons in the Aquatic Environment.* Plenum Press. New York. 559-565.

Blumel S, Knackmuss H. J, Stolz A. (2002) Molecular cloning and characterization of the gene coding for the aerobic azoreductase from *Xenophilus azovorans* KF46F. Appl. Microbiol. Biotechnol. 68, 3948-3955.

Boer C. G, Obici L, de Souza C. G, Peralta R. M. (2004) Decolorization of synthetic dyes by solid state cultures of Lentinula (Lentinus) edodes producing manganese peroxidase as the main ligninolytic enzyme. Bioresour Technol. 94(2), 107-12.

Bornhorst J. A, Falke J. J. (2000) Purification of proteins using polyhistidine affinity tags. Methods Enzymol. 326, 245-54.

Bradford M. M. (1976) A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brahimi-Horn M. C, Lim Liany S. L, Mou D. G. (1992) Binding of textile azo dyes by *Mirothecium verrucaria* Orange II, 10B (blue) and RS (red) azo dye uptake for textile wastewater decolorization. J. Ind. Microbiol. 10, 245–261.

Brazilian National Genome Project Consortium. The complete genome sequence of *Chromobacterium violaceum* reveals remarkable and exploitable bacterial adaptability (2003) ProcNatl Acad Sci U S A. 100 (20), 11660-5.

Buschmann H. J, Gardberg A, Schollmeyer E. (1994) Decoloration of textile wastewater through formation of inclusion complexes with dyes. Textil veredelung, 29, 58-60.

Caldinelli L, Iametti S, Barbiroli A, Fessas D, Bonomi F, Piubelli L, Molla G, Pollegioni L. (2008) Relevance of the flavin binding to the stability and folding of engineered cholesterol oxidase containing noncovalently bound FAD, ProteinSci. 17 (3) (2008) 409–419.

Carias C.C, Novais J.M, Martins-Dias S. (2008) Are *Phragmites australis* enzymes involved in the degradation of the textile azo dye acid orange 7? Bioresour. Technol. 99 (2), 243-251.

Carliell C. M, Barclay S. J, Naidoo N, Buckley C. A, Mulholland D. A, Senior E. (1995) Microbial decolourisation of a reactive azo dye under anaerobic conditions. Water S.A. 21, 61-69.

Centre for Science and Environmen (CSE) (2004) 'Not a Non-Issue', *Down to Earth*, 12, 19.

Chang J. S, Chou C, Lin Y. C, Lin P. J, Ho J. Y, Hu T. L. (2001) Kinetic characteristics of bacterial azo-dye decolorization by *Pseudomonas luteola*. Water Res. 35, 2841-50.

Chen H, Hopper S. L, Cerniglia C. E. (2005) Biochemical and molecular characterization of an azoreductase from *Staphylococcus aureus*, a tetrameric NADPH-dependent flavoprotein. Microbiology.151, 1433-41.

Chen H, Wang R. F, Cerniglia C. E. (2004) Molecular cloning, overexpression, purification, and characterization of an aerobic FMN-dependent azoreductase from *Enterococcus faecalis*. Protein Expr. Purif. 34, 302-310.

Chen H. (2006) Recent advances in azo dye degrading enzyme research. Curr. Protein Peptide Sci. 7, 101-111.

Chenna R, Sugawara H, Koike T, Lopez R, Gibson T. J, Higgins D. G, Thompson J. D. (2003) Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res. 31, 3497-500.

Chinwetkitvanich S, Tuntoolvest M, Panswad T. (2000) Anaerobic decolorization of reactive dyebath effluents by a two stage UASB system with Tapioca as co-substrate. Water Research. 34, 2223-2232.

Choy K. K. H, McKay G, Porter J.F. (1999) Sorption of acid dyes from effluents using activated carbon Resour. Conserv. Recy. 27, 57-71.

Chung K. T, Stevenes S. E. (1992) The production of azo dyes by intestinal microflora. Crit. Rev. Microbiol.18, 175-190.

Chung. K. T, Stevens Jr. S. E. (1993) Degradation azo dyes by environmental microorganisms and helminthes. Environmental toxicology and chemistry. 12, 2121-2132.

Coughlin M. F, Kinkle B. K, Bishop P.L. (1999) Degradation of azo dyes containing amino naphthol by *Sphingomonas* sp. strain ICX. Journal of Industrial Microbiology and Biotechnology. 23, 341-346.

Crini G, Lichtfouse E. (2016) Green adsorbents for pollutant removal. Environmental chemistry for a sustainable world.

Cui D, Li G, Zhao D, Gu X, Wang C, Zhao M. (2012) Purification and characterization of an azoreductase from *Escherichia coli* CD-2 possessing quinone reductase activity. Process Biochem. 47, 544-49.

Dantz D, Otyakmaz O, Buschmann H. J, Schollmeyer E. (1998) Separation of aromatic compounds with the supramolecular ligand cucurbituril. 91, 305-314.

Demoss R. D, Happel M. E. (1959) Nutritional requirements of *Chromobacterium violaceum*, J Bacteriol. 77, 137- 41.

Deu E., Kirsch J. F. (2007) Cofactor-directed reversible denaturation pathways: the cofactor-stabilized Escherichia coli aspartate aminotransferase homodimer unfolds through a pathway that differs from that of the apoenzyme. Biochemistry. 46, 5819-29.

Divyapriya S, Dimi D, Deepthi K. P. (2014) Biochemical effect of industrial effluence on germinating seeds of *Cicer arientum*. Int. J. Pharm. Pharm. Sci. 6, 538-542.

Dong X, Zhou J, Liu Y. (2003) Peptone–induced biodecolorization of reactive Brilliant blue (KN-R) by *Rhodocycus gelatinosus* XL-1, Process Biochem. 39, 89–94.

Dos Santos A. B, Cervantes F. J, van Lier J.B. (2007) Review paper on current technologies for decolourisation of textile wastewaters: Perspectives for anaerobic biotechnology. Bioresource Technology. 98 (12), 2369-2385.

Duddempudi P. K, Nakashe P, Blanton M. P, Jansen M. (2013) The monomeric state of the proton-coupled folate transporter represents the functional unit in the plasma membrane. FEBS J. 280, 2900-15.

Duhovny D, Nussinov R, Wolfson H. J. (2002) Efficient Unbound Docking of Rigid Molecules Proceedings of the 2'nd Workshop on Algorithms in Bioinformatics (WABI) Rome, Italy. 2452, 185-200.

Egboka B. C, Nwankwor G. I, Orajaka I. P, Ejiofor A. O. (1989) Principles and problems of environmental pollution of groundwater resources with case examples from developing countries. Environ Health Perspect. 83, 39-68.

Ekici P, Leupold G, Parlar H. (2001) Degradability of selected azo dye metabolites in activated sludge system. Chemosphere. 44, 721– 8.

Eslam M, Amoozegar M. A, Asad S. (2016) Isolation, cloning and characterization of an azoreductase from the halophilic bacterium *Halomonas elongate*. Int J Biol Macromol. 85, 111-6.

Field J.A, Stams A.J.M, Kato M, Schraa G. (1995) Enhanced biodegradation of aromatic pollutants in co cultures of anaerobic and aerobic bacterial consortia. Antonie van Leeuwenhoek 67, 47-77.

Forgacs E, Cserhati T, Oros G. (2004) Removal of synthetic dyes from wastewaters: a review. Environment International. 30(7), 953-971.

Fu Y, Viraraghavan T. (2000) Removal of a dye from an aqueous solution by fungus *Aspergillus niger*. Water Qual. Res. J. Canada. 35 (1), 95-111.

Fu Y, Viraraghavan T. (2001) Fungal decolorization of dye wastewaters: a review. Bioresour Technol. 79(3), 251-62.

Gadd, G. M. (1990) Biosorption. Chemistry and Industry. 421–426.

Gahr F, Hermanutz. F, Oppermann. W. (1994) Ozonation – An Important Technique To Comply With New German Laws For Textile Wastewater Treatment, Water Sci Technol, 30 (3), 255-263.

Gardossi L, Poulsen P. B, Ballesteros A, Hult K, Svedas V. K, Vasic-Racki D, Halling P. J. (2010) Guidelines for reporting of biocatalytic reactions. Trends Biotechnol. 28 (4), 171-180.

Ghosh D. K, Mandal A, Chaudhuri J. (1992) Purification and partial characterization of two azoreductases from *Shigella dysenteriae* type 1. FEMS Microbiol Lett. 77, 229-33.

Giardina P, Faraco V, Pezzella C, Piscitelli A, Vanhulle S, Sannia G. (2010) Laccases: a never-ending story. Cell. Mol. Life Sci. 67, 369-385.

Glick B. R. (2010) Using soil bacteria to facilitate phytoremediation, Biotechnol. Adv. 28, 367-374.

Gogate P. R, Pandit A. B. (2004) A reviews of imperative technologies for wastewater treatment II: hybrid methods. Advances in Environmental Research. 8, 553.

Golka K, Kopps S, Myslak Z. W. (2004) Carcinogenicity of azo colorants: influence of solubility and bioavailability. Toxicol. Lett. 151, 203-210.

Gonzalez-Gutierrez L. V, Escamilla-Silva E. M. (2009) Reactive red azo dye degradation in a UASB bioreactor: mechanism and kinetics. Eng Life Sci 9, 311-316.

Gorga F. R, Lienhard G. E. (1982) Changes in the intrinsic fluorescence of the human erythrocyte monosaccharide transporter upon ligand binding. Biochemistry. 21, 1905-8.

Grove T. H, Ishaque A, Levy H. R. (1976) Glucose-6-Phosphate Dehydrogenase from *Leuconostoc mesenteroides*. Archives Of Biochemistry And Biophysics. 177, 307-316.

Guex N, Peitsch M. C. (1997) SWISS-MODEL and the Swiss-Pdb Viewer: an environment for comparative protein modelling. Electrophoresis. 18, 2714-23.

Guivarch E, Trevin S, Lahitte C, Oturan M.A. (2003) Degradation of azo dyes in water by Electro-Fenton process. Environmental Chemistry Letters. 1(1), 38–44.

Gumiero A, Murphy E. J, Metcalfe C. L, Moody P.C.E, Raven E. L. (2010) An analysis of substrate binding interactions in the heme peroxidase enzymes: a structural perspective. Arch. Biochem. Biophys. 500, 13-20.

Haddaji D, Bousselmi L, Saadani O, Nouairi I, Ghrabi-Gammar Z. (2015) Enzymatic degradation of azo dyes using three macrophyte species: *Arundo donax*, *Typha angustifolia* and *Phragmites australis*, Desalin. Water Treat. 53,1129-1138.

Hao J. O, Kim. H, Chiang. Pen-Chi. (2000) Decolorization of Wastewater. Critical Reviews in Environmental Science and Technology. 30(4), 449-505.

Hashem R. A, Samir R, Essam T. M, Ali A. E, Amin M. A.(2018) Optimization and enhancement of textile reactive Remazol black B decolorization and detoxification by environmentally isolated pH tolerant *Pseudomonas aeruginosa* KY284155. AMB Express. 8(1), 83.

Havel R. J, Fielding C. J, Olivecrona J. T, Shore V. G, Fielding P. E, Egelrud T. (1973) Cofactor Activity of Protein Components of Human Very Low-Density Lipoproteins in the Hydrolysis of Triglycerides by Lipoprotein Lipase from Different Sources. Biochemistry. 12, 1928-1833.

Hayat H, Mahmood Q, Pervez A, Bhatti Z. A, Baig S. A. (2015) Comparative decolorization of dyes in textile wastewater using biological and chemical treatment. Sep. Purif. Technol. 154 149-153.

Hsueh C. C, Chen B. Y, Yen C. Y. (2009) Understanding effects of chemical structure on azo dye decolorization characteristics by *Aeromonas hydrophila*. J Hazard Mater.167(1-3), 995-1001.

Hunger K. (2003) Industrial Dyes. Chemistry, Properties, Applications. Weinheim: Wiley-VCH.

Ince N. H, Gonenc D. T. (1997) Treatability of a textile azo dye by UV/H_2O_2. Environ. Technol. 18, 179-185.

Irwin J. J, Shoichet B. K. (2005) ZINC a free database of commercially available compounds for virtual screening. J Chem Inf Model. 45, 177-82.

Jagat R, Shivani D, Archana G. (2017) Efficient approach to enhance whole cell azo dye decolorization by heterologous overexpression of *Enterococcus sp*. L2 azoreductase (azoA) and Mycobacterium vaccae formate dehydrogenase (fdh) in different bacterial systems. International Biodeterioration & Biodegradation. 124, 91-100.

Jingxian Q, Michael S, Dirk T. (2016) Biochemical characterization of an azoreductase from *Rhodococcus opacus* 1CP possessing methyl red degradation ability. Journal of Molecular Catalysis B: Enzymatic. 130, 9–17.

Kalyani D, Dhiman S. S, Kim H, Jeya M, Kim I.W, Lee J. K. (2012) Characterization of a novel laccase from the isolated *Coltricia perennis* and its application to detoxification of biomass. Process Biochem. 47, 671-678.

Kant R. (2012) Textile dyeing industry an environmental hazard. Natural Science.4, 22-26.

Karcher S, Kornmuller A, Jekel M. (1999) Effects of alkaline-earth cations on the removal of reactive dyes with cucurbituril. Acta hydrochim. 27, 38-42.

Karcher S, Kornmuller A, Jekel M. (1999) Removal of reactive dyes by sorption/complexion with cucurbituril. Water Sci. Technol. 40, 425-433.

Karthikeyan K, Nanthakumar K, Shanthi K, Lakshmanaperumalsamy P. (2010) Response surface methodology for optimization of culture conditions for dye decolorization by a fungus, *Aspergillus niger* HM11 isolated from dye affected soil. Iranian J. Microbiol. 2, 213–222.

Kelley L. A, Mezulis S, Yates C. M, Wass M. N, Sternberg M. J. (2015) The Phyre2 web portal for protein modelling, prediction and analysis. Nat Protoc.10, 845 - 58.

Khandare R.V, Kabra A.N, Awate A.V, Govindvar S.P. (2013) Synergistic degradation of diazo dye Direct Red 5B by *Portulaca grandiflora* and *Pseudomonas putida,* Int. J. Environ. Sci. Technol. 10, 1039-1050.

Khandare R.V, Kabra A.N, Kurade M.B, Govindwar S.P. (2011) Phytoremediation potential of *Portulaca grandiflora* Hook. (Moss-Rose) in degrading a sulfonated diazo reactive dye Navy Blue HE2R (Reactive Blue 172). Bioresour. Technol. 102, 6774-6777.

Khandare R.V, Kabra A.N, Tamboli D.P, Govindwar S.P. (2011) The role of *Aster amellus* Linn in the degradation of a sulfonated azo dye Ramazol Red: a phytoremediation strategy. Chemosphere. 82, 1147-1154.

Khataee A.R, Movafeghi A, Vafaei F, Lisar S.S.Y, Zarei M. (2013) Potential of the aquatic fern *Azolla filiculoides* in biodegradation of an azo dye: modeling of experimental results by artificial neural networks. Int. J. Phytoremediation, 15, 729-742.

Knapp J. S, Newby P. S, Reece L. P. (1995) Decolorization of dyes by wood-rotting basidiomycete fungi. Enzyme and Microbial Technol. 17,664-668.

Kodach L.L, Bos C. L, Duran N, Peppelenbosch M. P, Ferreira C.V, Hardwick J. C. (2006) Violacein synergistically increases 5-fluorouracil cytotoxicity, induces apoptosis and inhibits Akt-mediated signal transduction in human colorectal cancer cells. Carcinogenesis 27, 508 -16.

Konstantinous I. K, Albanis T. A. (2004) TiO2-assisted photocatalytic degradation of azo dyes in aqueous solution: kinetic and mechanistic investigations: A review. Applied Catalysis B: Environmental. 49, 1-14.

Krajewska B. (2004) Application of chitin- and chitosan-based materials for enzyme immobilizations: a review. Enzyme and Microbial Technology. 35, 126–139.

Kumar M. N. V. R, Sridhari T. R, Bhavani K. D, Dutta P. K. (1998) Trends in color removal from textile mill effluents Colorage. 40, 25-34.

Kumar R, Tiwari K, Dubey V. K. (2017) Methionine aminopeptidase 2 is a key regulator of apoptotic like cell death in *Leishmania donovani*. Sci. Rep. 7, 95.

Laskowski R. A, Rullmannn J. A, MacArthur M. W, Kaptein R, Thornton J. M. (1996) AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. J Biomol NMR. 8, 477-86.

Laskowski R. A. (2009) PDBsum new things, Nucleic Acids Res. 37, 355-9.

Lehmann G, Pierchalla P. (1998) Tattooing dyes. Derm Beruf Umwelt. 36, 52-56.

Lin S. H, Lin C. M. (1993) Treatment of textile waste effluents by ozonation and chemical coagulation. Water Res. 27, 1743-1748.

Liu G, Zhou J, Lv H, Xiang X, Wang J, Zhou M, Qv Y. (2007) Azoreductase from *Rhodobacter sphaeroides* AS1.1737 is a flavodoxin that also functions as nitroreductase and flavin mononucleotide reductase, Appl Microbiol Biotechnol.76, 1271-9.

Liu Z.J, Chen H, Shaw N, Hopper S.L, Chen L, Chen S, Cerniglia C.E, Wang B.C. (2007) Crystal structure of an aerobic FMN-dependent azoreductase (AzoA) from *Enterococcus faecalis*. Arch Biochem Biophys. 463, 68-77.

Lobenstein-Verbeek C. L, Jongejan J. A, Frank J, Duine J. A. (1984) Bovine serum aminoxidase: a mammalian enzyme having covalently bound PQQ as prosthetic group, FEBS. Lett. 170, 305-9.

Lucas M. S, Dias A. A, Sampaio A, Amaral C, Peres J. A. (2007) Degradation of a textile reactive Azo dye by a combined chemical-biological process: Fenton's reagent-yeast. Water Res. 41(5), 1103-9.

Maddhinni V.L, Vurimindi H.B, Yerramilli A. (2006) Degradation of azo dye with horse radish peroxidase (HRP). J. Indian Inst. Sci. 86,507-514.

Mahmood S, Khalid A, Arshad M, Mahmood T, Crowley D. E. (2016) Detoxification of azo dyes by bacterial oxidoreductase enzymes. Crit Rev Biotechnol. 36(4), 639-51.

Mateo C, Palomo J. M, Lorente G. F, Guisan J. M, Lafuente R. F. (2007) Improvement of enzyme activity, stability, and selectivity via immobilization techniques. Enzyme and Microbial Technology. 40, 1451–1463.

McMullan G, Meehan C, Conneely A, Kirby N, Robinson T, Nigam P, Banat I. M, Marchant R, Smyth W.F. (2001) Microbial Decolorization and degradation of textiles dye. appl Microbiol Biotechnol. 56, 81-87.

Mester T, Field J.A. (1998) Characterization of a novel manganese peroxidase hybrid isozyme produced by *Bjerkandera* species strain BOS55 in the absence of manganese. J. Biol. Chem. 273, 15412-15417.

Misal S. A, Lingojwar D. P, Shinde R. M, Gawai K. R. (2011) Purification and characterization of azoreductase from alkaliphilic strain *Bacillus badius*, Process Biochem. 46, 1264-1269.

Mishra G, Tripathy M. (1993) A critical review of the treatments for decolourization of textile effluent Colourage, 40, 35-38.

Mohan S.V, Prasad K. K, Rao N. C, Sarma P. N. (2005) Acid azo dye degradation by free and immobilized horseradish peroxidase (HRP) catalyzed process, Chemosphere. 58, 1097-105.

Mohan S.V, Rao N.C, Srinivas S, Prasad K.K, Karthikeyan J. (2002) Treatment of simulated Reactive Yellow 22 (azo) dye effluents using Spirogyra species. Waste Manag. 22, 575-82.

Morris G. M, Huey R, Lindstrom W, Sanner M. F, Belew R. K, Goodsell D. S, Olson A. J. (2009) AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. J Comput Chem. 30, 2785-91.

Moutaouakkil A, Zeroual Y, Zohra Dzayri F, Talbi M, Lee K, Blaghen M. (2003) Purification and partial characterization of azoreductase from *Enterobacter agglomerans.* Arch Biochem Biophys. 413, 139-46.

Muthunarayanan V, Santhiya M, Swabna V, Geetha A. (2011) Phytodegradation of textile dyes by Water Hyacinth (*Eichhornia crassipes*) from aqueous dye solutions. Int. J. Environ. Sci. 1 (7), 1702-1717.

Nachiyar C. V, Rajakumar G. S. (2005) Purification and characterization of an oxygen insensitive azoreductase from *Pseudomonas aeroginosa*. Enzyme Microb Technol. 36, 503-509.

Najar I. N, Sherpa M. T, Das S, Verma K, Dubey V. K, Thakur N. (2018) *Geobacillus yumthangensis* sp. nov., a thermophilic bacterium isolated from a north-east Indian hot spring. Int J Syst Evol Microbiol. 68, 3430-3434.

Nakanishi M, Yatome C, Ishida N, Kitade Y. (2001) Putative ACP phosphodiesterase gene (acpD) encodes an azoreductase. J. Biol. Chem. 276, 46394-46399.

Namboodri C. G, Walsh W. K. (1996) Ultraviolet light/hydrogen peroxide system for decolorizing spent reactive dye bath waste water. Am. Dyes. Rep. 15–25.

Nigam P, Armour G, Banat I.M, Singh D, Marchant R. (2000) Physical removal of textile dyes and solid state fermentation of dye-adsorbed agricultural residues. Bioresour. Technol. 72, 219-226.

Nilratnisakorn S, Thiravetyan P, Nakbanpote W. (2008) Synthetic reactive dye wastewater treatment by narrow-leaved cattail: Studied by XRD and FTIR. Asian J. Energy Environ. 9, 231-252.

O'Neill C, Hawkes F. R, Hawkes D.L, Lourenco N.D, Pinheiro H.M, Delee W. (1999) Colour in textile effluents – sources, measurement, discharge consents and simulation: a review. J Chem Technol Biotechnol. 74, 1009-1018.

Ogutveren U. B, Kaparal S. (1994) Colour removal from textile effluents by electrochemical destruction. J. Environ. Sci. Health A. 29, 1-16.

Ooi T, Shibata T, Sato R, Ohno H, Kinoshita S, Thuoc T. L, Taguchi S. (2007) An azoreductase, aerobic NADH-dependent flavoprotein discovered from *Bacillus sp*.: functional expression and enzymatic characterization. Appl Microbiol Biotechnol. 75, 377-86.

Parray Z. A, Ahamad S, Ahmad F, Hassan M. I, Islam A. (2019) First evidence of formation of pre-molten globule state in myoglobin: A macromolecular crowding approach towards protein folding in vivo. Int J Biol Macromol.126, 1288-1294.

Patil P, Desai N, Govindwar S, Jadhav J.P, Bapat V. (2009) Degradation analysis of Reactive Red 198 by hairy roots of *Tagetes patula* L. (Marigold). Planta. 230 (4), 725-735.

Paul T, Baskaran D, Pakshirajan K, Pugazhenthi G. (2019) Continuous bioreactor with cell recycle using tubular ceramic membrane for simultaneous wastewater treatment and bio-oil production by oleaginous *Rhodococcus opacus*. Chemical Engineering Journal. 367, 76-85.

Pelegrini R, Peralto-Zamora P, Andrade A. R. de, Reyers J, Duran N. (1999) Electrochemically assisted photocatalytic degradation of reactive dyes. App. Catal B-Environ. 22, 83-90.

Peralto-Zamora P, Kunz A, Gomez de Morales S, Pelegrini R, De Capos Moleiro P, Reyes J, Duran N. (1999) Degradation of reactive dyes I. A comparative study of ozonation, enzymatic and photochemical processes. Chemosphere. 38, 835-852.

Pinheiro H. M, Touraud E, Tomas O. (2004) Aromatic amin from azo dye reduction: status review with emphasis on direct UV spectrophotometric detection in textile industries waste water. Dyes Pigm. 61, 121-139.

Polman A, Brekenridge C. R. (1996) Biomass-mediated binding and recovery of textile dyes from waste effluents. Tex. Chem. Colour. 28,31-35.

Prasanna V, Gopal B, Murthy M. R, Santi D. V, Balaram P. (1999) Effect of amino acid substitutions at the subunit interface on the stability and aggregation properties of a dimeric protein: role of Arg 178 and Arg 218 at the Dimer interface of thymidylate synthase. Proteins. 34, 356-68.

Punj S, John G.H. (2009) Purification and identification of an FMN-dependent NAD(P)H azoreductase from *Enterococcus faecalis* Curr Issues Mol Biol. 11, 59-65.

Qi J, Paul C. E, Hollmann F, Tischler D. (2017) Changing the electron donor improves azoreductase dye degrading activity at neutral pH. Enzyme Microb Technol. 100, 17-19.

Raghavacharya C. (1997) Colour removal from industrial effluents – a comparative review of available technologies Chem. Eng. World, 32, 53-54.

Raghu S, Ahmed Basha. C (2007) Chemical or electrochemical techniques, followed by ion exchange, for recycle of textile dye wastewater. Journal of Hazardous Materials. 149, 324–330.

Raghukumar C, Chandramohan D, Michel Jr F.C, Reddy C. A. (1996) Degradation of lignin and decolorization of paper mill bleach plant effluent (BPE) by marine fungi. Biotechnol. Lett. 18 (1), 105-106.

Rajaguru P, Fairbairn L. J, Ashby J, Willington M. A, Turner S, Woolford L. A, Chinnasamy N, Rafferty J. A. (1999) Genotoxicity studies on the azo dye Direct Red 2 using the in vivo mouse bone marrow micronucleus test. Mutat Res. 444(1), 175-80.

Ramalho P. A, Helena Cardoso M, Cavaco-Paulo A, Teresa Ramalho M. (2004) Characterization of Azo reductase in a novel *Ascomycete Yeast* strain. Appl. Environ. Microbiol. 70, 2279-2288.

Rao K.L.L.N, Krishnaiah K, Ashutush (1994) Colour removal from a dye stuff industry effluent using activated carbon. Indian J. Chem. Technol. 1, 13-19.

Rao M. A, Scelza R, Acevedo F, Diez M. C, Gianfreda L. (2014) Enzymes as useful tools for environmental purposes. Chemosphere. 107.145-162.

Rauf. M. A, Ashraf. S. S. (2009) Radiation induced degradation of dyes-An overview. Journal of Hazardous Materials. 166, 6–16.

Rizwana M, Darshan M, Nilesh D. (2014) Phytoremediation of textile waste water using potential wetland plant: ecosustainable approach. Int. J. Interdiscip. Multidiscip. Stud. 1 (4), 130-138.

Robinson T, McMullan G, Marchant R, Nigam P. (2001) Remediation of dyes in textile effluent: a critical review on current treatment technologies with a proposed alternative. Bioresour Technol.77, 247-55.

Rosu C. M, Vochita G, Mihasan M, Avadanei M, Mihai C. T, Gherghel D.(2019) Performances of *Pichia kudriavzevii* in decolorization, biodegradation, and detoxification of C.I. Basic Blue 41 under optimized cultural conditions, Environ Sci Pollut Res Int. 26, 431- 445.

Ryan A. (2017) Azoreductases in drug metabolism. Br. J. Pharmacol. 174, 2161-2173.

Santos A. B. dos, Cervantes F. J, Van Lier J. B. (2007) Review paper on current technologies for decolourisation of textile wastewaters: perspectives for anaerobic biotechnology. Bioresour. Technol. 98. 2369-2385.

Saratale R. G, Saratale G. D, Chang J. S, Govindwar S. P. (2011) Bacterial decolorization and degradation of azo dyes: a review. J. Taiwan Inst. Chem. Eng. 42, 138-157.

Sathishkumar P, Kamala-Kannan S, Cho M, Kim J. S, Hadibarata T, Salim M. R, Oh B. T. (2014) Laccase immobilization on cellulose nanofiber: the catalytic efficiency and recyclic application for simulated dye effluent treatment. J. Mol. Catal. B Enzym. 100, 111-120.

Schoneck R, Billaut-Mulot O, Numrich P, Ouaissi M. A, Krauth-Siegel R.L. (1997) Cloning, sequencing and functional expression of dihydrolipoamide dehydrogenase from the the human pathogen *Trypanosoma cruzi*, Eur. J. Biochem. 243, 739-747.

Sen P, Atmaca G.Y, Erdogmus A, Dege N, Genc H, Atalay Y, Yildiz S. Z. (2015) The Synthesis, Characterization, Crystal Structure and Photophysical Properties of a New Meso-BODIPY Substituted Phthalonitrile. J Fluoresc. 25(5), 1225-34.

Singh A. N, Singh S, Dubey V. K. (2013) Immobilization of Procerain B, a Cysteine Endopeptidase, on Amberlite MB-150 Beads. Plos one. 8, e66000.

Singh A. R, Joshi S, Arya R, Kayastha A. M, Saxena J. K. (2010) Guanidine hydrochloride and urea-induced unfolding of *Brugia malayihexokinase*. Eur Biophys J. 39, 289-297.

Singh K, Arora S. (2011) Removal of synthetic textile dyes from wastewaters: a critical review on present treatment technologies. Cri. Rev. Environ. Sci. Technol. 41, 807-878.

Singh R. L, Singh P. K, Singh R. P. (2015) Enzymatic decolorization and degradation of azo dyes a review. International Biodeterioration & Biodegradation. 104, 21–31.

Slokar Y. M, Le Marechal A. M. (1997) Methods of decolouration of textile wastewaters. Dyes Pigments, 37, 335-356.

Solis M, Solis A, Perez H. N, Manjarrez N, Flores M. (2012) Microbial decolouration of azo dyes: A review. Process Biochemistr. 47(12), 1723-1748.

Song Z.Y, Zhou J.T, Wang J, Yan B, Du C.H. (2003) Decolorization of azo dyes by *Rhodobacter sphaeroides*. Biotechnol. Lett. 25, 1815-1818.

Spadaro J. T, Gold M. H, Renganathan V. (1992) Degradation of azo dyes by the lignin-degrading fungus *Phanerochaete chrysosporium*. Appl EnviroMicrobiol. 58, 2397-401.

Stockert J. C, Horobin R. W, Colombo L. L, Blazquez-Castro A. (2018) Tetrazolium salts and formazan products in Cell Biology: Viability assessment, fluorescence imaging and labeling perspectives. Acta Histochem. 120, 159-167.

Stolz A. (2001) Basic and applied aspects in the microbial degradation of azo dyes. Appl. Microbiol. Biotechnol. 56, 69–80.

Sun J, Kweon O, Jin J, He G. X, Li X, Cerniglia C. E, Chen H. (2017) Mutation network-based understanding of pleiotropic and epistatic mutational behavior of *Enterococcus faecalis* FMN-dependent azoreductase. Biochem Biophys Rep. 12, 240-244.

Sun Q, Gan N, Zhang S, Zhao L, Tang P, Pu H, Zhai Y, Gan R, Li H. (2019) Insights into protein recognition for γ-lactone essences and the effect of side chains on interaction via microscopic, spectroscopic, and simulative technologies. Food Chem.8, 127-135.

Sureshvarr K, Bharathiraja B, Jayakumar M, Jayamuthunagai J, Balaji L. (2010) Removal of azo dye compounds from paper industries wastes using phytoremediation methodology. Int. J. Chem. Sci. 8 (1), 687-700.

Suthar M. K, Verma A, Doharey P. K, Singh S. V, Saxena J. K. (2013) Single tryptophan of disordered loop from *Plasmodium falciparum* purine nucleoside phosphorylase: involvement in catalysis and microenvironment. Appl Biochem Biotechnol. 170, 868-79.

Suzuki Y, Yoda T, Ruhul A, Sagiura W. (2001) Molecular cloning and characterization of the gene encoding azoreductase from *Bacillus* sp. *OY 1-2* isolated from soil. Journal of Biological Chemistry. 246, 9059-9065.

Tahir U, Yasmin A, Khan U. H. (2016) Phytoremediation: Potential flora for synthetic dyestuff metabolism. Journal of King Saud University – Science 28(2), 119-130.

Talarposhti A. M, Donnelly T, Anderson G. K. (2001) Color removal from a simulated dye wastewater using a two-phase anaerobic packed bed reactor. Water Res.35, 425-432.

Tarlan E, Dilek F. B, Yetis U. (2002) Effectiveness of algae in the treatment of a wood-based pulp and paper industry wastewater. Bioresour. Technol. 84, 1–5.

Tiwari K, Kumar R, Dubey V. K. (2016) Biochemical characterization of dihydroorotase of *Leishmania donovani*: Understanding pyrimidine metabolism throμgh its inhibition. Biochimie. 131, 45-53.

Torbati S, Movafeghi A, Khataee A. R. (2015) Biodegradation of C.I. Acid Blue 92 by *Nasturtium officinale*: study of some physiological responses and metabolic fate of dye. Int. J. Phytoremediation. 17, 322-329.

Van der Zee F. P, Villaverde S. (2005) Combined Anaerobic-Aerobic Treatment of Azo Dyes—A Short Review of Bioreactor Studies. Water Research. 39, 1425-1440.

Venkatesha B, Udgaonkar J. B, Rao N. A, Savithri H. S. (1998) Reversible unfolding of sheep liver tetrameric serine hydroxymethyltransferase. Biochim Biophys Acta. 1384, 141-52.

Verma A, Chandra S, Suthar M. K, Doharey P. K, Siddiqi M. I, Saxena J. K. (2016) $NADP^+$ binding effects tryptophan accessibility, folding and stability of recombinant *B. malayi* G6PD. Int J Biol Macromol.85, 645-54.

Verma K, Saha G, Kundu L. M, Dubey V. K. (2019) Biochemical characterization of a stable azoreductase enzyme from *Chromobacterium violaceum*: Application in industrial effluent dye degradation. International Journal of Biological Macromolecules. 121, 1011-1018.

Von Der Lehr N, Johansson S, Wu S, Bahram F, Castell A, Cetinkaya C, Hydbring P, Weidung I, Nakayama K, Nakayama K. I, Soderberg O, Kerppola T. K, Larsson L. G. (2003) The F-box protein Skp2 participates in c-Myc proteasomal degradation and acts as a cofactor for c-Myc-regulated transcription. Mol Cell. 5, 1189-200.

Wackett L. P, Ellis L. B. M. (1999) Predicting biodegradation Environ. Microbiol. , 119-124.

Wang C, Yediler A, Lienert D, Wang Z, Kettrup A. (2003) Ozonation of an azo dye C.I. Remazol Black 5 and toxicological assessment of its oxidation products. Chemosphere. 52, 1225-1232.

Wang C. J, Hagemeier C, Rahman N, Lowe E, Noble M, Coughtrie M, Sim E, Westwood I. (2007) Molecular cloning, characterisation and ligand-bound structure of an azoreductase from *Pseudomonas aeruginosa*. J Mol Biol. 373(5), 1213-28.

Wang X. T, Chan T. F, Lam V. M, Engel P. C. (2008) what is the role of the second "structural" NADP+-binding site in human glucose 6-phosphate dehydrogenase. Protein Sci. 8, 1403-11.

Wang X. T, Lam V. M, Engel P. C. (2006) Functional properties of two mutants of human glucose 6-phosphate dehydrogenase, R393G and R393H, corresponding to the clinical variants G6PD Wisconsin and Nashville. Biochim Biophys Acta. 62, 767-74.

Wardell S. E, Kwok S. C, Sherman L, Hodges R. S, Edwards D. P. (2005) Regulation of the amino-terminal transcription activation domain of progesterone receptor by a cofactor-induced protein folding mechanism. Mol Cell Biol. 20, 8792-808.

Willmott N, Guthrie J, Nelson G. (1998) The biotechnology approaches to color removal from textile effluent. JSDC. 114, 38-41.

Wong P. K, Yuen P. Y. (1996) Decolorization and biodegradation of methyl red by *Klebsiella pneumonia* RS-13. Water Res. 30, 1736– 44.

World Bank annual report. 1998.

Wu B, Wawrzynow A, Zylicz M, Georgopoulos C. (1996) Structure-function analysis of the Escherichia coli GrpE heat shock protein. EMBO J. 15, 4806-16.

Xu Y, Lebrun R.E. (1999) Treatment of textile dye plant effluent by nanofiltration membrane. Separ. Sci. Technol. 34, 2501-2519.

Yang Y, Wyatt II D.T, Bahorsky M. (1998) Decolorization of dyes using UV/H_2O_2 photochemical oxidation. Text. Chem. Color. 30, 27-35.

Yerramilli A, Chary N. S, Dasary S. (2005) Decolourization of Industrial Effluents – Available Methods and Emerging Technologies, Reviews in Environmental. Science and Biotechnology. 4(4), 245-273.

Young L, Yu J. (1997) Ligninase-catalyzed Decolorization of synthetic dyes. Water Res. 31 (5), 1187-1193.

Zahran S. A, Ali-Tammam M, Hashem A. M, Aziz R. K, Ali A. E. (2019) Azoreductase activity of dye-decolorizing bacteria isolated from the human gut microbiota. Sci Rep. 9, 5508.

Zenno S, Koike H, Kumar A. N, Jayaraman R, Tanokura M, Saigo K. (1996) Biochemical characterization of NfsA, the *Escherichia coli* major nitroreductase exhibiting a high amino acid sequence homology to Frp, a *Vibrio harveyi* flavin oxidoreductase. J Bacteriol. 178, 4508 -14.

Zhang J, Feng M, Jiang Y, Hu M, Li S, Zhai Q. (2012) Efficient decolorization/degradation of aqueous azo dyes using buffered H_2O_2 oxidation catalyzed by a dosage below ppm level of chloroperoxidase. Chem. Eng. J. 191, 236-242.

Zhou J. L, Banks C. J. (1991) Removal of humic acid fraction by *Rhizopus arrhizus*: uptake and kenetic studies. Environ. Technol. 12, 859-869.

Zimmermann T, Kulla H, Leisinger T. (1982) Properties of purified orange II-azoreductase, the enzyme initiating azo dye degradation by *Pseudomonas KF46.* European Journal of Biochemistry. 129, 197-203.

Zollinger H, (1987) Synthesis, Properties and Application of Organic Dye and Pigments. *Color Chemistry*, VCH New York. 92- 102.

Zollinger H. (2004) Color Chemistry Synthesis, Properties, and Application of Organic Dye and Pigments. Angewandte Chemie International Edition 43, 5291-5292.

PUBLICATIONS

- **Kamalesh Verma**, Gundappa Saha, Lal Mohan Kundu, Vikash Kumar Dubey, Biochemical characterization of a stable azoreductase enzyme from *Chromobacterium violaceum*: Application in industrial effluent dye degradation**.** International Journal of Biological Macromolecules 121 (2019) 1011–1018.
- **Kamalesh Verma,** Debanjan Kundu, Lal Mohan Kundu, Ashish Kumar Singh, Vikash Kumar Dubey, Folding and stability of recombinant azoreductase enzyme from *Chromobacterium violaceum*. ***Enzyme and Microbial Technology*** 131 (2019) 109433.
- **Kamalesh Verma,** Lal Mohan Kundu, Vikash Kumar Dubey, Degradation of dye-waste from textile industries with immobilized azoreductase in continuous bead pack reactor. **(Submitted)**
- Ishfaq Nabi Najar, Mingma Thundu Sherpa, Sayak Das, **Kamalesh Verma**, Vikash Kumar Dubey, Nagendra Thakur, *Geobacillus yumthangensis* sp. nov., a thermophilic bacterium isolated from a north-east Indian hot spring. International Journal of Systematic and Evolutionary Microbiology 68 (11) (2018) 3430-3434.
- Soumi Das, **Kamalesh Verma**, Vikash K dubey, Lal Mohan Kundu, Fabrication of 5-Flurouracil Conjugated peptide nanoparticles as a versatile, Stimuli-Responsive dual drug Carrier with high antiproliferation activity**.** ***Bioorganic Chemistry*** 94(2020) 103440.

CONFERENCE PROCEEDINGS AND WORKSHOPS

- **Kamalesh Verma,** Lal Mohan Kundu, Vikash Kumar Dubey, Studies on degradative property of *Chromobacterium violaceum* azoreductase for the removal of effluent dyes. International conference on Advances in Biotechnology and Biotherapeutics (ICABBS-2017) Organized by Sathyabama University Chennai, India.ISBN: 978-93-83409-30-3.
- **Kamalesh Verma**, Lal Mohan Kundu, Vikash Kumar Dubey, Role of azoreductase enzyme from *Chromobacterium violaceum* for the removal of effluent dyes. International Conference on Biotechnology and Biological Sciences (BIOSPECTRUM-2017), 25-26 August, 2017 at University of engineering and management Kolkata.
- **Kamalesh Verma**, Lal Mohan Kundu, Vikash Kumar Dubey, Studies on azoreductase enzyme from *Chromobacterium violaceum*: application in industrial effluent dye degradation. International Conference on "Emerging Trends in Biotechnology for Waste Conversion (ETBWC-2017). 8-10 October, 2017 organised by BRSI at NEERI Nagpur.
- **Kamalesh Verma**, Lal Mohan Kundu, Vikash Kumar Dubey, Characterization of azoreductase enzyme from *Chromobacterium violaceum* for the removal of effluent dyes "National Conference on Recent advancements in Environmental Research"(RAER 2017) Organized by Centre for the Environment, Indian Institute of Techonology Guwahati Assam India.
- **Kamalesh Verma**, Lal Mohan Kundu, Vikash Kumar Dubey, Exploring the role of azoreductase enzyme from *Chromobacterium violaceum* in degradation of effluent dyes from textile industries, Research Conclave- 2017, organized by Indian Institute of Techonology Guwahati Assam India.
- **Kamalesh Verma**, Lal Mohan Kundu, Vikash Kumar Dubey, Exploring the role azoreductase from *Geobacillus kaustophilus* in degradation of effluent dyes from textile industries "National Conference on Recent advancements in Environmental Research"(RAER 2016) Organized by Centre for the Environment, Indian Institute of Techonology Guwahati Assam India.
- **Kamalesh Verma**, Lal Mohan Kundu, Vikash Kumar Dubey, Studies on stable azoreductase enzyme from *Chromobacterium violaceum*: application in industrial effluent dye degradation. National conference in Recent Advancement in Biochemical Engineering and Biotechnology (RABEB-2019) 15- 16 March, 2019 organized by School of Biochemical Engineering, IIT (BHU) Varanasi.

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OBJECTIVE

To contribute my efforts and skills in research to solve the mankind problem in long term.

EDUCATION

Year of Completion	Level of Study	Percentage/CPI
August, 2019 (***Thesis submitted***)	**Ph.D** Indian Institute of Technology Guwahati, Guwahati, Assam	Nil
June 2013	**Master of Science** **(**Molecular Biology and Biotechnology**)** Tezpur University, Assam India	**7.17/10** (First Division)
July 2010	**Bachelor of Science** **(**Botany**,** Chemistry, Zoology,**)** MGPG College Gorakhpur (DDU Gorakhpur University Gorakhpur)	**63.27 %** (First Division)
May 2006	**Uttar Pradesh Board** **(**English, Hindi, Physics, Chemistry, Biology) GSVS Intermediate College, Maharajganj, UP	**65.20 %** (First Division)
May 2004	**Uttar Pradesh Board** (English, Hindi, Science, Mathematics, S. Science, Drawing) NSKS Inter College Moulaganj Maharajganj, UP	**64.17%** (First Division)

AWARDS AND FELLOWSHIPS

- Qualified national level **GATE** (Graduate Aptitude Test in Engineering) examination in Life Science 2013.
- Qualified national level **CBEE (Combine Biotechnology Entrance Examination**) conducted by JNU (Jawaharlal Nehru University) AIR 639 and Receive fellowship from DBT for Master Studied

- **Qualified National Eligibility Test NET (I)-2016** in the subject of Agricultural Biotechnology conducted by Agricultural Scientists Recruitment Board, Indian Council of Agricultural Research.
- **Best presentation Award,** Regional Seminar on Eco-Restoration for Development in North East India, Date 22nd and 23rd April, 2013, Organized by Cotton College, Cotton University, Guwahati, Assam, India.
- **Best Poster award in research conclave 2017** organized by Indian Institute of Technology Guwahati, Guwahati Assam, India.

RESEARCH PAPER

1. **Kamalesh Verma**, Gundappa Saha, Lal Mohan Kundu, Vikash Kumar Dubey, Biochemical characterization of a stable azoreductase enzyme from *Chromobacterium violaceum:* Application in industrial effluent dye degradation**.** ***International Journal of Biological Macromolecules*** 121 (2019) 1011–1018. (**IF: 4.57**)
2. **Kamalesh Verma,** Debanjan Kundu, Lal Mohan Kundu, Ashish Kumar Singh, Vikash Kumar Dubey, Folding and stability of recombinant azoreductase enzyme from *Chromobacterium violaceum*. ***Enzyme and Microbial Technology*** 131 (2019) 109433. . (**IF: 3.65**)
3. Ishfaq Nabi Najar, Mingma Thundu Sherpa, Sayak Das, **Kamalesh Verma**, Vikash Kumar Dubey and Nagendra Thakur, *Geobacillus yumthangensis* sp. nov., a thermophilic bacterium isolated from a north-east Indian hot spring. ***International Journal of Systematic and Evolutionary Microbiology*** 68 (11) (2018) 3430-3434. (**IF: 3.10**)
4. Archana Singh, Anuj Kumar Borah, Kamalakshi Deka, Akash Protim Gogoi, **Kamalesh Verma**, Pankaj Barah, Sougata Saha, Arginylation regulates adipogenesis by regulating expression of PPARγ At transcript and protein level. ***BBA - Molecular and Cell Biology of Lipids*** 1864 (2019) 596–607. (**IF: 4.97**)
5. Soumi Das, **Kamalesh Verma**, Vikash K dubey, Lal Mohan Kundu, Fabrication of 5-Flurouracil Conjugated peptide nanoparticles as a versatile, Stimuli-Responsive dual drug Carrier with high antiproliferation activity**.** ***Bioorganic Chemistry*** 94(2020) 103440 . (**IF: 3.92**)
6. **Kamalesh Verma,** Lal Mohan Kundu, Vikash Kumar Dubey, Continuous bioreactor for the degradation of azo dyes using the immobilized azoreductase on bead. ***Communicated***

7. **Kamalesh Verma,** Himali horo, Lal Mohan Kundu, Vikash Kumar Dubey, Azoreductase mediated synthesis of gold nano particles: its characterization and application into nanotherapeutic and dyes degradation. ***Communicated***

BOOK CHAPTER

- Barbie Hazarika and **Kamalesh Verma,** Eco-restoration in North East India under the influences of climate change by compairing plant species. **ISBN: 978-93-81563-40-3**

WORKSHOP AND CONFERENCES ATTENDED

1. Regional Seminar on Eco-Restoration for Development in North East India, Date 22nd and 23rd April,2013, Cotton College, Guwahati , Assam
   Title of the Presented Paper: "***Eco restoration in North East India under the influence of climate change by comparing plant species***"
2. Training Workshop program on **Confocal Laser Scanning Microscopy**, 3-5 February 2016 to be organized by **Guwahati Biotech Park** in association with ***Leica Microsystems.***
3. Participated in Conference on Bioinformatics and Computer aided Drug Design Organized by DBT- Bioinformatics Infrastructure Facility, Department of Bioscience and Bioengineering **Indian Institute of Techonology Guwahati Assam India.**.
4. Presented a Poster in "National Conference on Recent advancements in Environmental Research" Organized by **Centre for the Environment, Indian Institute of Techonology Guwahati Assam India**
5. Presented a Poster in **International conference** on Advances in Biotechnology and Biotherapeutics(ICABBS-2017) Organized by **Sathyabama University Chennai, India.**
6. Presented a Poster in Research Conclave- 2017, organized by **Indian Institute of Techonology Guwahati Assam India.**
7. Organize a National conferences On the topic of Recent Developments In Medical Biotechnology And Structure Based Drug Designing (RDMBSBDD-2015) organized by Department of Biosciences and Bioengineering, **Indian Institute of Technology Guwahati, Assam.**
8. Kamalesh Verma, Lal Mohan Kundu, Vikash Kumar Dubey, Role of azoreductase enzyme from *Chromobacterium violaceum* for the removal of effluent dyes. International Conference on Biotechnology and Biological Sciences (**BIOSPECTRUM- 2017),** 25-26 August, 2017 at **University of engineering and management Kolkata**.
9. Kamalesh Verma, Lal Mohan Kundu, Vikash Kumar Dubey, Studies on azoreductase enzyme from Chromobacterium violaceum: application in industrial effluent dye degradation. International Conference on "Emerging Trends in Biotechnology for Waste Conversion **(ETBWC-2017)**. 8-10 October, 2017 organised by **BRSI at NEERI Nagpur.**

10. Kamalesh Verma, Lal Mohan Kundu, Vikash Kumar Dubey, Studies on stable azoreductase enzyme from *Chromobacterium violaceum:* application in industrial effluent dye degradation. National conference in Recent Advancement in Biochemical Engineering and Biotechnology (RABEB-2019) 15- 16 March, 2019 organized by **School of Biochemical Engineering, IIT (BHU) Varanasi**.

TECHNICAL EXPOSURE

- **Molecular Biology:** Genomic DNA/Plasmid Isolation, PCR, Primer designing, competent cell preparation, Cloning of gene in to different vector, transformation in to host, Selection of clone and it expression, Agarose Gel Electrophoresis, RNA isolation, cDNA Synthesis, Cell Culture, Cryopreservation, Electroporation etc.
- **Protein Biochemistry:** Recombinant protein production, Purification of proteins with Ni-NTA Affinity, Gel filtration chromatography and Ion exchange chromatography, Enzyme Kinetics, Native & SDS-PAGE Gel electrophoresis, UV-visible Spectroscopy, Dialysis, Crystallization setup etc.
- **Bioinformatics:** Sequence analysis, alignment, Homology modeling, Docking studies, Primer designing etc.
- **Analytical instruments:** HPLC, Low pressure column Chromatography (EKTA prime), FPLC, FTIR, TLC, Ultra-sonicator, Lyophilizer, Gas chromatography, Fluorescence microscope, Multimode Elisa Reader, Confocal Microscope (Leica) etc.
- **Computer Skill:** MS words, Power point, operating system: Windows, Linux etc.

Membership

Life time membership of **Bioinformatics and Drug Discovery Society** (BIDDS).

Extra Curricular Activity

- Represented Patkai Mens Hostel, Tezpur University at Annual Meet 2012 in March Past event.
- Served NSS (National service Scheme) for two years at MPGP College, Gorakhpur.
- Served as Volunteer for many events.

References

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DECLARATION

I hereby declare that the information furnished in this resume are true and correct to the best of my knowledge and belief.

Date: 18.01.2020

Place: GUWAHATI

KAMALESH VERMA